Food Toxicants Analysis

Techniques, Strategies and Developments

Edited by Yolanda Picó

Preface

As a consequence of the adoption of strict regulations, the science of food toxicants analysis has shown a rapid development in recent years. The number of related papers is increasing daily. New analytical techniques are being developed and the existing ones optimized. Analysis systems have been fully automated. A compilation of these new analytical methods is needed for food chemists.

This book covers different aspects of the analytical food toxicology: the emerging analytical techniques, the applications to detect food allergens, genetically modified organisms and novel ingredients (including those of functional foods), and the quality assurance to guarantee the reliability of the analysis. Attention is paid on natural toxins in food, plants and animals, cancer modulating substances, microbial toxins in foods (algal, fungal, and bacterial), and all groups of contaminants, such as pesticides, persistent organic pollutants, metals, packaging materials, hormones and animal drug residues.

The book contains 19 chapters covering the emerging topics in food toxicants analysis, and it is organized in four sections. The first of them describes current status of the regulatory framework, including the key principles of EU, US, and Mercosur food law, food safety regulations, and the main mechanisms of enforcement.

The second section covers the validation and quality assurance in food toxicants analysis and comprises a general discussion on the use of risk analysis. Historically, food safety risk analysis has been considered a useful decision tool; however, it has become a necessity to solve problems and to select priorities. In recent years, considerable attention has also been directed towards improving and ensuring the quality of analytical data on toxicants in food. Whether the data are used for assessing risk from exposure, for food control or food monitoring standard for trading purposes, it is crucial that contaminants be identified correctly and the quantitative date be consistent.

The third section addresses the new issues of food toxicant analysis including the food allergens and the genetically modified organisms (GMOs), which because of their characteristics require a special use of the analytical techniques. Allergens pose a serious risk to the consumers, making the development of effective detection methods a priority for the food industry. This part reviews the range of analytical techniques available, including enzyme linked immunoassays (ELISA), proteomics, polymerase chain reaction (PCR) and dipsticks and their use to detect specific allergens such as nuts, dairy, and wheat products. The use of GMOs –their release, importation and, particularly, their utilisation as food or food ingredients –is regulated in the European Union by a set of meticulous procedures. Methods are thus necessary, not only to detect the eventual presence of a GMO in a food matrix but also to identify the specific GMO and to quantify the amount of GMOs in different food ingredients.

The last part of the book discusses the “screening and chromatographic methods”, which is one of the most important topics. This group includes the wide variety techniques employed to determine substances such as pesticides residues, veterinary drugs, food additives, plant toxins, microbial toxins, environmental contaminants (dioxins, PCBs, polybrominated

biphenyls ethers, etc), and other toxics formed during the food processing (Heterocyclic amines, PAH, or acrylamide). There is a need for sensitive and selective analytical methods met by the use of modern techniques and approaches, which are being evaluated to screen, quantify and/or confirm chemical residues. These advanced technologies include hyphenated gas (GC) or liquid (LC) chromatography/mass spectrometry (MS) (or tandem mass spectrometry -MS^n), supercritical fluid extraction (SFE), aqueous microdyalisis, solid phase extraction (SPE) and other techniques.

In addition, the book leads with the newest techniques on the field of inorganic analysis. Cases of severe poisoning caused by metal compounds, especially compounds of mercury, lead, cadmium, arsenic, chromium, and nickel, and the proved carcinogenicity of some metal ions have brought about an intensive development of research to improve analytical methods. In their determination, there are various instrumental methods actually in use: atomic absorption spectrometry (AAS), atomic emission spectrometry (AES), potentiometry with membrane electrodes (ion selective), polarography, voltametry, capillary electrophoresis, ionic chromatography, the newest one, the induced coupled plasma-mass spectrometry (ICP-MS), etc... In selecting an analytical method, it should be taken into account not only the purpose of analysis and type of tested samples, but also sensitivity, duration, and possibilities of measurement automation. Nitrates and nitrites are commonly quantified, since nitrites are added as preservative and colour enhancer. Nitrites are also of concern since they can react with secondary amines to form carcinogen nitrosamines.

This volume is addressed to serve as general reference for post-graduate students as well as a practical reference for a wide range of experts: biologists, bromatologists, toxicologists, chemists, agronomists, hygienist, and everybody who need to use the analytical techniques for evaluating food safety. Each chapter contains enough references to the literature to help as an effective resource for a more detailed research.

The contributing authors from throughout the world are strong leaders in all fields of food analysis, in both industry and academic institutions. I congratulate them and thank them for the effort and collaboration as well as their excellent contributions.

Contributor contact details

Sam Adeloju
School of Applied Sciences & Engineering
Monash University,Gippsland Campus
Churchill
Victoria 3842, Australia

Suleyman Akman
Technical University Istanbul, Faculty of Science & Letters, Department of Chemistry
TR-34469 Istanbul

Elke Anklam
Commission European Communities
DGJ Joint Research Centre
Inst Reference Materials & Measurements, Retieseweg,
B-2440 Geel

Damià Barceló
Environmental Chemistry
IIQAB-CSIC,
Jordi Girona, 18
S-08034 Barcelona

Cristina Blasco
Laboratori de Bromatologia i Toxicologia
Facultat de Farmàcia, Universitat de València
Av. Vicent Andrés Estellés s/n
S-46100 Burjassot, Valencia

Sara Bogialli
Department of Chemistry
Universita degli studi di Roma "La Sapienza"
Ple Aldo Moro 5
I-00185 Roma

Jim Bridges
Research for Sustainability, Liddington Lodge, Guildford, Surrey, GU33AE, UK

Olga Bridges
Research for Sustainability, Liddington Lodge, Guildford, Surrey, GU33AE, UK

Tomas Čajka
Institute of Chemical Technology
Faculty of Food and Biochemical Technology
Department of Food Chemistry and Analysis
Techniká 3
CZ-16628 Prague

Antonio Di Corcia,
Department of Chemistry
Universita degli studi di Roma "La Sapienza"
Ple Aldo Moro 5
I-00185 Roma

Francesco Cubadda
National Centre for Food Quality and Risk Assessment
Istituto Superiore di Sanità (National Health Institute)
Viale Regina Elena, 299,
I-00161 Rome

Birsen Demirata-Ozturk
Technical University Istanbul, Faculty of Science & Letters, Department of Chemistry
TR-34469 Istanbul

Susanna Eerola
National Veterinary and Food Research Institute EELA
Department of Chemistry
P.O. Box 45
FIN-00581 Helsinki

Marinella Farré
Environmental Chemistry
IIQAB-CSIC,
Jordi Girona, 18
S-08034 Barcelona

Dimitrios J. Fletouris,
Department of Food Hygiene and Technology
School of Veterinary Medicine

Aristotle University of Thessaloniki
G-54124 Thessaloniki

Agnes Fekete
GSF-National Research Center for Environment and Health
Institute for Ecological Chemistry
Ingoldstädter Ladstraβe 1
D-85764 Neuherberg

Jana Hajšlová
Institute of Chemical Technology
Faculty of Food and Biochemical Technology
Department of Food Chemistry and Analysis
Techniká 3
CZ-16628 Prague

Susan L. Hefle
Food Allergy Research & Resource Program
University of Nebraska
Lincon
USA 68583-0919 Nebraska

Arne Holst-Jensen
Section of Feed and Food Microbiology
National Veterinary Institute
Ullevaalsveien 68
P.O. Box 8156 Dep.
0033 Oslo, Norway

Marika Jestoi
National Veterinary and Food Research Institute EELA
Department of Chemistry
P.O. Box 45
FIN-00581 Helsinki
marika.jestoi@eela.fi

Ivan R. Kennedy
Faculty of Agiculture, Food and Natural Resources
University of Sydney
NSW 2006, Australia

N. Alice Lee
School of Chemical Engineering and Industrial Chemistry
University of New South Wales
Sydney, NSW 2052, Australia

Wolfgang Luf
Institute of Milk Hygiene, Milk Technology and Food Science
University of Veterinary Medicine
Veterinärplatz 1
A 1210 Vienna

J.G.J. Mol
Cluster Leader Pesticides and Contaminants
RILKILT-Institute of Food Safety
PO Box 230
6700 AE Wageninge, The Netherlands

Manuela Nazzari
Department of Chemistry
Universita degli studi di Roma “La Sapienza”
Ple Aldo Moro 5
I-00185 Roma

Peter Paulsen
Institute of Meat Hygiene, Meat Technology and Food Science
University of Veterinary Medicine
Veterinärplatz 1
A 1210 Vienna

Yolanda Picó
Laboratori de Bromatologia i Toxicologia
Facultat de Farmàcia, Universitat de València

Av. Vicent Andrés Estellés s/n
46100 Burjassot, Valencia, Spain

Mervi Rokka
National Veterinary and Food Research Institute EELA
Department of Chemistry
P.O. Box 45
FIN-00581 Helsinki

Philippe Smith-Kopplin,
GSF-National Research Center for Environment and Health
Institute for Ecological Chemistry
Ingoldstädter Ladstraβe 1
D-85764 Neuherberg

Frans J. M. Smulders
Institute of Meat Hygiene, Meat Technology and Food Science
University of Veterinary Medicine
Veterinärplatz 1
A 1210 Vienna

Steve L. Taylor
Food Allergy Research & Resource Program
University of Nebraska
Lincon
USA 68583-0919 Nebraska

Nilgun Tokman
Technical University Istanbul, Faculty of Science & Art, Department of Chemistry
TR-34469 Istanbul

Adrianus J. Van Hengel
Commission European Communities
DGJ Joint Research Centre
Inst Reference Materials & Measurements,
Retieseweg,
B-2440 Geel

Maria Varadi
Head of Unit
Central Food Research Institute (KEKI)
P.O. Box 393
H-1537 Budapest

Roger Wood
Food Standards Agency
c/o Institute of Food Research,
Norwich Research Park,
Colney,
Norwich NR4 7UA,UK

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 1

The international regulation of chemical toxicants in food. Codex Alimentarius

Maria Varadi

Central Food Research Institute, Herman Ottó u. 15., Budapest 1022, Hungary

1. General principles of Codex Alimentarius

The Codex Alimentarius Commission is the international body established in 1962 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to implement the Joint FAO/WHO Food Standards Programme. The Statutes of the Codex Alimentarius Commission have been approved by the Governing bodies of the FAO and WHO, it is a subsidiary body of these two parent organizations.

The aim of the Food Standards Programme is to protect the health of consumers and to ensure fair practices in the food trade, to promote coordination of all food standards work undertaken by international governmental and non-governmental organizations, to determine priorities and initiate and guide the preparation of draft standards through and with the aid of appropriate organizations, to finalize standards, and, after acceptance by governments, publish them in a Codex Alimentarius either as regional or world-wide standards, to amend published standards, after appropriate survey in the light of developments.

The Codex Alimentarius (Latin, meaning Food Law or Code) is a collection of international food standards, codes of practice, guidelines and other recommendations in a uniform manner. It is a reference point for facilitating international trade and resolving trade disputes in international law. It includes standards for all the principal foods, whether processed or raw. Materials for further processing into foods are included to the extent necessary to achieve the purposes of the Codex Alimentarius. It contains provisions relating to the hygienic and nutritional quality of food, microbiological norms, provisions for food additives, pesticide residues, contaminants, labelling and presentations, and methods of analysis and sampling.

The food standards, guidelines and other recommendations of Codex Alimentarius shall be based on the principle of sound scientific analysis and evidence, involving a thorough review of all relevant information in order that the standards assure the quality and safety of the food supply.

Creating Codex documents, the international trade agreements are taken into consideration. They are presumed to meet the requirements of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) and Agreement on Technical Barriers to Trade (TBT). Both the SPS and TBT Agreements acknowledge the importance of harmonizing standards

internationally so as to minimize or eliminate the risk of sanitary, phytosanitary and other technical standards becoming barriers to trade. With a view to harmonization in food safety, the SPS Agreement has identified and chosen the standards, guidelines and recommendations established by the Codex Alimentarius Commission for food additives, veterinary drug and pesticide residues, contaminants, methods of analysis and sampling, and codes and guidelines of hygienic practice.

2. Operation of Codex Alimentarius

The Codex Alimentarius Commission is responsible for making proposals and consulting with the Directors-General of the FAO and WHO on all matters pertaining to the implementation of the Joint FAO/WHO Food Standards Programme.

The Commission normally meets every year, alternately at FAO headquarters in Rome and at WHO headquarters in Geneva. Between sessions, the Executive Committee acts as the Executive organ of the Commission. In the Executive Committee an adequate representation is ensured by the various geographical areas of the world to which the Members of the Commission belong. Membership of the Commission is open to all Member Nations and Associate Members of FAO and WHO, which are interested in international food standards. Non-Governmental Organizations can also participate in the work of the Codex Alimentarius in order to ensure expert information, advice and to harmonize intersectoral interest among the various sectoral bodies concerned in a country, and to represent the public opinion.

The Commission is empowered to establish two kinds of subsidiary body: the Coordinating Committees and Codex Committees. Coordinating Committees for regions or groups of countries coordinate food standards activities in the region, including the development of regional standards.

Codex Committees, including General Subject Committees and Commodity Committees, are responsible for initiating, preparing, discussing and elaborating the standards, guidelines, etc. General Subject Committees are sometimes called as horizontal committees, because they develop all-embracing concepts and principles applying to foods in general, specific foods or groups of foods, endorse or review relevant provisions in Codex commodity standards. These Committees also develop standards, codes of practice or other guidelines for either general application or in specific cases where the development of a complete commodity standard is not required and set maximum limits for additives and contaminants. The Committee on Pesticide Residues and the Committee on Residues of Veterinary Drugs in Foods prepare MRLs for these two categories of chemicals used in agricultural production.

The Commodity Committees, so-called vertical Committees, are responsible for developing standards for specific foods or classes of food. (E.g. Committee on Fats and Oils, on Fish and Fishery Products, on Milk and Milk Products, on Fresh Fruits and Vegetables, on Processed Fruits and Vegetables etc.). Codex Commodity standards contain sections on hygiene,

labelling and methods of analysis and sampling. Provisions of Codex General Standards, Codes or Guidelines are incorporated into Codex Commodity Standards by reference unless there is a need for doing otherwise. In this case the responsible Committees need to endorse deviations from the general provisions. It should be fully justified and supported by available scientific evidence and other relevant information.

Because the Commission realized that its rather inflexible committee structure was not able to cope with the demand for standards and guidelines across an ever-widening range of subjects established a third type of subsidiary body called ad hoc Intergovernmental Task Forces. They exist for a fixed period of time with very limited terms of reference. (E.g. Task Force on Food Derived from Biotechnology, 1999-2003, 2005-2009).

The work of the Codex Alimentarius is supported by FAO/WHO expert bodies and expert meetings and consultations, which are independent of the Commission. The experts invited to participate, are pre-eminent in their specialty, have the highest respect of their scientific peers, and they are impartial and indisputably objective in their judgement. Experts contribute in their own capacity and not on behalf of a government or institution.

Two such groups, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meetings on Pesticide Residues (JMPR), have for many years produced internationally acclaimed data. JECFA was established to consider chemical, toxicological and other aspects of contaminants and residues of veterinary drugs in foods for human consumption. The Codex Committee on Food Additives and Contaminants (CCFAC) and the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVD) identify food additives, contaminants and veterinary drug residues that should receive priority evaluation and refer them to JECFA for assessment before incorporating them into Codex standards. JMPR recommends maximum residue limits (MRLs) for pesticide and environmental contaminants in specific food products to ensure the safety of foods containing residues. Methods of analysis and sampling are also recommended by JMPR. The Codex Committee on Pesticide Residues (CCPR) identifies those substances requiring priority evaluation. After JMPR evaluation, CCPR discusses the recommended MRLs and forwards them to the Commission for adoption as Codex MRLs.

In 2000 JEMRA, the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment, began its work. JEMRA aims to optimize the use of microbiological risk assessment as the scientific basis for risk management decisions that address microbiological hazards in foods.

Codex encourages other scientifically based intergovernmental organizations to contribute to the joint FAO/WHO work. E.g. the International Atomic Energy Agency (IAEA), the World Organisation for Animal Health (OIE), the International Standardization Office (ISO), provide advices for different Codex Committees.

3. Decision making and enforcement mechanisms in Codex Alimentarius

Most standards take a number of years to develop. The Codex standards process consists of eight steps. Before a decision is made for developing a new standard or other text, first at a Committee level a discussion paper, then a project proposal is prepared which is discussed by the Committee. At step 1 the project proposal is critically reviewed by the Executive Committee taking into consideration the criteria and priorities established by the Commission. At steps 2-3 a draft is prepared and circulated to member countries and all interested parties for comments. At step 4 the draft and the comments are discussed at Committee level. At step 5 the Commission makes a decision that the draft should go to finalization. Then the draft is also endorsed by the relevant General Subject Committees. At steps 6-7 the draft is sent again to governments and interested parties for comment and is finalized by the relevant Committee. Then it is submitted to the Commission for adoption, as a formal Codex text (step 8). The standard, guideline or other text is then published by the Codex Secretariat.

4. Codex standard for contaminants and toxins in foods *CODEX STAN 193-1995 (Rev.1-1997)*

4.1. Scope

This Standard contains the main principles and procedures that are recommended by the Codex Alimentarius concerning contaminants and toxins in foods and feeds, and lists the maximum levels of contaminants and natural toxicants in foods and feeds which are recommended by the CAC to be applied in international trade.

4.2. Definition of terms

4.2.1. Contaminant

Codex Alimentarius defines a contaminant as follows:

"Any substance not intentionally added to food, which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or as a result of environmental contamination. The term does not include insect fragments, rodent hairs and other extraneous matter".

The standard does not include the substances, which are within the terms of reference of CCPR, CCRVDF and CCFH, as well as contaminants and processing aids having no public health significance.

4.2.2. Natural toxins

The Codex definition of a contaminant implicitly includes naturally occurring toxicants, which comprisse.

Mycotoxins, which are produced as toxic metabolites of certain microfungi that are not intentionally added to food.

Microbial toxins are those that produced by algae and may be accumulated in edible aquatic organisms such as shellfish (phycotoxins) are also included in this standard. Mycotoxins and phycotoxins are both subclasses of contaminants.

Inherent natural toxicants that are implicit constituents of foods resulting from a genus, species or strain ordinarily producing hazardous levels of a toxic metabolite(s), i.e. phytotoxins are not generally considered within the scope of this standard. They are, however, within the terms of reference of the CCFAC and will be dealt with on a case-by-case basis.

4.2.3. Maximum level

The ***Codex Maximum Level (ML)*** for a contaminant in a food or feed commodity is the maximum concentration of that substance which is legally permitted in that commodity by the CAC.

A ***Codex Guideline Level (GL)*** is the maximum level of a substance in a food or feed commodity, which is acceptable in international trade. When the GL is exceeded, governments should decide whether and under what circumstances the food should be distributed within their territory or jurisdiction.

4.3. General principles regarding contaminants in food

4.3.1. General

Foods and feeds can become contaminated by various causes and processes. Contamination generally has a negative impact on the quality of the food or feed and may imply a risk to human or animal health.

Contaminant levels in foods shall be as low as reasonably achievable. For ensuring this purpose the following actions are very important: preventing the contamination of raw materials at the source, and applying appropriate technology in agricultural and food production, handling, storage, processing, packaging and distribution. It is necessary to elaborate a Code of Practice and Good Manufacturing Practice as well as Good Agricultural Practice for the specific contamination problem.

For all contaminants, which may be present in more than one food or feed item, a broad approach is applied for the assessment of risks and for the development of recommendations and measures, including the setting of maximum levels.

4.3.2. Principles for establishing maximum levels (MLs) in foods and feeds

Maximum levels are to be determined only for those foods in which the amount of contaminants is significant for the total exposure of the consumer. It should be based on sound scientific principles leading to levels that are acceptable world wide.

4.3.3. Specific criteria

The following criteria should be taken into account for preparing the Codex General Standard for Contaminants in Food: Toxicological information, Analytical data, Intake data, Fair trade considerations, Technological considerations, Risk assessment and risk management considerations.

MLs shall be set as low as reasonably achievable. Providing it is acceptable from the toxicological point of view, MLs shall be set at a level that is (slightly) higher than the normal range of variation in levels in foods that are produced with current adequate technological methods in order to avoid undue disruptions of food production and trade. Where possible, MLs shall be based on GMP and/or GAP considerations in which the health concerns have been incorporated as a guiding principle to achieve contaminant levels as low as reasonably achievable.

Proposals for MLs in products shall be based on data from various countries and sources, encompassing the main production areas/processes of those products, as far as they are engaged in international trade. When there is evidence that contamination patterns are sufficiently understood and will be comparable on a global scale, more limited data may be enough.

MLs should not be lower than a level which can be analyzed with methods of analysis that can be readily applied in normal product control laboratories, unless public health considerations necessitate a lower detection limit, which can only be controlled by means of a more elaborate method of analysis. In all cases, however, a validated method of analysis should be available with which a ML can be controlled.

4.4. Codex procedure for establishing standards for contaminants in food

4.4.1. Procedure for preliminary discussion about contaminants in the CCFAC

An initial discussion may be held based on oral contributions, but preferably on the basis of a note containing relevant and adequate information.

4.4.2. Procedure for risk management decisions in the CCFAC regarding contaminants

An evaluation by JECFA of the toxicological and other aspects of a contaminant and subsequent recommendations regarding the acceptable intake and maximum levels in foods shall be the main basis for decisions to be discussed by the CCFAC. In the absence of

recommendations by JECFA, decisions may be taken by CCFAC when sufficient information from other sources is available to the Committee and the matter is considered urgent.

4.5. Format of the standard for contaminants in food

4.5.1. Types of presentation for the standards

The General Standard for Contaminants in Foods contains two types of presentation for the Standards: Schedule I in which the standards are listed per contaminant in the various food categories, and Schedule II in which the contaminant standards are presented per food (category). For each session of the CCFAC, a working document shall be prepared in which the complete list of Codex Standards for contaminants in foods (both proposed and agreed) is presented in the form of Schedule I. The list of Codex contaminant standards for individual foods or food categories is presented according to an agreed food categorization system.

4.5.2. Food categorization system

It has a logical structure, which enables a clear and systematic presentation of the (proposed) MLs. It contains (references to) product definitions and definitions of the part of the product which is analyzed and to which the ML refers. It contains codes for the food categories and the individual foods, so that data can be stored and retrieved in a convenient way.

The Codex General Standard for Contaminants and Toxins in Foods (GSC) uses the system which is developed in the framework of the CCPR, as it is also suitable for contaminants. It is adopted for characterizing the various food and feed groups and the individual commodities. This system is especially elaborated regarding primary agricultural commodities, but needs further extension regarding processed products.

Where necessary, new (sub)group codes or commodity codes are therefore introduced. Where appropriate and possible, the descriptive texts accompanying the food categories do or should also contain indications about the concentration or dilution factor in the processed commodities mentioned in relation to the primary product(s) involved. In that way, a first estimate can be made of the possible carryover of contaminants from primary products to the various processed products. The specific distribution of a contaminant in the primary product and the behaviour during processing are complicating factors.

4.5.3. Description of the food categorization system of the GSC

The first part contains the categorization system as developed and maintained by the CCPR. It consists of 5 classes, covering primary food commodities of plant, resp. animal origin, primary feed commodities and processed commodities of plant, resp. animal origin. The classes are subdivided in 19 types and 93 groups, which are identified by code numbers and letters.

Reference is made to Vol. 2 of the Codex Alimentarius, section 2 (1993), in which this system is described, and to CX/PR 92/6 (in which a different kind of group numbering was introduced).

The second part of the food categorization system for the GSC is developed and maintained by the CCFAC, and is complementary to the system described in the first part. It is mainly directed to processed, derived and multi-ingredient foods and encompasses all those types and groups and commodity descriptions that are necessary to assign food categorization codes to existing or planned Codex MLs for contaminants.

4.6. Review and revision of the standard

The contaminant provisions for this Standard shall be reviewed on a regular basis and revised as necessary in the light of revisions of toxicological advice by JECFA or of changed risk management views, residue management possibilities, scientific knowledge or other important relevant developments.

Specific attention shall be given to the review of existing Maximum Levels and Guideline Levels and to their possible conversion to Maximum Levels.

4.7. Other codex standards and guidelines regarding contaminants in food

The Codex has been continuously creating and revising standards, guidelines, which define maximum limits and analytical methods for contaminants. Tables 1-4 provide some examples of these documents.

Table 1. Codex Standards for maximum contaminant levels

Number	Revision	Name
CODEX STAN 230-2001	1-2003	Maximum levels for lead
CODEX STAN 232-2001	-	Maximum level for aflatoxin M1 in milk
CODEX STAN 235-2003	-	Maximum level for patulin in apple juice and apple juice ingredients and other beverages
CODEX STAN 248-2005	-	Maximum levels for cadmium
CAC/MRL 02-2005	-	Maximum residue limits for veterinary drugs in foods

Table 2. Codex Guideline levels for contaminants

Number	Revision	Name
CAC/GL 5-1989	-	Guideline levels for radionuclides in foods following accidental nuclear contamination for use in international trade
CAC/GL 7-1991	-	Guideline levels for methylmercury in fish
CAC/GL 6-1991	-	Guideline levels for vinyl chloride monomer and acrylonitrile in food and packaging material
CAC/GL 39-2001	-	Codex maximum level for cadmium in cereals, pulses and legumes

Table 3. Other Codex Guidelines

Number	Revision	Name
CAC/GL 28-1995	*1-1997*	Food control laboratory management: recommendations
CAC/GL 33-1999	-	Recommended methods of sampling for the determination of pesticide residues for compliance with mrls
CAC/GL 40-1993	*1-2003*	Guidelines on good laboratory practice in residue analysis
CAC/GL 49-2003	-	Harmonized iupac guidelines for single-laboratory validation of methods of analysis

These documents are available on the official website of CODEX Alimentarius (http://www.codexalimentarius.org/).

In addition to the above standards and guidelines, numerous other publications such as proceedings of joint FAO/WHO meetings, reports, etc., provide a source of relevant and up-to-date information for decision makers, producers and food processors as well as consumers.

Table 4. Codex Gereral Standards

Number	Revision	Name
CODEX STAN 1-1985	*1-1991*	General standard for the labelling of prepackaged foods
CODEX STAN 193-1995	*1-1997*	Codex general standard for contaminants and toxins in foods
CODEX STAN 228-2001	-	General codex methods for contaminants
CODEX STAN 229-1993	*1-2003*	Analysis of pesticide residues: recommended methods
CODEX STAN 234-1999	-	Recommended methods of analysis and sampling

References

World Health Organization and Food and Agriculture Organization of the United Nations: Understanding the Codex Alimentarius (2005)

Codex Alimentarius Commission, Procedural Manual, Fifteenth Edition (2005)

Codex Alimentarius, General Requirements, Volume 1A, Second Edition (Revised, 1999)

Codex Alimentarius, Pesticide Residues in Food – Maximum Residue Limits, Volume 2B (2000)

Codex Alimentarius, Pesticide Residues in Food, Methods of Analysis and Sampling, Volume 2A Part 1, Second Edition (2000)

Codex Alimentarius, Residues of Veterinary Drugs in Foods, Volume 3, Secon Edition (Revised 1995)

Chapter 2

Different legislations on toxicants in foodstuffs

Peter Paulsen, Wolfgang Luf and Frans J. M. Smulders

Department of Veterinary Public Health and Food Science, University of Veterinary Medicine,Veterinärplatz 1, A 1210 Vienna, Austria

1. Food toxicants and Food safety

1.1. Nature of toxicants

Safety is the primary requirement to be met before foods can be placed on the market. A variety of agents can compromise food safety. These agents can, according to the hazard analysis and critical control point (HACCP) schemes, be grouped into physical, biological and chemical hazards [1]. Foodborne toxicants, i.e. chemical compounds causing adverse health effects to the consumer, generally fall in the latter two categories. Among the toxicants of biological origin, microbial toxins (e.g. those formed by *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens*, the variety of mycotoxins), as well as toxins formed by plants (algal toxins in bivalves) and animals (tetrodotoxin in fish), and finally, chemicals formed by enzymatic reactions (histamine) are of relevance. These toxins may naturally be present in the raw food or may be formed by the activity of contaminant bacteria. Chemical hazards may also (naturally or as a result of contamination) be present in the (raw) food, or in some of the ingredients. Moreover, some additives have toxic potential *per se*. Some toxic compounds are known to be formed during food processing (acrylamide, benzo-a-pyrene).

Despite a number of “new” food safety issues as BSE (bovine spongiform encephalopathy) and antimicrobial resistant bacteria, chemical hazards still represent a major issue in the consumers´ perception of food safety [2]. Some chemical hazard are not directly adverse to human health, but may trigger public health threats, as antimicrobial residues do for the emergence of antibiotic-resistant bacteria [3,4].

“Natural” or “traditional” products or processing techniques (e.g. nitrite curing [5], special smoking techniques [6]) are not necessarily free from potentially toxic compounds, and some food additives are approved, because, when used in appropriate concentrations their protective effects are more important than their (potential) adverse health effects.

1.2. Occurrence and control of toxicants along the food chain

A variety of toxicants can be introduced or formed at practically all stages of food production. Even a quite simple scenario, where one contaminated raw material is further processed, can be difficult to control when this raw material is processed to animal feed by a

number of different companies upon being distributed to a number of farms. This “spreading” could be observed in the “dioxin crisis” in the European Union (EU) in 1999 [7], where contaminated fat was produced in one rendering plant, which supplied nine feed establishments. This feed was used in 1828 food-producing farms, i.e.102 cattle farms, 239 dairy farms, 431 poultry farms and 1056 pig farms. Notably, this incident was a major motivation to have a new view of food safety in the EU (“White Paper on Food Safety”).

Thus, it is essential, that, throughout the complex process of food production, distribution, and preparation (“food chain”), each stakeholder pro-actively takes preventive measures to control risks associated with food toxicants; and that he can rely on the preceding stakeholders having done the same. Process control, preventive measures, appropriate documentation, information flow and rapid communication are main pillars for food safety. For a variety of reasons, testing foods for toxicants by official authorities on a routine basis can be done to a limited extent only, and will often be employed merely to verify that process control and prevention strategies were operational.

This has important consequences for food safety legislation. With respect to compounds with (potential or factual) toxicity, legislation will have to cover: registration/approval of chemicals and regulations on their use; establishment of limits; monitoring programs; guidelines for the prudent use of chemicals in the food sector and prevention of contamination [2]. These legal provisions shall ensure not only domestic consumers´ protection, but also are also oriented towards international trade agreements. This requires some harmonisation at the regulatory level, which includes: mutual recognition of legislation, codes of practices and enforcement procedures; demonstration that these measures provide an equivalent level of food safety; and reference standards [8].

This chapter gives an introduction into the structure of food safety/toxicant legislation, with a focus on the EU and the USA.

2. European Union

The EU legislation on food safety is complex. Despite the efforts in harmonizing and simplifying legislation, differences between member states may exist due to differences in the implementation of EU Directives, or because of national derogations to (principally binding) EU Regulations. The structures at EU level: legislative bodies (Commission, Standing Committee on the Food Chain and Animal Health), expert groups (European Food Safety Authority) and enforcement agencies (Food and Veterinary Office) are paralleled by comparable institutions in the member states. Cooperation between EU and national authorities is governed by Council Directive 93/5/EEC, as amended, with a number of implementing decisions.

Changes in legislation result in repealing or amending existing legal texts. Although these texts are made publically available (under the framework of the “Official Journal”; in print or

at the EU web server at http://europa.eu), sometimes it is not easy to follow what documents are valid. Thus, the CONSLEG service at the web server provides (non- binding) consolidated legislation also.

EU legislation can be addressed in three main sections: a) the general framework represented by the EU White Paper on Food Safety, b) Food toxicants as addressed in the "new" EU food hygiene legislation, and c) legal texts regarding specific toxicants.

2.1. The EU White Paper on Food Safety

Quite recently, the EU Commission has defined food safety as a key policy priority, to ensure free movement of safe and wholesome food on the internal market. Thus, a new approach has been chosen, following the principles laid down in the EU White Paper on Food Safety [COM (1999) 719 final], and the Green Paper on Food Law [COM (1997) 176 final]. The principles of this approach [9] are:

- Food safety policy has to include the whole food chain from producers to consumers ("from stable to table"), in a uniform and consistent manner in all member states.
- The responsibilities for all stakeholders in the food chain are clearly defined. In particular, the primary responsibility for production of safe foods rests with the feed producers, farmers and food business operators. The consumer, being the ultimate stakeholder in the food chain, is assigned some responsibility for safe storage, handling and processing of food.
- National authorities implement appropriate official controls, to ensure that these responsibilities are met. National control systems are inspected by the European Commission. This ensures that the level of food safety is uniform in all member states, and enables free movement of food in the Community.
- Traceability of feed and food serves food safety in two ways, when health risks are identified in food: first, the risk can be traced back to its origin, and, second, the identification and withdrawal of suspicious batches is facilitated.
- Food safety policy will be based on risk analysis, which means, that decisions on food safety are based on a scientific evaluation of the risk (identification and characterization of the hazard, and assessment of the actual risk associated with it), with measures that effectively manage the risk, and that the relevant information is exchanged between all involved parties ("risk communication").
- In case of potential health or environmental risks, which (due to a lack of scientific data) cannot be fully evaluated, measures will be taken based on the precautionary principle. The conditions for application and the resulting measures are clearly defined [The precautionary principle; COM (2000) 1 - final], to avoid that this principle is abused to justify arbitrary decisions or protectionist measures.
- Food safety legislation also considers other (non-safety) issues related with food production, where necessary. This includes animal welfare, ecology, sustainable

management of natural resources, but also consumers´ expectations of food quality, and characteristics of different food types.

- The European Food Safety Authority provides independent scientific advice on all aspects relating to food safety.
- Food safety must be dealt with in a transparent way - which means that relevant information must be accessible not only for EU authorities and consumers, but also for third countries.

Food safety policy is based on scientific advice. Relevant information is generated in national laboratories and institutions as well as in European reference laboratories. Emphasis is put on cooperation and information exchange between these institutions.

Given this community framework for the development and operation of national control systems, Member States have to ensure that there are only small variations in the manner in which Community legislation is being implemented and enforced. The Food and Veterinary Office (FVO) determines whether or not national implementation is done correctly and also performs checks of registered importers in third countries. Controls on imports at the borders of the Community will cover all feed and foodstuffs.

The European Food Safety Authority (EFSA) was created with a view to risk assessment and risk communication. The EFSA is independent from economic and political bodies, and provides information (e.g. food safety, animal and plant health, GMOs, nutrition) for the decision-makers (EU Commission, national authorities) on a level of excellence. It is also responsible for operation of rapid alert systems, ensures communication and dialogue with consumers on food safety and health issues as well as networking with national agencies and scientific bodies. The EFSA cooperates with EU reference laboratories and Joint Research Centers as well as with national laboratories.

Finally, the process of risk assessment and decisions on risk management must be transparent and information must be made accessible to the consumer. Also, consumers need to be informed on improvements in food safety standards, and there must be an information system in case of emerging food safety risks. Adequate and clear labelling of foods can keep the consumers informed about food constituents (e.g. allergens), which are a hazard for specific consumer groups only.

Based on these considerations, the White Paper on Food Safety proposed a number of implementing “actions”.

Figure 1 summarizes the fundamentals of the EU´s food safety approach and the institutional framework.

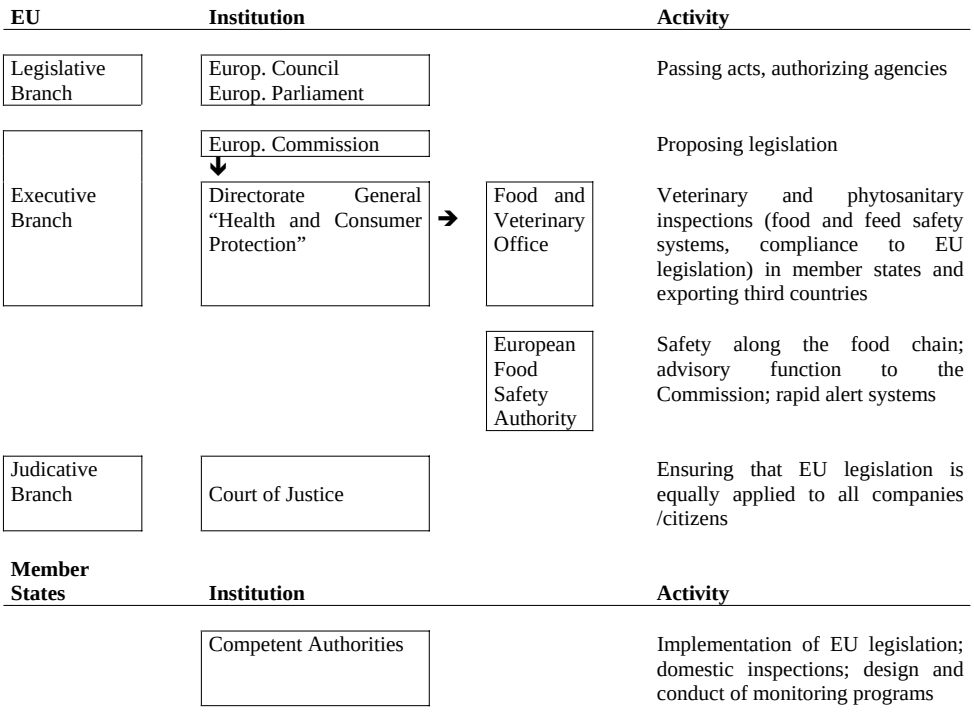


Figure 1: Schematic diagram of EU Institutions and Agencies Concerned with Food Safety and Food Toxicants

2.2. Food toxicants as addressed in the "new" EU food hygiene legislation and related legal texts

The Regulation (EC) No 178/2002 ("General Food Law") constitutes the base for feed and food hygiene legislation. The essentials of this regulation are:

- Decisions on food safety (legislation and control = risk management) are based on risk assessment, or, when data are insufficient for risk assessment, and on a provisional basis, the precautionary principle (articles 6,7)
- Food/feed imports must comply with EU food law or with equivalent conditions. In turn, food and feed exports must comply with EU standards, unless otherwise requested by the importers (articles 11,12).
- To ensure a high level of consumers health protection, only safe foods are allowed to be put on the market (article 14). The production of safe foods implies that only safe feedstuffs were used (article 15).

- The feed and food business operators bear the responsibility that the legal requirements are met. Official controls ensure that the responsibilities are correctly served (article 17).
- The feed and food business operators must establish a system which allows to trace back all components to their origin ("one step down - one step up", article 18). When necessary, these data are made accessible to the competent authority.
- The primary responsibility for feed and food rests with the producers; it is, therefore, also their duty to withdraw unsafe (batches of) feed or food from the market (articles 19,20).
- The objectives and structure of the EFSA are laid down (articles 22-49).
- Food- or feedborne health risks are reported to the Commission via a (internet based) rapid alert system, which disseminates the information to member states and the competent authorities. By this system, the member states also inform the Commission on measures taken to control or prevent foodborne health risks. Special procedures are provided for emergencies (articles 50-57), when serious risks cannot be controlled by the concerned member states. In this case, the Standing Committee on the Food Chain and Animal Health may adopt measures. Measures taken directly by the Commission are provisional. When necessary, the Commission, with participation from the EFSA, sets up a crisis unit, to collect and evaluate information and to identify options to control the health risk.

With the beginning of 2006, three main regulations came into force, concerning the hygiene of foodstuffs [Regulation (EC) No 852/2004], specific hygiene rules [Regulation (EC) No 853/2004], and specific rules for the organization of official controls on products of animal origin intended for human consumption [Regulation (EC) No 854/2004]. These regulations implement the general considerations on food safety described above, starting from primary production. There are only few exceptions (e.g. primary production for private domestic use, or domestic preparation, handling or storage of food for private domestic consumption) and provisions for flexibility for traditional products/processes or in case of regional constraints. The identification and control of food hazards shall be based on HACCP principles, or, when not feasible in the area of primary production, on guides of good practice. To reduce contamination of foods, already existing legislation laying down Community procedures for contaminants in food [Council Regulation (EEC) No 315/93] has to be considered as well as new standards have to be defined.

Regulation (EC) No. 852/2004 explicitly addresses food toxicants with respect to primary production (Annex I). Thus, food business operators must comply with community and national legislation, including "measures to control contamination arising from …. veterinary medicinal products, plant protection products and biocides …". The correct use of feed additives and veterinary medicinal products, as well as the correct use of plant protection products and biocides has to be ensured and documented (e.g. in animal production:

treatment, veterinary medicinal product, withdrawal period, and dates of administration) and by the food business operator (Part A). The compliance with food hygiene legislation and the implementation of HACCP are strengthened by EU guidances as well as national guides to good hygiene practice. "Recommendations for Guides to Good Hygiene Practice" (Part B) should include "appropriate information on hazards that may arise in primary production and associated operations and actions to control hazards, including relevant measures set out in Community and national legislation or national and Community programmes. Examples of such hazards and measures may include: (a) the control of contamination such as mycotoxins, heavy metals and radioactive material; (b) the use of water, organic waste and fertilisers; (c) the correct and appropriate use of plant protection products and biocides and their traceability; (d) the correct and appropriate use of veterinary medicinal products and feed additives and their traceability;…".

General hygiene requirements for food businesses other than primary production (Annex II) lay down that surfaces (of rooms, equipment...) coming in contact with food must be non-toxic. The food business operator must not use raw materials or ingredients, other than live animals (or any other material used in processing products) for food production, when these materials are known to be contaminated with "toxic, decomposed or foreign substances" (or when it is reasonable to expect that this is the case) "to such an extent that, even after the food business operator had hygienically applied normal sorting and/or preparatory or processing procedures, the final product would be unfit for human consumption". Food must be prevented from contamination and storage conditions must ensure that no microbiological toxins are formed.

The regulation (EC) No 853/2004 provides additional specific legislation for the hygiene of food of animal origin. It is the responsibility of the food business operator that

- animals for slaughter are accompanied by information about "veterinary medicinal products or other treatments administered to the animals within a relevant period and with a withdrawal period greater than zero, together with their dates of administration and withdrawal periods...";
- wild game are not marketed as food when environmental contamination is suspected;
- with respect to gelatin and collagen, residue limits defined for a number of metals (As, Pb, Cd, Hg, Cr, Cu, Zn) are not exceeded;
- toxic fish (families Tetraodontidae, Molidae, Diodontidae and Canthigasteridae) are not put on the market;
- bivalve molluscs contain no marine toxins [poisonous substances accumulated by bivalve molluscs, in particular as a result of feeding on plankton containing toxins: PSP, ASP, okadaic acid, yessotoxins, azaspiracids – for recognized and alternative testing methods, see Commission Regulation (EC) No 2074/2005] exceeding certain limits. A number of measures assures that these requirements are met: the bivalve molluscs must originate from registered, classified relaying and production areas; the presence of toxin-producing plankton in production and relaying waters, of biotoxins

in live bivalve molluscs and the presence of chemical contaminants [with respect to limits laid down in Commission Regulation (EC) No 466/2001] in live bivalve molluscs is to be monitored. Detailed provisions are made for sampling to obtain representative results; frequency and intensity of sampling are based on risk assessment.

Official control for feedstuffs and food are specified in Regulation (EC) No 882/2004. The regulation (EC) No 854/2004 provides specific rules for the organization of official controls on products of animal origin intended for human consumption. These controls shall verify that food business operators comply with hygiene rules, fulfill criteria and targets laid down in Community legislation, and maintain a functional self control system. HACCP-based procedures are audited, on whether they can guarantee, to the extent possible, that products of animal origin comply with Community legislation on residues, contaminants and prohibited substances. Referring recommendations for various slaughter animal species have been designed by Bolton et al. [10].

When Guides on Good Hygiene Practice are used, their correct application is audited. In fresh meat inspection, the official veterinarian has to conduct sampling in the framework of the detection of unauthorized substances or products and the control of regulated substances, in particular within the framework of the National Residue Plans referred to in Council Directive 96/23/EC.

2.3. Legal texts dealing with specific substances with possible or factual adverse health effects

A number of legal provisions address specific toxic substances in foods. With respect to drugs (Council Directive 96/23/EC), pesticides [authorized substances: Directive 91/414/EEC; banned substances: Council Directive 79/117/EEC; maximum residue levels : European Parliament and Council Regulation (EC) No 396/2005], food additives [approved substances: Council Directive 89/107/EEC; exemptions for national traditional products: European Parliament and Council Directive 94/34/EC; approval of novel food/ novel food additives: Regulation (EC) No 258/97 and GMOs: Regulation (EC) No 1829/2003], a prior authorization system for placing products on the market exists. Their use has to be documented. Control of these documentations and monitoring programs shall ensure that food businesses comply with legislative requirements.

Some bacterial toxins and marine biotoxins are addressed in Regulation (EC) No 2073/2005 and Regulations (EC) No 853, 854/2004, respectively.

2.3.1. Toxins of bacterial origin

Legal provisions and limits exist for staphylococcal enterotoxins (cheeses, milk powder and whey powder) and histamine (in fishery products from species associated with high tissue levels of histidine - *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae,*

Scombresosidae), Regulation (EC) No 2073/2005. This regulation uses the term "criterion" to concisely contain:

- Which is the agent of concern?
- What limit applies at what stage of the food chain (e.g. food placed on the market during shelf-life)?
- What analytical method to be used (ISO reference methods)?
- Consequences when results are not in compliance.

2.3.2. Contaminants, residues, food additives and substances formed during food processing

EU legislation on food contaminants is laid down in a framework regulation, i.e. Council Regulation (EEC) No 315/93. No foodstuffs containing a toxicologically unacceptable amount of a contaminant may be marketed. Contaminants, i.e. any substance unintentionally added to food and present therein in the form of a residue from production, manufacture, processing, preparation, treatment, packaging, transport or storage or as a result of environmental contamination, must be kept at the lowest possible levels. To harmonize different legislation in EU member states, and to prevent the risk of distortion of competition, measures were introduced by Commission Regulation (EC) No 466/2001, setting maximum levels for certain contaminants in foodstuffs. Maximum levels are set for environmental contaminants (lead, cadmium, mercury, dioxins, nitrate), process contaminants (3-monochloropropane-1,2diol: 3-MCDP), mycotoxins (aflatoxins, ochratoxin A, patulin) and inorganic tin. This legislation is periodically reviewed, and revised/amended, if necessary.

Major amendments to this regulation are (listing not exhaustive):

- Commission Regulation (EC) No 123/2005 to establish maximum levels for ochratoxin A, and Commission Directive 2005/5/EC defining sampling methods and analytical procedures for purposes of official control;
- Commission Regulations 257/2002, 472/2002 and 2174/2003 on aflatoxins in nuts, dried fruit, cereals, maize and milk and Commission Directive 98/53/EC defining sampling methods and analytical procedures for purposes of official control;
- Commission Regulation (EC) No 1425/2003 addressing the daily uptake limits for patulin;
- Commission Regulation (EC) No 78/2005 with maximum levels for lead, cadmium and mercury in fish;
- Commission Regulation (EC) No 242/2004, to establish limits for inorganic tin in canned foods and beverages;
- Commission Regulation (EC) No 1822/2005, with definitions of the periods when maximum limits for nitrate in fresh spinach apply, and derogations for certain member states;
- Commission Regulation (EC) No 655/2004, establishing limits for nitrate concentrations in food for babies and infants;

- Commission Regulation (EC) No 208/2005 for maximum levels of polycyclic aromatic hydrocarbons (in particular, benzo(a)pyrene) in foodstuffs (oils and fats for human consumption, foods for babies, infants, young children, smoked meat/meat products, muscle tissue of smoked fish/fishery products), and Commission Directive 2005/10/EC on sampling and analytical methods for purposes of official control;
- Commission Directive 2005/4/EC on sampling and detection methods for heavy metals and 3-MCPD.

Related legislation includes:

- Regulation (EC) No 2375/2001 specifying tolerable weekly doses for the foodborne uptake of dioxins and dioxin-type polychlorobiphenyls (PCB);
- Regulation (EC) No 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in products of plant and animal origin. The maximum "default" pesticide residue level in foodstuffs was set to 0.01 mg/kg. This MRL applies to foodstuffs pesticides, when no other limits are set explicitly. Directive 2002/63/EC establishes Community methods of sampling for the official control of pesticide residues in and on products of plant and animal origin.
- In Council Directive 96/22/EC the use of certain substances having a hormonal (estradiol 17ß and its ester-like derivatives) or thyrostatic action (stilbenes, stilbene derivatives, their salts and esters) and of beta-agonists in stockfarming was prohibited, and the use of hormones (progesterone, testosterone, trenbolone acetate, zeranol and melengestrol acetate) provisionally banned. Council Directive 96/23/EC of 29 April 1996 establishes measures to monitor certain substances and residues thereof in live animals and animal products. From the practical viewpoint, some shortcomings in the current system have been addressed by Stephany [11]: The reliability of the results could be improved; although sufficient analytical capacity exists for analysis of hormonal residues in non-edible sample material, analyses of edible products are far less common; reliable data on the levels on xenobiotic as well as natural hormones in different foods are scarce, which makes a risk assessment based approach (exposure assessment) complicated.
- Council Directive 96/23/EC lays down measures to monitor certain substances and residues thereof (forbidden substances, as stilbenes, antithyroid agents, steroids, resorcylic acid lactones inluding zeranol and beta-agonists; veterinary drugs and contaminants: antibacterial substances and a number of other veterinary drugs; organochlorine and organophosphorous compounds, chemical elements and mycotoxins) in live animals and animal products. Based on general rules for sampling plans, the member states develop national sampling plans, which are subject to approval by the commission.

Food additives, i.e. substances which are added during production of food and are a part of the finished product, but usually are not consumed as food itself, are subject to prior

authorization, with exception of minerals, trace elements etc. [12]. Based on Council Directive 89/107/EEC,

- additives, the use of which is authorized, are explicitly listed ("positive list");
- the use of additives is regulated, with respect to: foodstuffs to which these substances may be added; conditions of use;
- maximum levels (this requires also a classification of the technological function of the compound) of additives in marketed foodstuffs, or, alternatively, the *quantum satis* usage (dosage is an issue of Good Manufacturing Practice, e.g. some acids, gases, starch, cellulose and derivatives), are laid down;
- substances used as solvents or carriers, and their purity, are defined.

Sweeteners, colours and other additives ("miscellaneous directive") are addressed in European Parliament and Council Directives 94/35, 94/36 and 95/2/EC (as amended), respectively. Additional Directives (Commission Directives 95/31/EC; 95/45/EC; 96/77/EC) deal with purity criteria for these substances.

The provisions for approval and use of additives have been reviewed by Hammer and Honikel [5]: evidence must exist that the use of this particular compound has demonstrable advantages for the consumer and that there is a need for it (with respect to keepability; fulfilment of special dietary needs; preservation of the nutritional or sensory food quality, without any risk of deceiving the consumer).

The authorization of novel food and novel food additives is addressed in Regulation (EC) No 258/97.

New food additives can be allowed by member states for domestic products for a two year transitional period provided the Commission has been notified. The EU wide approval (i.e. inclusion in the "positive list") requires a review by EFSA and a final decision on authorization (or disapproval) by the Commission. Flavourings are not fully harmonized in the EU; however, a register on all flavourings authorized in the member states exists.

The Member states can - under some provisions - restrict the use of additives to foodstuffs produced according to traditional methods (Directive 94/43/EC). Directive 89/107/EEC on food additives has been amended by a number of Directives on sweeteners, colours and other compounds in foodstuffs [12].

This scheme is relatively rigid, and for a number of additives, the technological need is unclear or not well defined [6]. For some substances, the classification is somewhat arbitrary (e.g. nitrite as preservative, ignoring the antioxidative properties of this compound [5]). Maximum limits are usually oriented towards toxicological limits, not taking into account that the technologically necessary concentrations may be substantially lower (nitrite [5]). There has also been concern that limits for certain additives might be mistaken as optimum values.

Implications for fresh meat [Regulation (EC) No. 853/2004] have been discussed by Paulsen and Smulders [13]: The addition of organic acids naturally occurring in meat (lactic

acid) with a view to suppress microbial contaminants, does not comply with the definition of fresh meat, but results in a meat product.

Only extraction solvents listed in Council Directive 88/344/EEC (as amended) can be authorized by the member states.

Materials coming into contact with food must not release substances endangering human health (Council Directive 2002/72/EC). In particular, restrictions are laid down for vinyl chloride monomer (Council Directive 78/142/EEC; analytical procedures laid down in Directives 80/766/EEC and 81/432/EEC) and epoxy derivatives, as BADGE (2,2-bis(4-hydroxyphenyl)propane bis(2,3-epoxypropyl)-ethers), BFDGE (bis(hydroxyphenyl)methane bis(2,3-epoxypropyl)-ethers) and NOGE (novolac glycidyl ethers) (Commission Directive 2002/16/EC). Basic rules for testing the migration of constituents of plastic materials coming in contact with food are laid down in Council Directive 82/711/EEC.

Recent developments in “active” (packaging releases compounds to the food to improve shelf-life) and “intelligent” (packaging material contains indicators for product deterioration) packaging are addressed in Regulation (EC) No 1935/2004. Authorization is based on the expertise of the European Food Safety Authority.

A variety of substances may induce food allergy or intolerance. Only in a few cases, such compounds are undesired food components (e.g. histamine). In this context, consumer protection can be achieved best by exhaustive labelling, giving full information on the composition of foods, especially with respect to additives and allergens (Directive 2003/89/EC).

2.4. Legal requirements for detection methods

For a number of chemical compounds and bacteria, legal limits for food have been defined. This is underpinned by legislation on detection methods, to ensure that results are correct and reliable as well as comparable between laboratories. Maximum residue levels of certain compounds are usually based on toxicological evaluation (dose-response models, exposure assessment) and technological needs, while in case of substances the presence of which is generally considered as undesirable, the limits should be set as low as reasonably achievable and in accordance with the attainable sensitivity of the analytical method of detection of these substances. Therefore the current strategy of the EU is to clearly define the criteria for analytical methods, which have to be fulfilled. This so called “criteria approach” establishes quality control principles, limits, validation procedures of both screening and confirmatory methods. With respect to chemical compounds, this novel concept is increasingly replacing that of stringent standardization of reference and routine methods, based on the consideration that analytical procedures are improving at a higher rate than the standardization process goes on. The Commission Decision 2002/657/EC includes performance criteria and requirements for appropriate analytical methods. Guidelines for implementation of this Decision are summarized in the document “SANCO/2004/2726rev1”.

Depending on the nature of the toxicant, limits and thus, requirements for sensitivity of the analytical procedure, are quite different, as can be seen in Table 1.

For toxicants of bacterial origin [Regulation (EC) No 2073/2005], "criteria" still include reference methods, rather than performance standards. The use of validated (EN/ISO 16140) alternative methods, however, is explicitly allowed.

Table 1: EU limits for selected contaminants in foodstuffs

Max. level in the range of	Substance	Example
g/kg	Nitrate	Spinach; max. levels 2000-4500 mg NO_3/kg[1]
mg/kg	Histamine	certain fishery products; limits 100-400 mg/kg[2]
	Inorganic tin	canned foods and beverages; max. permitted levels 50-200 mg/kg[3]
	3-MCPD	soy sauce; max. level 20 mg/kg[1]
µg/kg	Heavy metals	various foods, Pb, Cd, Hg max. levels 50–1000 µg/kg[1]
	Pesticides	foodstuffs, "default" MRL 10 µg/kg[4]
	Aflatoxin B_1	groundnuts, nuts, dried fruit; max. levels 0.1-8 µg/kg[1]
Ng/kg	Aflatoxin M_1	milk, max. level. 50 ng/kg[1];
		infant milk, dietary food for babies; max. level 25 ng/kg[5]
	Dioxins	various foods of animal origin (muscle, liver, milk) and vegetable oil; max. levels 0.75-6 ng/kg fat (expressed as WHO toxic equivalents)[6]

1 … Reg. (EC) No 466/2001; 2 …Reg. (EC) No 2073/2005; 3 … Reg. (EC) No 242/2002; 4 … Reg (EC) No 396/2005; 5 … Reg. (EC) No 863/2004; 6 … Reg. (EC) No 2375/2001.

3. USA

3.1. Historical aspects

First attempts for a federal legislation on food safety date back to ca. 1880 [14]. From 1906 to 1914, federal legislation on adulterated or misbranded foods and drinks, meat inspection, a "positive list" of food colorants and labelling of food were issued. This legislation was revised from 1938 onwards ("Federal Food, Drug and Cosmetic Act"). Based on the work of the Delaney committee, from 1958 onwards, the Congress issued legislation on pesticides, food additives and colour additives. It is the responsibility of the producers to prove that any

"new" (post-1958) food additives are safe. From the viewpoint of the regulatory authorities, the "Delaney clause" prohibits the approval of any food additive inducing cancer in animals or humans.

Guidelines to the food industry with respect to the toxicity of food ingredients were issued in 1949 and 1982.

3.2. Organizational framework

The legal framework is represented by Congress statutes defining the level of consumer protection and the authority of the food safety organizations [15]. The major food safety authorizing statutes include:

- Federal Food, Drug, and Cosmetic Act;
- Federal Meat Inspection Act;
- Poultry Products Inspection Act;
- Egg Products Inspection Act;
- Food Quality Protection Act;
- Public Health Service Act;
- Statutes on the procedures to be followed by regulatory agencies (freedom of information, decision-making).

This legislation is implemented in the form of regulations by various federal organizations. Additionally, guidelines explaining the view of the agencies are issued. In case of non-compliance of companies, both agencies and companies can appeal to court for settling disputes.

A number of federal organizations are involved in food safety [14]: The Food Safety and Inspection Service (FSIS) ensures that meat, products of poultry and eggs are safe, wholesome and correctly labelled. For other types of food and food additives, drugs used in and feed used for animals for food production, the Food and Drug Administration (FDA) is responsible. Pesticides (residues, tolerance levels, safe use) are dealt with by the Environmental Protection Agency (EPA). Protection against plant and animal pests and diseases is the responsibility of the Animal and Plant Health Inspection Service (APHIS). Various other agencies have missions dealing with food safety issues in research, education, prevention and surveillance. Within these organizations, specialised branches exist, e.g. the Center for Food Safety and Applied Nutrition (CFSAN) of the FDA.

Legal documents are published in the Federal Register (www.gpoaccess.gov). Permanent legislation is sorted according to subject into 50 "titles" in the Code of Federal Regulations, CFR. The CFR is updated once per calendar year. Of particular relevance are the titles 21 (Food and Drugs) , 9 (Animals and Animal Products), 40 (Protection of Environment).

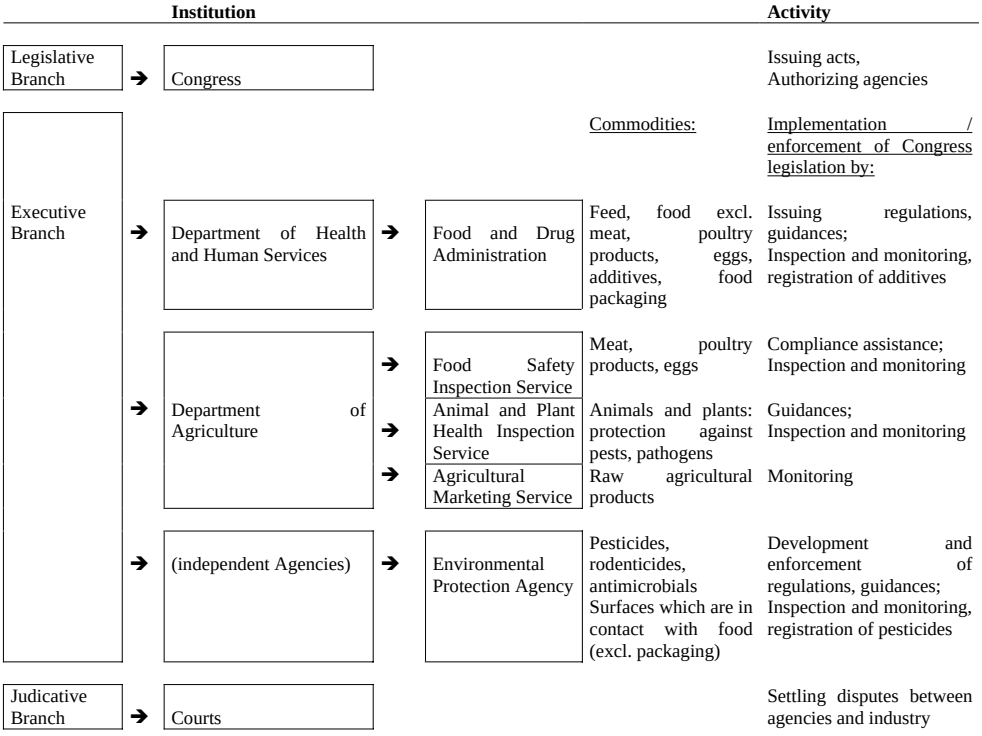


Figure 2. Schematic Diagram of US Federal Institutions Concerned with Food Safety and Food Toxicants

This organizational structure is not comparable with the EU system. While the EU recently tends to base food safety on EU regulations, i.e. laws applicable directly in the member states, the congress legislation is the framework of the activity of a number of agencies, which issue binding legal texts as well as non-binding guidances. The number of US federal agencies with similar responsibilities sometimes has caused problems; in the EU, the corresponding structures have been streamlined, with the EFSA and the FVO as major institutions. Figure 2 summarizes the US organization framework.

3.3. Principles of food safety

The key principles of the US food safety system [16] are that

- foods placed on the market must be wholesome and safe;
- food safety policy is based on scientific evaluation of hazards; in particular the principles of risk analysis, and, when necessary, the precaution principle apply;

- government has enforcement responsibility;
- food producers, retailers etc. are expected to comply with food safety legislation;
- the regulatory process is transparent for the public.

3.4. Specific legislation

Drugs administered to animals producing food are subject to prior approval of FDA, which also operates monitoring programmes.

Pesticides are subject to approval and registration according to the Federal Insecticide, Fungicide, and Rodenticide Act, under Title 40 of the CFR by the EPA before they can be used on food crops. The criteria for approval are laid down in the Federal Food Drug and Cosmetic Act. The EPA sets limits for residues of pesticides in raw agricultural commodities and foods under the Federal Insecticide, Fungicide, and Rodenticide Act. This previously caused problems, with respect to the Delaney clause, which demanded a zero tolerance for carcinogenic substances. In practice, a number of pesticides were approved and (risk - benefit assessment based) limits were established in raw agricultural products (§408). Their residues in foods, however, were considered as "additives" (§409), and thus fell into the zero tolerance requirement. This "Delaney paradox" was first discussed in 1987 [17]. The Food Quality Protection Act of 1996 allowed to solve this problem by re-definition of the term "food additive", in a way that allowed risk-assessment based tolerances for pesticide residues in food. This was paralleled by a re-assessment of pesticide tolerances or exemptions, with a focus on the safety of infants and children. This re-assessment and subsequent revoking, suspending or modifying of tolerances would occasionally result in foods on the market, which complied with regulations at the time of production (i.e. lawful use of pesticides), but not with "new" limits (based on dietary risk considerations) issued during shelf-life of the food. For such transitional situations, the FDA declared to apply the "Channels of Trade Policy" [18]. Monitoring programmes for domestic produce as well as for imports are operated by the FSIS (meat and poultry), the Agricultural Marketing Service (raw agricultural products) and the FDA (other food and feed). The results are published yearly.

FDA has issued guidances for industry concerning heavy metals, chemical contaminants in foods (acrylamide, dioxins - polychlorinated biphenyls, furan, nitrofuran, ethyl carbamate, radonuclides), other residues (quinolones, malachite green), and natural toxins (mycotoxins), as well as a number of supporting documents.

Substances which are intended as food additives or come into contact with foods need either to be notified before marketed, or undergo a petition - approval process.

Additives and colours fall into three categories:

- Substances which have been used for many years without any indication of adverse effects. These compounds are given the "generally recognized as safe" status ("GRAS") and their use is limited by good manufacturing practice only. On request of interested parties, the GRAS status may be affirmed for certain compounds.

- Substances which have been in use as food additives and colors before 1958 and were approved via the Food Additive Amendment to the Food, Drug and Cosmetic Act.
- "New" additives, where the sponsor has to prove that the compound is safe for its intended use. This documentation is evaluated by the FDA, which -in case of approval- publishes a regulation prescribing safe conditions for the use of this compound. Re-assessment of already approved substances may be done by the FDA in the light of new scientific findings.

For colour additives for food for human use, Part 70 of the Title 21 gives general provisions, requirements for packaging and labelling, as well as criteria for the safety evaluation. Approved colour additives have to undergo a batchwise examination by the FDA they are put on the market (Part 74) or they may be exempt from prior certification (Part 73).

Substances coming into contact with foods require to undergo a notification process before marketing, as laid down in the Food and Drug Administration Modernization Act of 1997. This repealed the former petition-approval process. A Final Rule on the notification system was issued in 1992, and also a guidance for industry how to prepare such notifications. An inventory of effective notifications is provided by the FDA. This inventory includes at present ca. 550 compounds with their exact definition, intended use, and the notifier and the manufacturer.

Antimicrobials used directly in/on food or coming into contact with food surfaces are regulated under the Federal Food, Cosmetics, and Drugs Act, and the Antimicrobial Regulation Technical Corrections Act. Most antimicrobial uses on raw agricultural commodities, and permanent or semipermanent food contact surfaces (other than food packaging, i.e. cutting boards, countertops, cutlery …) are under the authority of EPA. Other applications are in the scope of the FDA, which issued guidance on this subject in 1999 [19]. While in the first case, a compound would be classified as "pesticide chemical", it is considered as food additive in the latter situation. However, even some of such food additives may fulfill the definition of a "pesticide" according to the Federal Insecticide, Fungicide, and Rodenticide Act, and need to be registered thereunder.

The petitioning-approval and notification requirements and the progress are available at the "Approval and Notification Programs" web page of the FDA/CFSAN (http://www.cfsan.fda.gov/~lrd/foodadd.html).

To facilitate understanding of the considerably different EU system, the USDA Foreign Agricultural Service prepared a review on the respective legislation from the point of view of the USA (GAIN E35162 report [20]).

4. Mercosur legislation

Mercosur is an alliance of South American countries (Argentina, Brasilia, Paraguay, Uruguay, Venezuela and associated countries), with the aim to form a strong internal market.

The most relevant partner in terms of trade (both export and import) is the EU [21]. In the EU Commission "Mercosur - European Community Regional Strategy Paper" of 2002 [22], support for establishment of veterinary and phytosanitary rules in Mercosur was explicitly addressed. By nature, these rules are / will be oriented on international (CAC, JEFCA) and national (USA, EU) regulations.

With respect to mycotoxins, Mercosur member states have established common limits (including sampling and analysis methods) for total aflatoxins in peanuts, maize, and products thereof, and aflatoxin M_1 limits in milk [23].

The control system for residues of veterinary drugs was reviewed by Fernandez Suarez [24]. It consists of:

- Rules on use and registration of veterinary medicinal products. Three relevant Mercosur Resolutions (Res.No 11/93; 29/93; 39/96) resemble to EU directives, in the way that they need national implementation.
- Implementation of residue control programs. Operational residue control programs are a prerequisite for food (meat, fish, aquaculture products) export in the USA and the EU. The MERCOSUR framework consists of four resolutions (Establishing priorities in residue control: Mercosur/GMC/Res.No 53/94; standards for maximum residue limits: Res.No. 75/94; validation of analytical methods: Res. No 57/94; sampling methods: Res.No 46/98). National residues control programs are based on the above mentioned framework. With respect to export to the EU, these national plans have to be approved by the EU (e.g. Decisions 485/2003/EC, 432/2004/EC).
- Definition of national authorities responsible for registration of veterinary medicinal products and for residue control programs.

Special regulations are established for substances with no acceptable daily intake / maximum residue level. These substances fall into three categories:

1. Veterinary products which may not be used in food animals (stilbenes, chloramphenicol, nitrofurans...): these substances are not authorized for marketing; live animals, feed and water are monitored for residues.
2. Natural anabolics (products of animal metabolism: estradiol, testosterone).
3. Synthetic growth promoters.

The use of the substances of the latter two groups is not allowed in all Mercosur countries. If allowed (Argentina), the prescription of these substances is classified, farms using no growth promoters are registered and have to operate with full traceability, animals and feed are monitored for residues. This ensures that requirements of meat importers (EU) can be met.

5. Asia-Pacific Region

In the Asia-Pacific region, several associations exist which aim to establish free trade areas. An equivalent level of food safety throughout the member states is a prerequisite for barrier-free food trade.

The Asia- Pacific Economic Cooperation (APEC) was founded in 1989 on an initiative of Australia, Japan and the USA and now includes Australia, Brunei, PR China, Hongkong, Indonesia, Japan, Canada, South Korea, Malaysia, Mexico, New Zealand, Papua - New Guinea, Peru, Philippines, Russia, Singapore, Taiwan, Thailand, USA, Viet Nam. APEC issues non-binding agreements only, and the member states establish individual action plans and give a yearly report on their activities. Subgroups of the Committee on Trade and Investment (CTI) are active in training, e.g. courses on risk assessment on chemical contaminants, natural toxicants, additives and pesticides, assessment of genetically modified organisms (GMOs), and pursue harmonization of regulations for fisheries and seafood products. Consolidated reports on the APEC Food System (AFS) are collated yearly (www.apec.org). An example of the way how risk assessment is implemented by the national authorities is the report “The application of risk analysis in food control - challenges and benefits” prepared by FSANZ (Food Standards Australia New Zealand) for the FAO/WHO [25].

A network of food scientists (Asia Pacific Food Analysis Network / Federation of Asian Chemical Societies- FACS) promotes food safety by information exchange, conferences, workshops [26].

The Association of South East Asian Nations (ASEAN) was founded in 1967 and currently comprises Thailand, Indonesia, Malaysia, Philippines, Singapore, Brunei, Viet Nam, Myanmar, Laos and Cambodia. Strengthening the food security arrangement was one of the major tasks of a “strategic plan of action” for the period 1999-2004. Cooperation projects on food safety are guided by the ASEAN ministers of agriculture and forestry [27].

6. Concluding Remarks

A variety of substances in food can cause adverse health effects to the consumer. Not all effects follow a dose-response-relation, and even traditionally used compounds may bear some toxic potential. Consequently, not only “new” toxicants have to be assessed, but several well-known substances need a re-assessment. Long term strategies are necessary for lowering the levels of various environmental contaminants, and improved and more sensitive analytical techniques are necessary to demonstrate that this aim is being fulfilled.

The establishment of limits or tolerances for toxicants in food, the provision of analytical capacity and the existence of enforcement measures is a prerequisite not only for consumer protection, but also necessary for trade. Apart from the common concept of tolerance levels,

the organization framework as well as the analytical methodology to pursue this aim may differ substantially between nations.

References

[1] ICMSF, Microorganisms in food, vol.4: Application of the Hazard Analysis Critical Control Point (HACCP) system to ensure microbiological safety and quality, Blackwell, London, 1988.

[2] S.M. Cahill, E. Boutrif, M. De Lourdes, G. Costarrica, Chemical residues in foods of animal origin : assessing risk and implementing control strategies. In: Food safety assurance and veterinary public health. vol.4, Ed. F.J.M. Smulders, Wageningen Academic Publishers, in press.

[3] M. Teuber: Veterinary use and antibiotic resistance in: Food Safety and Veterinary Public Health, vol. 2, Eds. F.J.M. Smulders, F.J.M., J.D. Collins, Wageningen Academic Publ., Wageningen, 2004, p. 229-241.

[4] A.D. Anderson, M. Nelson, L. Baker, S. Rossiter, F.J. Anulo, Public health consequences of use of antimicrobial agents in agriculture. In: Food safety and veterinary public health. vol 3, Eds. F.J.M. Smulders, J.D. Collins, Wageningen Academic Publ., Wageningen, 2005, p. 173-184.

[5] G. Hammer, K.O. Honikel, Food additives and public health. In: Food safety and veterinary public health. vol. 2, Eds. F.J.M. Smulders, J.D. Collins, Wageningen Academic Publ., Wageningen, 2004, p. 317-329.

[6] H.G. Classen, P.S. Elias, W.P. Hammes, M. Winter, Toxikologisch-hygienische Bewertung von Lebensmittelinhaltsstoffen und Zusatzstoffen. 2nd ed., Behrs´, Hamburg, 2001.

[7] W. Maier, European Union Food Regulations and Private Standards. Workshop held at the USDA, Nov. 3rd, 2005.

[8] A. Kabwit Nguz, Food safety and international trade: a challenge for developing countries - a review paper. University of Ghent, Belgium, undated. Available as: http://www.foodhaccp.com/internetjournal/ijfsv4-2.pdf

[9] Anonymous, General principles of food law - European Food Safety Authority Procedures for food safety. Available at: http://europa.eu.int/scadplus/printversion/en/lvb/f80501.htm. Last accessed: 16.02.2006, 2006a.

[10] D.J. Bolton, B. Maunsell, B. Howlett, Pork slaughter and processing food safety, Teagasc-The National Food Centre, Dublin, 2005.

[11] R.W Stephany, Hormones residue testing: An update in research and approaches, in: Food safety and veterinary public health. vol. 2, Eds. F.J.M. Smulders, J.D. Collins, Wageningen Academic Publ., Wageningen, 2004, p. 213-228.

[12] Anonymous, Authorised food additives: general scheme. Available at: http://www.europa.eu.int/scadplus/printversion/en/lvb/121067.htm.

[13] P. Paulsen, F.J.M. Smulders, Reduction of the microbial contamination of carcasses and meat cuts with particular reference to the application of organic acids, in Food Safety and Veterinary Public Health: vol. 2, Eds. F.J.M. Smulders, J.D. Collins, Wageningen Academic Publishers, Wageningen, 2004, pp. 177-199.

[14] FDA/CFSAN, FDA Backgrounder: Food Safety: A Team Approach.24.9.1998; available at: http://www.cfsan.fda.gov/~lrd/foodteam.html.

[15] Anonymous, United States Food Safety System. Available at: http://www.foodsafety.gov/~fsg/fssyst2.html.

[16] FDA/USDA, U.S. Food Safety System Country Report. 3.3.2000. Available at: http://www.foodsafety.gov/~fsg/fssyst2.html.

[17] D.U. Vogt, The Delaney Clause: The Dilemma of Regulating Health Risk for Pesticide Residues. 1992. Available at: www.ncseonline.org/nle/crsreports/pesticides/pest-3.cfm.
[18] FDA/CFSAN, Channels of Trade Policy for Commodities with Residues of Pesticide Chemicals, for which Tolerances Have Been Revoked, Suspended or Modified by the Environmental Protection Agency Pursuant to Dietary Risk Considerations. Final Guidance, May 2005. Available at: www.cfsan.fda.gov/~dms/pesguid2.html.
[19] FDA, Antimicrobial food additives - Guidance. July 1999. Available at: www.cfsan.fda.gov/~dms/opa-antg.html.
[20] USDA, GAIN E35162 report of the USDA Foreign Agricultural Service. EU-25 Food and Agricultural Import Regulations and Standards. European Union Report 2005. Dated 8-18-2005; 31pp.
[21] Anonymous, The EU´s relations with Mercosur. Available at: http://europa.eu.int/comm/external_relations/mercosur. Last accessed: 01-03-2006.
[22] Anonymous, Mercosur - European Community regional strategy paper 2002-2006. Available at: europa.eu.int/comm/external_relations/mercosur/02_06en.pdf.
[23] Anonymous, Worldwide regulations for mycotoxins in food and feed in 2003. Available at: ftp://ftp.fao.org/docrep/fao/007/y5499e/y5499e00.pdf.
[24] A. Fernandez Suarez: South America´s regulatory framework for the control of residues of veterinary drugs. 2004. Available at: http://www.fao.org//docrep/008/y5723e/y5723e01.htm. Last accessed: 16.02.2006.
[25] FAO-WHO, FAO/WHO regional conference on food safety for Asia and Pacific: The application of risk analysis in food control - challenges and benefits, CAP 04/3, FAO/WHO, Geneva.
[26] Asia Pacific Food Analysis Network / Federation of Asian Chemical Societies- FACS: www.fecs-as.org.
[27] Association of South East Asian Nations (ASEAN): www.aseansec.org.

Note: Legal texts are not included in Reference List. They are, however, quoted in a way that allows retrieval from the relevant web pages.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 3

Risk assessment of food additives and contaminants

Jim Bridges, Olga Bridges

Research for Sustainability, Liddington Lodge, Guildford, Surrey, GU33AE, UK

1. Risk assessment procedures

1.1. The need for risk assessments

There are many thousands of chemical substances in food, most of them are of natural origin. A number, however, are man-made and arise from the use of agrichemicals, or due to pollution of air, water or soil, or occur during food preparation/processing. In addition food may contain microbial and other biological contaminants and physical contaminants. A range of additives may also be added for a variety of purposes (e.g. enhance the flavour colour, improve stability etc).

The public rightly expect that the food they eat is wholesome (safe). Over the past 50 or so years, a number of major incidents have led to public and political concern about food safety. Among the substances highlighted have been: various pesticides, dioxins, polychlorinated biphenyls (PCBs), antibiotic residues and metals such as methyl mercury. However in political terms, an even greater impact on our current approach to food safety was identification of bovine spongiform encephalopathy (BSE) in bovine products.

A vital element in the assurance of the safety of food and drink products is a scientifically valid, transparent, objective and trusted means for evaluating the safety of food components. In the past, in many countries, food safety was managed by the departments of either health or agriculture of national governments. However in the last decade, as a consequence of incidents such as those indicated above, many countries have established independent food safety authorities at arms length from central government. A principal role of such agencies is to ensure that the evaluation of safety is demonstrably independent and that appropriate regulatory measures and monitoring procedures are in place to provide the public with the necessary confidence in the safety of the food and drink they consume. A variety of tools have been developed to assess food safety. Two such tools in particular are extensively written into legislation:

- Risk assessment (RA) which considers separately each food contaminant/ food additive
- Hazard and critical point analysis (HACCP) which identifies vulnerable points (i.e. high-medium risk stages) in food processing procedures.

This chapter concentrates on the discussion of the risk assessment process. Risk assessment can be described as a data driven process for determining the likelihood of an adverse event taking place. For the purpose of this chapter risk assessment is defined more specifically as ‘a process for establishing the likelihood of adverse effects to human health from exposure to chemical agents in food’.

In principle, risk assessment should be an objective process, but inevitably it involves both scientific and value judgments.

The process of risk assessment is gaining importance because:

1. It is a crucial source of information for the risk management process
2. It is a legal requirement for an ever increasing number of domains (both food and non-food)
3. The public is increasingly concerned about the risks to health from foods for example, genetically modified organisms (GMO’s) and novel foods and needs to understand how judgments on their safety are made.
4. New relevant technologies are becoming available with the potential to strengthen the science base for the risk assessment process

Risk assessment is generally recognized to comprise the following stages:

- hazard identification
- hazard characterisation
- exposure assessment
- risk characterisation

1.2. Hazard identification and characterisation

1.2.1. Use of animal tests

It is a fundamental principle that for toxicity to arise there must be significant exposure. There are two stages to hazard evaluation:

1. Identification of the adverse biological effects. In principle this can involve both in vitro and in vivo animal tests.
2. Examination of the relationship between each adverse effect and the exposure level. The currently available in vitro tests are less useful for this hazard characterisation.
3. Although the distinction between hazard identification and hazard characterisation is scientifically important for the purposes of this chapter they will be considered together.
4. The vast majority of information about the adverse effects of chemicals is derived from animal tests.

A series of tests have been developed over many years to evaluate the hazards of food additives and contaminants these include:

- acute toxicity tests,
- genotoxicity tests

- metabolism and toxicokinetics
- subacute and chronic toxicity tests
- lifetime (cancer bioassay) tests
- allergy and sensitization tests
- reproduction/teratogenicity tests
- special tests such as neurobehavioural tests

Tests are normally conducted in a relevant sequence and not all of these tests will be carried out for most food additives and contaminants. The selection of tests is driven principally by the legislative requirements of the importing countries. It is important that the tests are performed to a high standard (Good Laboratory Practice) and to well established protocols. The OECD is the body which leads in the validation and recognition of individual test protocols.

In most tests administration of an additive/contaminant of interest is generally at two or more dose levels (test groups) plus a control group. The doses of food additives/food contaminants used in animal tests tend to be much higher than the levels to which humans would be normally exposed. This is done in order to ensure that any potential adverse effects are manifested and also to minimize the number of animals used.

It is particularly important in long term studies to have a control group of animals that is treated in an identical manner to the test groups (apart from the administration of the additive/contaminant of interest). For example, any solvent used to dissolve the additive/contaminant of interest should also be given to the control group.

Chronic studies are often difficult to interpret because as the animals get older they tend to become more diseased. The challenge for the risk assessor is to differentiate between an increasing 'natural' disease burden due to ageing from disease caused by exposure to the chemical under study. This is fairly easy if the adverse effect such as a tumour is a rare one in the animal test species. However if the effect is very common in the control group it may be much more difficult to interpret the results.

Ideally, for risk assessment purposes, chronic data should be available from at least two animal species, (typically rats and mice), and in both sexes by the relevant exposure route which for food additives and food contaminant is administration in the diet. If the findings are in agreement between the two species there is a likelihood that the same or similar adverse effects will occur in man. Although it is evident that rodents are by no means an ideal model for human response to chemicals. However, if the findings in the two test species differ substantially the extrapolation to consumers can be very problematic. Another challenge for the risk assessor is to extrapolate from the high dose exposure used in the animal tests to the much lower exposures, which are likely to be experienced in humans. This is normally addressed by mathematic modeling or by building in safety factors to allow for uncertainty (see below).

1.2.2. Use of epidemiology data

Epidemiology studies are the primary source of direct information on the relationship between chronic exposure to chemicals, particularly mixtures of chemicals, and possible human adverse health effects. Although epidemiological information is highly desirable often it is not available. Even when there is some potentially relevant data regarding specific food, contaminants epidemiological findings may be difficult to interpret for the following reasons:

- The level of actual exposure of individuals/ small population groups to a contaminant over time is often very uncertain.
- Many other factors, apart from exposure to chemicals in food, (termed "*confounding factors*") may contribute to the incidence of a particular disease. These confounding factors need to be compensated for, as far as possible.
- The global market for many food products means that it is often difficult to identify an appropriate control group (i.e. one with no or a low exposure to the food additive or contaminant of interest).

There are two other important factors to consider in interpreting epidemiological findings:

- The methods used are intrinsically insensitive, unless quite large population groups are studied in order to identify a true adverse effect consequently negative findings need to be interpreted with care.
- Correlation of disease with the intake of a particular additive or contaminant may or may not indicate a cause- effect relationship. Clusters of disease can arise purely by chance. It means that criteria other than pure statistical significance are applied in order to interpret epidemiological findings.

In practice, hazard data in the great majority of additives and contaminants is derived from animal studies.

1.2.3. Use of other data

In addition to data from animal tests and human studies there has been a substantial development over the past few years in both in vitro (laboratory tests using cells/tissues). To date, the main value of in vitro tests has been in hazard identification and in the investigation of mechanisms of toxicity. In vitro tests have proved a valuable means for the identification of the genotoxicity (ability of chemicals to interact with the DNA of a cell causing semi-permanent or permanent change). In vitro tests are also available to assess irritancy but as yet have not yet found a wider application.

In silico methods (computer based methods) are still in their infancy for the risk assessment of food additives and food contaminants. They offer the potential to compare the toxicity of related structures and to therefore extrapolate from data rich to data poor additives and contaminants. However there is no adequate common validated data base on the known

adverse properties of chemicals in current use at present to enable the effective development of such in silico methods.

1.2.4. Dose considerations

Adverse effects are conventionally divided into:

1. Those with a threshold exposure level can be identified, below which toxic effects are unlikely
2. Those for which no threshold exposure can be identified. Most of these chemicals are genotoxic carcinogens, i.e. compounds that cause cancer and interact with the DNA of the cells in the organ in which the cancer is expressed.

The distinction between genotoxic carcinogens and chemicals with other properties has been very important from a regulatory viewpoint. For the majority of chemicals for which there is a threshold, a standard can be set below which adverse effects are unlikely. If no threshold exposure level can be identified, it must be assumed that there is a risk of cancer at any level, albeit the risk diminishes with reducing exposure. As a consequence, genotoxic carcinogens are banned for use as additives in most countries. For contaminants that are genotoxic and carcinogenic, the approach is to keep exposure as low as is achievable (ALARA Principle).

For various reasons, the outcome from a long term toxicity study may be uncertain in such situations other information should be sought. This may include:

- any information in humans such as data on the metabolic fate of the contaminant/additive of interest
- findings from mechanistic studies
- cross reading from chronic tests performed on related chemical structures.

1.3. Exposure assessment

Exposure assessment is a key part of the risk assessment process. At present, exposure assessment is often ‘the weakest link’ of a risk assessment process due to limited sampling and variations in exposure across areas of a country or between countries.

Three main practical questions have to be answered in investigating exposure to chemicals in foods:

1. how to determine quantitatively the presence of a chemical in individual foods and diets, including its fate during the processes within the food production chain;
2. how to determine the consumption patterns of the individual foods containing the relevant chemicals;
3. How to integrate both, the likelihood of consumers eating large amounts of the given foods and of the relevant chemical being present in these foods at high levels.

The techniques used for the evaluation of these three aspects have been critically reviewed elsewhere [1]. A number of possible options for improvements have been suggested, for example:

- development of a pan-European food composition database
- harmonisation of food consumption survey methods
- evaluation of probabilistic models
- development of models to assess exposure to food allergens.

It is most important that exposure assessment is based on reliable analytical data. In the absence of direct analytical data some estimate of approximate consumer exposure levels can be made based on knowledge of their origin. Intelligence gathering on the exposure values used for substances being assessed by national/EU/other international bodies can be employed to supplement this approach

To overcome such problems risk assessors resort frequently to worst case assumptions for the daily/weekly intake of particular food additives/contaminants. However, there is a danger that as a result the conclusions of the exposure assessment will be totally unrealistic. Quantitative uncertainty analysis is a device that is gaining acceptance as a means to address the propagation of uncertainties in a more scientific manner along with the distribution of risks and benefits. Estimates of exposure to contaminants is more challenging than that for food additives. Moreover, exposure to many contaminants occurs not just in food but via other media. All too often this important issue is not considered by the food regulatory agencies.

1.4. Risk characterization

Risk characterization draws on the information in hazard identification and characterization to establish the risk to consumers at particular levels of intake of individual food additives and contaminants. This can then be compared with the actual exposure levels.

For many chemicals a variety of effects may be observed at high doses. The first step in analysis of the data is to determine the critical effect(s). Namely the effects, of biological concern, that occur at the lower exposure doses.

The second step is to identify whether a clear non-observable effect level (NOEL) can be identified for the critical effect. If this is the case it is appropriate to consider the setting of a standard for the additive/contaminant. To select an appropriate standard that will ensure a high level of protection of the health of consumers the following should be considered:

- the quality of the data (is the data clearly described, were the studies performed to good laboratory practice, was the control group adequate)
- the comprehensiveness of the data (what could have been missed, are the same findings found in both sexes, more than one species, is there any reliable human data)
- what is known about the toxicology of closely related substances (it is consistent with the findings)

In addition to the above considerations, there are two other important issues that need to be addresses:

- How should we allow for the possibility that man could be more sensitive than the experimental animals to the adverse effects of the food additive/contaminant ?
- How should we allow for the possibility than some consumers may have a substantially greater sensitivity to the food additive/ contaminant than the general population ?

Typically the NOEL is divided by a factor of ten to allow for possible differences in sensitivity between man and animals and a further ten to allow for possible substantial variation in sensitivity among the consumer population. The numerical value of these safety (uncertainty) factors may be varied according to the information available however some commonly the NOEL/100 is used to set a standard. For food additives, this is termed the acceptable daily intake (ADI) while for food contaminants the term tolerated daily intake (TDI) or tolerated weekly intake (TWI) is used.

This value may then be used to assign particular maximum amount to a contaminant/additive in individual food types. In the case of pesticide and drug residues the term maximum residue limit (MRL) is used to describe the maximum amounts. MRL's take into account the amount of particular foods that are consumed by the population.

1.4.1. The issue of carcinogens

For contaminants that are carcinogenic and genotoxic a different strategy has to be employed. The European Food Safety Authority (EFSA) and JECFA (WHO/FAO) have developed an approach based on the estimation of a bench mark dose to determine the margin of exposure (MOE). The bench mark dose is defined as the dose which in a lifetime cancer bioassay test produces 10% of tumours. Both JECFA and EFSA have identified that if the 'margin of exposure' is greater than 10,000 the exposure situation should be viewed as 'of low health concern'. If the MOE is substantially smaller than 10,000 exposure reduction measures should be considered. It could be, however, that risk managers will still look for exposure reduction even if the MOE is above 10,000.

It can be concluded for the above that the procedure for the determination of the risk from exposure to food additives and contaminants is well established issue which is not well addressed. However risk assessors continue to face a major challenge of how to assess the total impact of food contaminants/food additives on consumer health.

Let us consider just one example carcinogens in food. Diet is considered by many specialists to be a major route of exposure to carcinogens. Chemicals of concern include nitrosamines, aromatic and heterocyclic amines and polycyclic hydrocarbons, along with many carcinogenic natural products [2].

To illustrate the complexity of the actual exposure situation we can take just one group of chemical carcinogens in food, the aromatic amines.

Among the aromatic amines, aniline has been found as a component in fresh fruit and vegetables in concentrations of 0.6 to 30.9 mg/kg. Toluidine has been identified in kale, celery, and carrots (1.1-7.2mg/kg). Amines may also be created by the cooking process. For example, Sugimura et al. [3] discovered the formation of mutagens (heterocyclic amines) at the surface of cooked meat or fish. Various components present in food may modify either the formation of these amines or the effects that they produce. The formation heterocyclic aromatic amines during cooking can be decreased by natural and, synthetic antioxidants, by tryptophan or proline, or by removing creatine prior to frying or grilling. The amounts of heterocyclic amines in cooked foods are small, but other components in diet such as w-6-polyunsaturated foods can have a powerful promoting effect in the target organs. On the other hand the action of heterocyclic amines may be decreased by the presence of antioxidants and other anticarcinogenic substances contained in many vegetables and fruits, as stated by Ribero and Salvadori [4].

It is evident that the key issue is what is the total impact of these components on health. Can synergy or additive effects occur? As yet, we do not have sufficient knowledge to evaluate this.

1.5. Methods for evaluating the risk from 'data poor' additives and contaminants

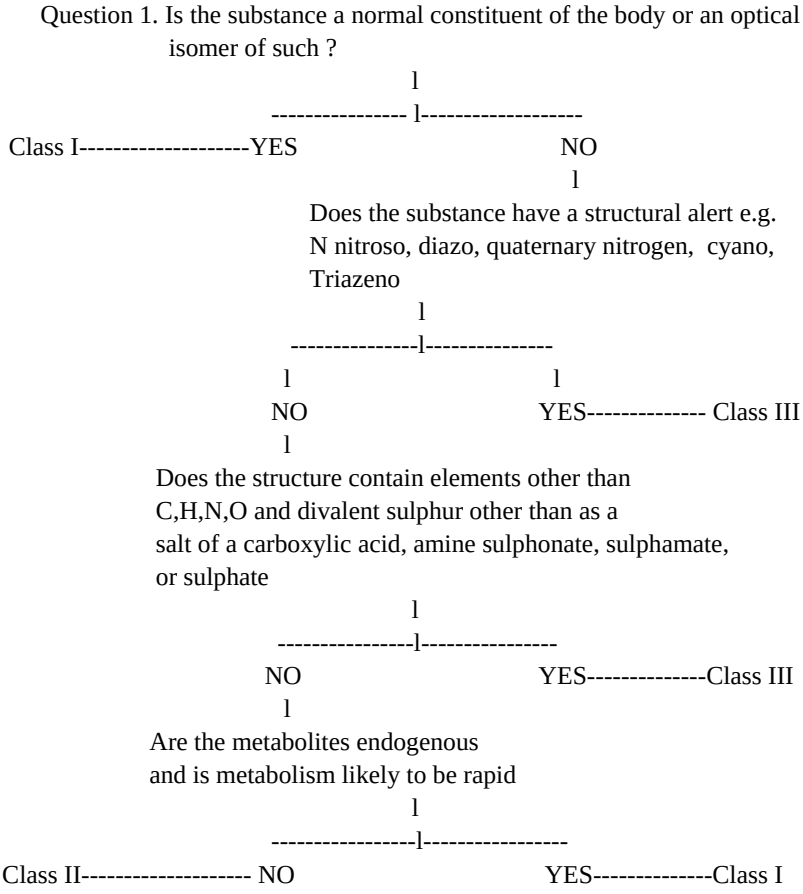
Food contains many thousands of chemicals of natural and synthetic origin. It has to be recognized that for many contaminants and food additives the hazard information is either poor or, in a number of cases, non-existent. Such substances for risk assessment purposes are often termed 'data poor'. To avoid unnecessary testing, a strategy known as the 'threshold of toxicological concern' (TTC) has been developed, in which the decision on whether testing is required is based on:

- The physicochemical characteristics of the additive/contaminant
- The estimated levels of exposure via the diet.

The approach is a practical one. Despite this there is considerable reticence about its adoption by regulatory authorities. This is expected to change as more data becomes available to validate TTC. It is already in use by the USFDA for assessing the risk of contamination of food by various food packaging materials. JECFA have also adopted the approach for food flavourings.

JECFA use the TTC approach to determine whether there is a need for a more detailed risk assessment. The approach was developed by JECFA because of the need to establish the safety of the 3000 or more food flavourings in current use. For many of these flavourings there is no toxicological data available. Consequently, assessment is based on the initial characterisation of exposure. The method is based on chemical properties. The value of the TTC for each class is based on the published literature, which is used to determine the NOEL for the most potent chemical in a particular class (the class assignment is based on a range of chemicals with some shared structural properties). A safety factor is then added for

uncertainty in order to set a level below which adverse effects for any chemical in the class is most unlikely. JECFA have identified three chemical classes (I, II, III) each with its own threshold.



Schemme 1. Process for assignment of substances to one of the three classes

Class I substances are those with structures and related data suggesting a low order of oral toxicity. A TTC value 1800ug per day has been set for this class.

Class III substances are those that tend to be complex structures they may have structural features (termed structural alerts) that have been associated with particular toxic effects e.g. azo, secondary amines, nitroso etc. TTC value 90ug per day.

Class II substances have intermediary properties between class I and class III. TTC value 540ug per day.

For those substances for which the exposure levels are below the relevant TTC level no further investigation is needed. For a substance whose exposure levels are above the TTC some assessment of their adverse toxicological properties is appropriate (see section 1.2.3 for list of possible tests). The higher the level, the more urgent the requirement for further information. The TTC approach is of course highly dependent on reliable exposure data and hence on good quality analytical data.

1.6. Regulation of food additives and contaminants

There are three approaches that may be used by regulatory authorities to identify acceptable food additives:

1. The positive list, which only includes additives that have been specifically approved on the basis that the available data is sufficient to ensure safety in use. The availability of such a list should not be taken to mean that substances not on the list represent any danger to health. They may not be on the list because they have not been submitted for approval or because there is insufficient data to make a judgment on their safety.
2. The negative list. This identifies specific substances that may not be used as food additives because of specific adverse properties that have been identified or are suspected.
3. The GRAS (generally regarded as safe) list. This list covers substances, particularly natural ones, that have been in use for a very long period of time but for which there is typically limited or no direct data to confirm their safety directly.

The lead international body for advice on the safety of food additives and contaminants is the Codex Alimenterius –a joint body of WHO and FAO which is based in Rome. This body brings together committees of international experts who evaluate the available data on both food additives and contaminants and recommend where appropriate ADI values. Although the conclusions of these committees (JECFA/ JECFC) are not mandatory on the member countries generally they are adopted.

1.7. Overall conclusions on the risk assessment process

Risk assessment draws on a number of scientific disciplines. Although the process of risk assessment is strongly based on the available scientific data inevitably judgment is also important. The quality of the process is therefore very dependant on the willingness of high quality experienced scientists to participate in risk assessments.

It is evident that the estimate of the acceptable daily intake (ADI) is strongly influenced by:

- the judgment of risk assessors on the relative significance of particular findings
- the extent to which the risk assessors err on the side of caution (conservatism) where they identify uncertainty regarding the meaning of particular findings.

Increasing attempts are being made at the international level to harmonise risk assessment practices.

Typically in setting an acceptable daily intake, and consequently the maximum residue levels for particular foods, little or no attention is paid to the feasibility of analysis of the additive/contaminant at the proposed level. This is unfortunate and points to the need for analytical chemists to be more involved in the risk assessment process in the future.

2. The case of Sunset Yellow

In order to show how the risk assessment procedures are applied two case histories are discussed below:

- the risk assessment of the food dye Sunset Yellow
- the risk assessment of a contaminant of sunset yellow (Sudan 1).

2.1. Food dyes and their regulation

A variety of colours have been used to make food more attractive and appetizing for centuries. Historically colourants derived from naturally occurring minerals, plants, and animals were used, along with spices, for these purposes [5].

At the end of the 19th century the discovery of synthetic dyes, whose colours were more intense and more stable than the natural dyes started a new era in the production of consumer goods? The overall use of colourants world wide now amounts to hundreds of thousands of tons per year [6].

Sunset yellow is an azodye. An azo dye comprises two amines (normally aromatic amines) joined together through their amino groups by means of an azo bond. Azo dyes are grouped into several types, depending on the nature of the amine that is incorporated in their structure[7]. During the 20th century gradually restrictions had been placed on the dyes permitted for food use and also on the levels of impurities [8].

It became a matter of public concern that abuse of these additives was taking place when, in 1950, some children became ill after eating popcorn with high levels of colourants. A seminal piece of legislation was introduced in the USA in the 1960's known as the Delany Amendment that banned all carcinogenic substances from food. This legislation did not distinguish between genotoxic and non-genotoxic carcinogens.

The Color Additive Amendment of 1960 eliminated the 'harmless per se' interpretation that had previously applied to many colourants. It resulted in a list of only nine permitted synthetic colorants for food, drugs and cosmetics. The list was reduced to eight with the delisting of FD&C Red No.3 in 1998. These include 5 azodyes: FD&C yellow No5, FD&C yellow No6, Orange B, citrus red No2, FD&C red No red 40 and three others FD&C Blue No1, FD&C Green no3 and FD&C Blue No2. It should be noted that the last dye approved in the USA for food uses was in 1971 (FD&C red No 40). It has been argued by the FDA that since the three

primary colours are included in the approved list there is no need for any other dyes. The Color Additive Amendment also set up a category of exempt colourants which were not subject to the rigorous requirements of the certified colorants. There are 26 colorants in this category and they comprise most of the preparations which would be called ‘natural’ . For all of these dyes an ADI has been set. It is forbidden to exceed this ADI in foods.

In contrast to the USA the European Union Member States have adopted the common principle in respect to food that carcinogenic substances must be eliminated from food or (where this is impractical) the levels must be made as low as is reasonably achievable (this is known as the ALARA Principle). In the absence of clear data to demonstrate that a substance is non-genotoxic, a default position is adopted that it may be genotoxic (now described as the Precautionary Principle). In 1962 the EU set a guideline on which colourants could be used in food and the purity standards for food colorants that must be adhered. In addition, many Member States have their own guidelines that permit particular colourants for specific foods. In 1994 a new EU guideline was agreed (the Register of the European Community No. L 237). Special emphasis was placed on the concept that non-processed food and certain other food products ‘must be free from food additives’. A number of other foods were identified for which the colourants permitted were strictly limited. Member States were required to introduce systems which- monitor the use and application of food colourants.

2.2. Sunset Yellow FCF

One of the most widely used azo dyes has been Sunset yellow (6-Hydroxy-5-[(4-sulphophenyl)azo]-2-naphthalene-sulphonic acid). This dye is produced by coupling diazotized 4-aminobenzene sulphonic acid with 2-naphthol-6-sulphic acid. It is related structurally to the Sudan series of dyes .

In the USA, a large-scale production of Sunset Yellow started in the 1920s and is now used worldwide in bakery and confectionary products, desserts, soft drinks and cereals [9]. In addition, a proportionally smaller amount of Sunset Yellow has been used in drugs, cosmetics and stationary products.

The legislation allows for a substantial quantity of contaminants in Sunset Yellow. This means that batches of Sunset Yellow may vary in their adverse properties. For example, the British Standard allows the following also to be present:

- 6-Hydroxy-5- [(4-sulfophenyl) azo]-2-naphthalenesulfonic acid, disodium salt.
- Subsidiary dyes (nature not specified)
- Matter volatile at 135 degrees centigrade
- Matter insoluble in water
- Matter soluble in diisopropyl ether
- Chloride and sulphate

2.2.1. Hazard identification and charcterisation- animal findings

In one animal study, 30 male and 30 female mice were given 0.2, 0.4, 8 or 1.6% Sunset Yellow FCF for 80 weeks with 60 male and 60 female animals as controls. There was no evidence of increased tumours in the mice given Sunset Yellow up to 1.6% [10]. Some kidney and urinary tract damage was observed but this was discounted since it was not dose related. In pigs no adverse effects of Sunset Yellow was found when the dye was administered at dose levels of 250, 500 and 1000mg/kg per day in the diet for 98 days. The no effects level in this study was therefore greater than 1000mg/kg/body weight [11]

A NOEL of 2% in the diet (equivalent to 500 mg/kg body weight/day) was identified in a seven year feeding study in dogs. A similar NOEL value was also identified (equivalent to 550mg/kgbody weight/day) in a long- term rat feeding study [12].

In a number of animal studies in the 1980s Sunset Yellow was found to be non-genotoxic [13] but there were indications of low levels of ether-extractable mutagenic impurities [14] bearing in mind the possible content of aromatic amines this finding is not surprising.

As Sunset yellow is partly reduced in the gut to two sulphonated aromatic amines consideration of its carcinogenic potential is essential. In the 1970's the International Agency on Research in Cancer (IARC) concluded that administered of sunset yellow to mice and rats by the oral and subcutaneous routes did not cause cancer. It commented that there was no evidence of carcinogenicity in both cases but the report acknowledged that these studies gave very little in the experimental details [15].

In 1981, the USA NTP programme reported their findings of carcinogenic bioassays in both rats and mice [16]. They found no compound related neoplastic or non-neoplastic lesions in rats following oral administration of sunset yellow. There was an increased incidence of hepatocellular carcinomas in mice at the lowest dose (12,500ppm) but there was no increase in the number of these tumours at the top dose (25000ppm) as well as considerable variability in the tumours themselves. The NTP Report therefore concluded that 'under the conditions of this bioassay there was no clear evidence of the carcinogenicity of FD and C Yellow no 6 (Sunset Yellow) in F344rats or B6CF1mice of either sex.

An 80-week study in mice also provided no evidence that Sunset Yellow is carcinogenic [10]

In another study, which demonstrated some adverse effects on reproductive and neurobehaviour due to Sunset Yellow FCF, mice were given the additive at 0 (control), 0.15, 0.30 and 0.60% (equivalent to 243, 493 and 999 mg/kg body weight per day) from 5 weeks in the FO generation to 9 weeks in the F1 generation [17]. In the low and middle-dose groups (regardless of sex) but not the upper dose the body weight of the offspring during the lactation period was increased. In the mid and upper dose males, swimming direction was affected as well as surface righting and negative geotaxis during the early lactation period was observed. In the females, the swimming head angle was also significantly affected. On the basis of this study an NOEL of 250mg/kg body weight per day would be appropriate.

2.2.2. Human data

It has been suspected for a considerable time that Sunset Yellow, on its own or in combination with other dyes, can cause allergic or pseudo-allergic reaction (PAR) [18]. This can be exacerbated in people who are already asthma or urticaria sufferers [19]. A number of drugs have food dyes such as Sunset yellow in them, which can provoke allergic reaction. Cross-sensitivity to sulphanilic acid has been cited as a possible cause [20].

In an earlier trial involving a hundred patients with urticaria (53 women and 47 men) were tested with 8 dyes, 7 preservatives and 7 antiphlogistica. Reactions were observed with the yellow dye tartrazine (20 out of 96) and Sunset Yellow (13 out of 86). 13 patients out of hundred reported immediate reactions to different foods[21]

These findings are not taken into account in the current ADI for Sunset Yellow

2.2.3. Assessment of intake

In Ireland it has been estimated that colours, emulsifiers and acids represent 18%, 13% and 12% of the total additives used. These three categories are the most commonly used additives. Colours are most frequently used in sauces [22].

As a consequence of the wide variety of products that may contain Sunset Yellow and the individual variation in their consumption reliable estimates of the total daily intake of this dye are difficult to obtain [23]. The Danish Budget Method estimates the maximum amount of the additive, based on the functional properties of the additive and the category of food [24]. This method can be modified to take into consideration the competition between different food additives with the same functional properties. It has been shown that the ADI for synthetic food dyes is exceeded in the diet of children and young people in a number of countries [25]. The extent to which the ADI for Sunset Yellow is exceeded is variable.

A related issue is the anticipation of trends in consumption over time.

One of the reasons for the uncertainty is under-reporting in food consumption surveys [26]. Individuals tend to under-report both their frequency of consumption and the portion size.

There are also well-known inaccuracies in surveys of food intake of young children. However, it has been noted that the colour of the food product can very strongly influence the food choices of children between the ages of 4 to 10 [27]. Dietary trends among American children were examined in the last three decades of the 20th century. It was shown that there were increases in intakes of soft drinks, sweets, popcorn/crisps/pretzels, which use a number of food additives, including colours [28].

In one double blind, placebo controlled study of 1873 children (age three) screened for the presence of hyperactivity, there was a considerable reduction in hyperactive behaviour in the group, which had no artificial colourings and benzoate preservatives in their diet for one week[23]. It has also been calculated that hypersensitivity reactions towards food additives are observed in between 2 and 7% of adult population [29].

It may be concluded that:

- The very limited exposure data on Sunset Yellow indicates that some individuals may consume levels close to or above the ADI. Although this is evidently undesirable it does not necessarily indicate a health risk
- There is no reason to assume that this situation will not continue unless there is firm regulatory action.

2.3. Consideration of Sudan1 - a contaminant of Sunset Yellow

Sudan 1 is of relevance here because it is a recognized toxic contaminant of sunset yellow. Sudan 1 is comprised of two amines linked by an azo bond, namely aniline and 2-hydroxy-1-naphthylamine (1-amino2-naphthol). In common with azodyes in general, Sudan 1 has a large number (almost 100) of alternative names. This is a source of potential confusion for manufacturers and regulators alike. Sudan 1 has low water solubility. It has been claimed, that the greater the insolubility of a dye in water the least likely it is to be toxic. This is almost certainly an oversimplification of the real situation for a number of reasons. It should be noted that a number of well-known genotoxic compounds such as benzo[a]pyrene, and 3-methylcholanthene are genotoxic and carcinogenic in spite of their very limited water solubility [30].

2.3.1. Sudan 1. Hazard identification and characterization.

In contrast to Sunset Yellow itself Sudan 1 a contaminant of Sunset Yellow is probably both carcinogenic and genotoxic.

lnvestigations of DNA adducts formed by treatment of Sudan 1 have been conducted and several adducts have been detected. Chromosomal aberrations were identified in vitro [31]

In bacterial studies using the Ames test, Sudan 1 was not mutagenic in TAIOO, TA1535 and TA1537 strains. Sudan 1 was also negative in TA1530 with the exception of a weak mutagenic response when liver S9 mix from hamsters was used as the source of metabolic activation. Three studies found Sudan 1 to be mutagenic in TA98, while one study reported a negative result. In a Comet genotoxicity assay, Sudan 1 produced DNA damage in the stomach and colon but not interestingly in the liver which appears to be the target site for carcinogenicity [32]

Although the picture is far from clear cut it must be concluded, on the basis of the above data that Sudan 1 is probably genotoxic although this activity appears to be of low potency. This low potency would account for the variability in results between studies.

It is probable that the critical first step for the initiation of genotoxicity is metabolic activation due to reduction of the azo bond of Sudan 1. Azo reduction takes place by means of the reductase activity of the gut microflora and also through the microsomal and cytosolic reductases present in the liver and the extrahepatic tissues. The dominant reductase activity is almost certainly due to the gut microflora. Although the direct evidence to support this for Sudan 1 itself is weak, for other azo dyes the vital role of the gut microflora has been clearly

demonstrated. The resultant aromatic amines (aniline and 2-hydroxyl-1-naphthol) are probably absorbed without further metabolism. They will therefore pass via the hepatic portal vein directly to the liver and thence to other tissues. Reactive metabolites could arise potentially from the further metabolism of the two constituent amines [33].

The International Agency for Research on Cancer (IARC), on the basis of published data classified Sudan 1 as a class three carcinogen (i.e. it is unclassifiable as to the carcinogenicity in humans[34].

2.3.2. Sudan 1. Risk characterization

The EFSA has endorsed a so called margin of exposure approach for assessing the risk from genotoxic carcinogens in food. Using this approach, the higher margin of exposure the lower should be the health risk [35]. .

To conduct such a calculation two factors must be identified:

- the exposure level (see above)
- the benchmark dose.

The bench mark dose is an estimate based on the animal carcinogenicity data of the oral dose administered for the lifetime of the animal that will produce 10% of tumours.

Using the published data (given in Table 1) [34] an approximate benchmark dose (BMDL) value can be calculated as 6 mg/kg body weight per day for male rats and 18 mg/kg body weight per day in female rats. Taking the most conservative of these two numbers i.e. 6mg/kg body weight per day the margin of exposure may be calculated:

6mg/2.5-25 ng equals 240,000 -2.400,000

Table 1. Dose-response relationship for tumour development in rats exposed to Sudan 1

Dose (mg/kg)		No of tumours	Corrected incidence %
Males	0	5	0
	25	10	10
	50	30	50
Females	0	2	0
	25	3	2
	50	10	17

EFSA proposed that a margin of exposure below 10,000 is regarded as of low concern in terms of the need for risk management [35].

Thus, the risk from the co-intake of this contaminant along with Sunset Yellow can be considered extremely small. However, it does emphasise the importance of recognizing and carrying out a proper risk assessment of potential contaminants of food additives.

3. Future food risk assessment

3.1. Societal trends

In many recent publications attention is drawn to the fact that in the 21st century new technologies, the sustainability agenda and /or changing climate will have a marked influence on our food. Moreover trends in consumer populations and habits and are emerging and these may take some time to stabilize or will simply continue to change. Inevitably, not all these trends will be consistent across the world and hence the need for risk assessment relevant to the needs of individual regions and countries will remain.

Some well established trends will continue. The most important ones are increasing urbanization and ethnic diversity. This can be observed across all continents together with increase in life expectancy and falling birth rates in the West.

There is a dichotomy of public opinion on what constitutes 'good food' of greater concern is the trend in the younger population is for decrease in knowledge and skills in basic food safety.

Another trend is a greater distrust of the quality of food produced from many suppliers. As more precise and accurate analytical methods are developed, more and more residues are bound to be identified this is unlikely to lessen public concerns. However, time spent in shopping for and preparing meals is decreasing. Consumer demand for new, exotic foods has also grown and we are eating out more than ever before. There is also a general increase in the consumption of alcohol and other mood modifying substances. The interactions in terms of human health between changing patterns of food consumption and changes in other life factors is poorly understood.

More people in the more affluent parts of the world are buying bio/organic food products and follow diets, which exclude specific groups of food, such as grains, red meat or saturated fats. Again the public health implications of these changes are uncertain.

The relative increase in cardiovascular disease, type- 2 diabetes, cancers of endocrine organs, antibiotic resistant infections observed in the 20th Century are likely to continue fro the foreseeable future. In each case, diet appears to be a contributory factor. The food industry in response is producing an increasing range of 'therapeutic' foods, with such claims as control of diabetes, or levels of cholesterol. The interaction of such foods with conventional medicines is another area of uncertainty.

In view of the public concerns over food safety the food industry is developing intelligent packaging as well as markers for product authentication. This too requires safety evaluations.

3.2. The future challenge for risk assessment

The above trends represent an additional challenge to risk assessors for in addition to the many novel substances that will be created more and more known chemicals will be identified in food and drink due to the ever improving analytical techniques. For many of these chemicals that will be identified there is likely to be little or no hazard data and in the case of contaminants in most cases no obvious 'owner' prepared to fund the necessary tests.

Greater emphasis on in vitro methods and in silico methods is inevitable but they must be used intelligently. The continual updating of the risk assessment paradigm in the light of new development in science is essential.

For the immediate future more effort needs to be taken to develop:

- the scientific basis for thresholds of toxicological concern
- better methodologies for assessing the risk from mixtures of chemicals
- methods for the identification of 'at risk' groups

It is evident that the overall objective of the risk assessment is to assess the total health impact of all the contaminants and additives present in food. As yet the methodology that would enable this is in its infancy.

3.3. Training needs

It is ironic bearing in mind the growing demand for risk assessments in both the food and non-food areas that there is an increasing shortage of high quality scientists with the expertise and willingness to carry out risk assessments in the food and non-food areas. In recent years the availability of formal training in the field has fallen almost in parallel with the growth in demand for risk assessments.

It is interesting to note that while the input from chemistry trained scientists is essential to risk assessment there is no recognized sub-discipline dealing with exposure aspects of risk assessment.

International collaboration is needed if risk assessment is to meet the demands placed upon it. Further use of the precautionary principle is not the way forward.

4. References

[1] R. Kroes, A.G. Renwick, M. Cheeseman, J. Kleiner, I. Mangelsdorf, A. Piersma, M. Schilter, J. Schlatter, F. van Schothorst, G. VOS and G. Wiirtzen, Food Chem. Toxicol. 42 (2004) 65.

[2] F.P. Guengerich and T. Shimada, Mutat. Res. 400 (1998) 201.

[3] T. Sugimura, History, present and future of heterocyclic amines, cooked food mutagens in: Heterocyclic Amines: Possible Human Carcinogens, Eds R.H. Adamson, J.A. Gustafsson, N. Ito, M. Nagao, T. Sugimura, K. Wakabayashi and Y. Yamazoe, Princeton Scientific Publishing, Princeton, NJ, 1995.

[4] A.C. Ribeiro AC and D.M.F. Salvadori, Mutat. Res. 544 (2003) 195.

[5] R. Gong, Y. Sun, J. Chen, H. Liu and C. Yang, Dyes Pigments 67 (2005) 175.

[6] P. Meller and H. Wallin, Mutat. Res. 462 (2000) 13
[7] R. Beneigni and L. Passerini, Mutat. Res.. 511 (2002) 191.
[8] L.H. Ahlstrom, C.S. Eskilsson and E. Bjorklund, Trends Anal. Chem. 24 (2005) 49.
[9] H.Y. Huang, C.L. Chuang, C.W. Chiu, M.C. Chung, Electrophoresis 26 (2005) 867.
[10] I.F. Gaunt, P.L. Mason, P. Grasso, I.S. Kiss, Food Cosmet. Toxicol. 12 (1974) 1.
[11] I.F. Gaunt, I.S. Kiss, P. Grasso, S.D. Gangolli, Food Cosmet. Toxicol. 7 (1969) 9.
[12] B.H. Ershoff, J. Nutr. 107 (1977) 822.
[13] J. Wever, R. Munzer and H.W. Renner, Environ. Mol. Mutagen. 13 (1989) 271.
[14] M.J. Prival, V.M. Davis, M.D. Peiperl, S.J. Bell, Mutat Res. 206 (1988) 247.
[15] IARC, Monograph on the evaluation of the carcinogenic risk of chemicals to man: some aromatic azo compounds. Vol 8, WHO, Lyon, 1975.
[16] NTP Program TR-208, Carcinogenesis bioassay of FD & C Yellow No6 (As No2784-94-0), 1981
[17] T. Tanaka, Toxicol. Ind. Health 12 (1996) 69.
[18] H. Mikkelsen, J.C. Larsen and F. Tarding, Arch. Toxicol. 1 (1978) 141.
[19] M. Jaworska, Z. Szulinska, M. Wilk and E. Anuszewska, J Chromatogr. A 1081 (2005) 42.
[20] C. Sortin de Leysat, M. Boone, A. Blondeel and M. Song, Dermatology 206 (2003) 379.
[21] P. Thune and A. Granholt, Dermatologica 151 (1975) 360.
[22] M.B. Gilsenan, J. Lambe and M.J. Gibney, Food Add. Contam. 19 (2002)1105.
[23] B. Bateman, J.O. Warner, E. Hutchinson, T. Dean, P. Rowlandson, C. Gant, J. Grundy, C. Fitzgerald and J. Stevenson, Arch. Dis. Child. 89 (2004) 506.
[24] M. Marchinski, Food Add. Contam. 15 (1998) 481.
[25] P. Rao, R.V. Bhat, R.V. Sudershan, T.P. Krishna and N. Naidu, Food Add. Contam. 21 (2004) 415.
[26] M.B. Gilsenan and M.J. Gibney, Food Add. Contam. 21 (2004) 195.
[27] Leon F, Couronne T, Marcuz MC and Koster EP, Food Qual. Prefer. 10 (1999) 93.
[28] E.C. Wilkinson, S.J. Mickle and J.D. Goldman, Fam. Econ. Nutr. Rev. 14 (2002) 56.
[29] M. Worm, I. Ehlers, W. Sherry and T. Zuberbier, Clin. Exp. Allergy 30 (2000) 407.
[30] IARC Publications list, WHO Lyon, France, 2006.
[31] J.L. Ivett, B.M. Brown, C. Rodgers, B.E. Anderson, M.A. Resnick and E. Zeiger, Environ. mol. Mutagen. 14 (1989) 165.
[32] S. Tsuda, N. Matsusaka, H. Madarame, S. Ueno, N. Susa, N. Ishida, N. Kawamura, K. Sekihashi and Y. Sasaki, Mutat. Res. 465 (2000) 1.
[33] K.T. Chung, S.E. Stevens and C.E. Cerniglia, Crit. Rev. Microbiol. 18 (1992) 175.
[34] IARC, Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Overall evaluation of carcinogenicity: an updating of IARC monographs 1987 1-42(Suppl 7)
[35] EFSA, Opinion of the Scientific Committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic (adopted 18th October 2005).

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 4

Quality Assurance

Roger Wood

Food Standards Agency, c/o Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK.

1. Introduction

It is now internationally recognised that for a laboratory to produce consistently reliable data it must implement an appropriate programme of quality assurance measures; this is particularly the case when trace constituents, such as natural toxicants, trace elements, allergens and GMOs, the subject of this book, are to be determined. However the same considerations apply to all analytical determinations. Amongst such measures is the need for the laboratory to demonstrate that the methods it uses are "fit-for-purpose" and in statistical control, and to participate in proficiency testing schemes. This chapter surveys some of the essential elements that a laboratory has to consider when wishing to implement a programme of quality assurance measures.

Whilst it is generally recognised that it is necessary for the above measures to be implemented, in the food sector control laboratories determining trace constituents in the European Union are also required to do so by legislative requirements. These are described below as they provide good guidance as to the quality assurance measures that all laboratories should follow to produce reliable data.

Other International Organisations, most notably the Codex Alimentarius Commission, are taking a similar approach as the European Union. The Codex approach is therefore also described in this chapter.

1.1. European Union - Food control Directives

Methods of analysis have been prescribed by legislation for a number of foodstuffs since the formation of the European Community, now European Union. However, the Union now recognises that the quality of results from a laboratory is equally as important as the method used to obtain the results. This is best illustrated by consideration of the Council Directive on the Official Control of Foodstuffs (OCF) which was adopted by the Community in June 1989 [EEC, 1989], and the Council Directive on Additional Measures Concerning the Food Control of Foodstuffs (AMFC) [EEC, 1993].

Both these directives have recently been repealed and replaced by the "Council Regulation 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules" (OFFC) [EC, 2004]. The majority of the provisions of this Regulation came into force on the 1st January 2006.

The essential elements of that Regulation in the laboratory quality area are given in Article 11 (and the associated Annex III) and in Article 12:

Article 11 (Methods of sampling and analysis):

1. Sampling and analysis methods used in the context of official controls shall comply with relevant Community rules or,
   (a) if no such rules exist, with internationally recognised rules or protocols, for example those that the European Committee for standardisation (CEN) has accepted or those agreed in national legislation; o
   (b) in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols.
2. Where paragraph 1 does not apply, validation of methods of analysis may take place within a single laboratory according to an internationally accepted protocol.
3. Wherever possible, methods of analysis shall be characterised by the appropriate criteria set out in Annex III.
4. The following implementing measures may be taken in accordance with the procedure referred to in Article 62(3)
   (a) methods of sampling and analysis, including the confirmatory or reference methods to be used in the event of a dispute;
   (b) performance criteria, analysis parameters, measurement uncertainty and procedures for the validation of the methods referred to in (a); and
   (c) rules on the interpretation of results.
5. The competent authorities shall establish adequate procedures in order to guarantee the right of feed and food business operators whose products are subject to sampling and analysis to apply for a supplementary expert opinion, without prejudice to the obligation of competent authorities to take prompt action in case of emergency.
6. In particular, they shall ensure that feed and food business operators can obtain sufficient numbers of samples for a supplementary expert opinion, unless impossible in case of highly perishable products or very low quantity of available substrate.
7. Samples must be handled and labelled in such a way as to guarantee both their legal and analytical validity.

ANNEX III (Characterisation of methods of analysis):

1. Methods of analysis should be characterised by the following criteria:
   (a) accuracy;
   (b) applicability (matrix and concentration range);
   (c) limit of detection;
   (d) limit of determination;
   (e) precision;

(f) repeatability;
(g) reproducibility;
(h) recovery;
(i) selectivity;
(j) sensitivity;
(k) linearity;
(l) measurement uncertainty;
(m) other criteria that may be selected as required.

2. The precision values referred to in 1(e) shall either be obtained from a collaborative trial which has been conducted in accordance with an internationally recognised protocol on collaborative trials (e.g. ISO 5725:1994 or the IUPAC International Harmonised Protocol) or, where performance criteria for analytical methods have been established, be based on criteria compliance tests. The repeatability and reproducibility values shall be expressed in an internationally recognised form (e.g. the 95% confidence intervals as defined by ISO 5725:1994 or IUPAC). The results from the collaborative trial shall be published or freely available.
3. Methods of analysis which are applicable uniformly to various groups of commodities should be given preference over methods which apply only to individual commodities.
4. In situations where methods of analysis can only be validated within a single laboratory then they should be validated in accordance with e.g. IUPAC Harmonised Guidelines, or where performance criteria for analytical methods have been established, be based on criteria compliance tests.
5. Methods of analysis adopted under this Regulation should be edited in the standard layout for methods of analysis recommended by the ISO.

Article 12 (Official laboratories):

1. The competent authority shall designate laboratories that may carry out the analysis of samples taken during official controls.
2. However, competent authorities may only designate laboratories that operate and are assessed and accredited in accordance with the following European Standards:
   (a) EN ISO/IEC 17025 on "General requirements for the competence of testing and calibration laboratories";
   (b) EN 45002 on "General criteria for the assessment of testing laboratories";
   (c) EN 45003 on "Calibration and testing laboratory accreditation system-General requirements for operation and recognition",

   taking into account criteria for different testing methods laid down in Community feed and food law.
3. The accreditation and assessment of testing laboratories referred to in paragraph 2 may relate to individual tests or groups of tests.

4. The competent authority may cancel the designation referred to in paragraph 1 when the conditions referred to in paragraph 2 are no longer fulfilled.

There is further information on the role and duties of Community and National Reference Laboratories. Although such laboratories have a limited role in the EU at the present time, it may be anticipated that the Commission will wish to expand both the areas covered and scope of such laboratories.

As a result of the adoption of the above Regulations legislation is now in place to ensure that there is confidence in food analysis laboratories. The legislation requirements serve as a valid model for the quality assurance measures that laboratories analysing for toxicants should follow.

1.2. Codex Alimentarius commission

The Codex Committee on Methods of Analysis and Sampling has also developed objective criteria for assessing the competence of testing laboratories involved in the official import and export control of foods. These were recommended by the Committee at its Twenty-first Session [FAO, 1997] and mirror the EU recommendations for laboratory quality standards and methods of analysis.

The Codex recommendations are that laboratories:

1. Comply with the general criteria for testing laboratories laid down in ISO/IEC Guide 25:1990 “General requirements for the competence of calibration and testing laboratories” [ISO, 1993],
2. Participate in appropriate proficiency testing schemes for food analysis which conform to the requirements laid down in “The International Harmonised Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories”, Pure & Appl. Chem. 65 (1993) 2132-2144 (as adopted for Codex purposes by the Codex Alimentarius Commission at its 21st Session in July 1995) [Thompson & Wood, 1993],
3. Whenever available, use methods of analysis which have been validated according to the principles laid down by the Codex Alimentarius Commission (these are identical to the requirements laid down by the EU in the Methods of Analysis and Sampling Directive [EEC, 1985]; and
4. Use internal quality control procedures, such as those described in the “Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories”, Pure & Appl. Chem, 67 (1995) 649-666 [Thompson and Wood, 1995].

The Committee noted that the compliance with the above criteria for laboratories involved in the official import and export control of foods needed to be assessed by suitable mechanisms. The bodies assessing the laboratories should comply with the general criteria for laboratory accreditation, such as those laid down in ISO/IEC Guide 58:1993, “Calibration and testing laboratory accreditation systems - General requirements for operation and recognition” [ISO, 1993].

The Codex requirements are becoming of increasing importance because of the acceptance of Codex Standards in the World Trade Organisation agreements.

The effect of the Official Feed and Food Control (OFFC) Regulation and the Codex requirements is that food control laboratories must consider the following aspects within the laboratory:

- the organisation of the laboratory,
- how well the laboratory actually carries out analyses, and
- the methods of analysis used in the laboratory.

All these aspects are inter-related, but in simple terms may be thought of as:

- becoming accredited to an Internationally Recognised Standard; such accreditation is aided by the use of internal quality control procedures,
- participating in proficiency schemes, and
- using validated methods.

These considerations will be addressed in turn, as will other quality assurance measures that a laboratory should consider, e.g. the use of recovery factors.

2. Accreditation

Although to be formally accredited is not a necessity to ensure that a laboratory will produce "quality data", an accredited laboratory is able to state that it has been third party assessed which does give additional assurance to its "customers".

The OFFC Regulation requires that all feed and food control laboratories should be accredited to the ISO Standard 17025 [ISO, 2005], the latest version of which was published in 2005. In the EU Member States Governments nominate the accreditation service to carry out the required accreditation under the Regulation. For example, in the UK the United Kingdom Accreditation Service (UKAS) is nominated to carry out the accreditation of official feed and food control laboratories for all the aspects prescribed in the Regulation. However, as the accreditation agency will also be required to comply to EN 45003 Standard and to carry out assessments in accordance with the EN 45002 Standard, any other accreditation agencies that are members of the European Co-operation for Accreditation of Laboratories (EA) may also be nominated to carry out the accreditation. Similar procedures are followed in the other Member States, all having developed or are developing equivalent organisations to UKAS. Laboratories carrying out official control analyses in the toxicants areas will have to be accredited. Other (non-control) laboratories may wish to be seen to be of the same standard in order that confidence can be placed in their work.

In the UK it has been the normal practice for UKAS to accredit the scope of laboratories on a method-by-method basis. However, in the case of official food control laboratories undertaking non-routine or investigative chemical analysis, it is accepted that it is not practical to use an accredited fully documented method in the conventional sense, i.e. which

specifies each sample type and analyte. Such laboratories must have a protocol defining the approach to be adopted including requirements for validation of methods and internal quality control. Full details of procedures used, including instrumental parameters, must be recorded at the time of each analysis in order to enable the procedure to be repeated in the same manner at a later date. Thus, for official food control laboratories undertaking analysis, appropriate methods may be accredited on a generic basis with such generic accreditation being underpinned where necessary by specific method accreditation. This approach is particularly appropriate to laboratories carrying out toxicants work where the methodology may be continuously developed and where a laboratory may wish for any "research" activities to be regarded as being accredited as well as its defined methods used on a routine basis.

3. Internal quality control: harmonised guidelines for internal quality control in analytical chemistry laboratories

An integral part of the accreditation process is the introduction of acceptable Internal Quality Control (IQC) procedures. IQC is one of a number of concerted measures that analytical chemists can take to ensure that the data produced in the laboratory are of known quality and uncertainty. In practice this is determined by comparing the results achieved in the laboratory at a given time with a standard. IQC therefore comprises the routine practical procedures that enable the analyst to accept a result or group of results or reject the results and repeat the analysis.

IQC is undertaken by the inclusion of particular reference materials, "control materials", into the analytical sequence and by duplicate analysis.

ISO, IUPAC and AOAC INTERNATIONAL have co-operated to produce agreed protocols on the "Design, Conduct and Interpretation of Collaborative Studies" [Horwitz, 1988 and 1995] and on the "Proficiency Testing of (Chemical) Analytical Laboratories" [Thompson and Wood, 1993]. The Working Group that produced these protocols has prepared a further protocol on the internal quality control of data produced in analytical laboratories. The document was finalised in 1994 and published in 1995 as the "Harmonised Guidelines For Internal Quality Control In Analytical Chemistry Laboratories" [Thompson and Wood, 1995].

The use of the procedures outlined in the Protocol should aid compliance with the accreditation requirements specified above.

3.1. Basic concepts

The protocol sets out guidelines for the implementation of internal quality control (IQC) in analytical laboratories. IQC is one of a number of concerted measures that analytical chemists can take to ensure that the data produced in the laboratory are fit for their intended purpose. In practice, fitness for purpose is determined by a comparison of the accuracy achieved in a laboratory at a given time with a required level of accuracy. Internal quality control therefore

comprises the routine practical procedures that enable the analytical chemist to accept a result or group of results as fit for purpose, or reject the results and repeat the analysis. As such, IQC is an important determinant of the quality of analytical data, and is recognised as such by accreditation agencies.

Internal quality control is undertaken by the inclusion of particular reference materials, called "control materials", into the analytical sequence and by duplicate analysis. The control materials should, wherever possible, be representative of the test materials under consideration in respect of matrix composition, the state of physical preparation and the concentration range of the analyte. As the control materials are treated in exactly the same way as the test materials, they are regarded as surrogates that can be used to characterise the performance of the analytical system, both at a specific time and over longer intervals. Internal quality control is a final check of the correct execution of all of the procedures (including calibration) that are prescribed in the analytical protocol and all of the other quality assurance measures that underlie good analytical practice. IQC is therefore necessarily retrospective. It is also required to be as far as possible independent of the analytical protocol, especially the calibration, that it is designed to test.

Ideally both the control materials and those used to create the calibration should be traceable to appropriate certified reference materials or a recognised empirical reference method. When this is not possible, control materials should be traceable at least to a material of guaranteed purity or other well characterised material. However, the two paths of traceability must not become coincident at too late a stage in the analytical process. For instance, if control materials and calibration standards were prepared from a single stock solution of analyte, IQC would not detect any inaccuracy stemming from the incorrect preparation of the stock solution.

In a typical analytical situation several, or perhaps many, similar test materials will be analysed together, and control materials will be included in the group. Often determinations will be duplicated by the analysis of separate test portions of the same material. Such a group of materials is referred to as an analytical "run". (The words "set", "series" and "batch" have also been used as synonyms for "run".) Runs are regarded as being analysed under effectively constant conditions. The batches of reagents, the instrument settings, the analyst, and the laboratory environment will, under ideal conditions, remain unchanged during analysis of a run. Systematic errors should therefore remain constant during a run, as should the values of the parameters that describe random errors. As the monitoring of these errors is of concern, the run is the basic operational unit of IQC.

A run is therefore regarded as being carried out under repeatability conditions, i.e., the random measurement errors are of a magnitude that would be encountered in a "short" period of time. In practice the analysis of a run may occupy sufficient time for small systematic changes to occur. For example, reagents may degrade, instruments may drift, minor adjustments to instrumental settings may be called for, or the laboratory temperature may rise.

However, these systematic effects are, for the purposes of IQC, subsumed into the repeatability variations. Sorting the materials making up a run into a randomised order converts the effects of drift into random errors.

3.2. Scope of the guidelines

The guidelines are a harmonisation of IQC procedures that have evolved in various fields of analysis, notably clinical biochemistry, geochemistry, environmental studies, occupational hygiene and food analysis. There is much common ground in the procedures from these various fields. However, analytical chemistry comprises an even wider range of activities, and the basic principles of IQC should be able to encompass all of these. The Guidelines will be applicable in the great majority of instances although there are a number of IQC practices that are restricted to individual sectors of the analytical community and so not included in the guidelines.

In order to achieve harmonisation and provide basic guidance on IQC, some types of analytical activity have been excluded from the guidelines. Issues specifically excluded are as follows:

(i) Quality control of sampling. While it is recognised that the quality of the analytical result can be no better than that of the sample, quality control of sampling is a separate subject and in many areas is not yet fully developed. Moreover, in many instances analytical laboratories have no control over sampling practice and quality.

(ii) In-line analysis and continuous monitoring. In this style of analysis there is no possibility of repeating the measurement, so the concept of IQC as used in the Guidelines is inapplicable.

(iii) Multivariate IQC. Multivariate methods in IQC are still the subject of research and cannot be regarded as sufficiently established for inclusion in the Guidelines. The current document regards multianalyte data as requiring a series of univariate IQC tests. Caution is necessary in the interpretation of this type of data to avoid inappropriately frequent rejection of data.

(iv) Statutory and contractual requirements.

(v) Quality assurance measures such as pre-analytical checks on instrumental stability, wavelength calibration, balance calibration, tests on resolution of chromatography columns, and problem diagnostics are not included. For present purposes they are regarded as part of the analytical protocol, and IQC tests their effectiveness together with the other aspects of the methodology.

3.3. Internal quality control and uncertainty

A prerequisite of analytical chemistry is the recognition of “fitness for purpose”, the standard of accuracy that is required for an effective use of the analytical data. This standard is arrived at by consideration of the intended uses of the data although it is seldom possible to

foresee all of the potential future applications of analytical results. For this reason, in order to prevent inappropriate interpretation, it is important that a statement of the uncertainty should accompany analytical results, or be readily available to those who wish to use the data. This is now a specific requirement of the ISO 17025 Accreditation Standard.

Strictly speaking, an analytical result cannot be interpreted unless it is accompanied by knowledge of its associated uncertainty at a stated level of confidence. A simple example demonstrates this principle. Suppose that there is a statutory requirement that a foodstuff must not contain more than 10 mg/kg of a particular constituent. A manufacturer analyses a batch and obtains a result of 9 mg/kg for that constituent. If the uncertainty on the result expressed as a half range (assuming no sampling error) is 0.1 mg/kg (i.e. the true result falls, with a high probability, within the range 8.9 9.1) then it may be assumed that the legal limit is not exceeded. If, in contrast, the uncertainty is 2 mg/kg then there is no such assurance. The interpretation and use that may be made of the measurement thus depends on the uncertainty associated with it.

Analytical results should therefore have an associated uncertainty if any definite meaning is to be attached to them or an informed interpretation made. If this requirement cannot be fulfilled, the use to which the data can be put is limited. Moreover, the achievement of the required measurement uncertainty must be tested as a routine procedure, because the quality of data can vary, both in time within a single laboratory and between different laboratories. IQC comprises the process of checking that the required uncertainty is achieved in a run.

3.4. Recommendations in the guidelines

The following recommendations represent integrated approaches to IQC that are suitable for many types of analysis and applications areas. Managers of laboratory quality systems will have to adapt the recommendations to the demands of their own particular requirements. Such adoption could be implemented, for example, by adjusting the number of duplicates and control material inserted into a run, or by the inclusion of any additional measures favoured in the particular application area. The procedure finally chosen and its accompanying decision rules must be codified in an IQC protocol that is separate from the analytical system protocol.

The practical approach to quality control is determined by the frequency with which the measurement is carried out and the size and nature of each run. The following recommendations are therefore made. (The use of control charts and decision rules are covered in Appendix 1 to the guidelines.)

In all of the following the order in the run in which the various materials are analysed should be randomised if possible. A failure to randomise may result in an underestimation of various components of error.

(i) *Short (e.g., n<20) frequent runs of similar materials.* Here the concentration range of the analyte in the run is relatively small, so a common value of standard deviation can be assumed.

Insert a control material at least once per run. Plot either the individual values obtained, or the mean value, on an appropriate control chart. Analyse in duplicate at least half of the test materials, selected at random. Insert at least one blank determination.

(ii) *Longer (e.g., n>20) frequent runs of similar materials.* Again a common level of standard deviation is assumed.
Insert the control material at an approximate frequency of one per ten test materials. If the run size is likely to vary from run to run it is easier to standardise on a fixed number of insertions per run and plot the mean value on a control chart of means. Otherwise plot individual values. Analyse in duplicate a minimum of five test materials selected at random. Insert one blank determination per ten test materials.

(iii) *Frequent runs* containing similar materials but with a wide range of analyte concentration. Here we cannot assume that a single value of standard deviation is applicable.
Insert control materials in total numbers approximately as recommended above. However, there should be at least two levels of analyte represented, one close to the median level of typical test materials, and the other approximately at the upper or lower decile as appropriate. Enter values for the two control materials on separate control charts. Duplicate a minimum of five test materials, and insert one procedural blank per ten test materials.

(iv) *Ad hoc analysis*. Here the concept of statistical control is not applicable. It is assumed, however, that the materials in the run are of a single type.
Carry out duplicate analysis on all of the test materials. Carry out spiking or recovery tests or use a formulated control material, with an appropriate number of insertions (see above), and with different concentrations of analyte if appropriate. Carry out blank determinations. As no control limits are available, compare the bias and precision with fitness-for-purpose limits or other established criteria..

By following the above recommendations laboratories would introduce internal quality control measures which are an essential aspect of ensuring that data released from a laboratory are fit-for-purpose. If properly executed, quality control methods can monitor the various aspects of data quality on a run-by-run basis. In runs where performance falls outside acceptable limits, the data produced can be rejected and, after remedial action on the analytical system, the analysis can be repeated.

The guidelines stress, however, that internal quality control is not foolproof even when properly executed. Obviously it is subject to “errors of both kinds”, i.e., runs that are in control will occasionally be rejected and runs that are out of control occasionally accepted. Of more importance, IQC cannot usually identify sporadic gross errors or short term disturbances in the analytical system that affect the results for individual test materials. Moreover, inferences based on IQC results are applicable only to test materials that fall within

the scope of the analytical method validation. Despite these limitations, which professional experience and diligence can alleviate to a degree, internal quality control is the principal recourse available for ensuring that only data of appropriate quality are released from a laboratory. When properly executed it is very successful.

The guidelines also stress that the perfunctory execution of any quality system will not guarantee the production of data of adequate quality. The correct procedures for feedback, remedial action and staff motivation must also be documented and acted upon. In other words, there must be a genuine commitment to quality within a laboratory for an internal quality control programme to succeed, i.e., the IQC must be part of a complete quality management system.

4. Proficiency testing

The need for laboratories carrying out analytical determinations to demonstrate to their "customers" that they are doing so competently has become paramount. Thus laboratories are required to become accredited, to use fully validated methods and to successfully participate in proficiency testing schemes. Proficiency testing has assumed a far greater importance than previously and, during negotiation on the AMFC Directive, it was agreed that proficiency testing schemes will comply with the requirements of the ISO/IUPAC/AOAC INTERNATIONAL Harmonised Protocol For Proficiency Testing Of (Chemical) Analytical Laboratories [Thompson and Wood, 1993], thus eliminating the potential for different interpretations on the requirement for proficiency testing under the EN 45,000 Standards.

The essential elements of proficiency testing which have now been accepted within the Union and laboratories carrying out toxicants work must regard these as an integral part of their quality assurance activities. For that reason proficiency testing is described in detail below:

4.1. What is proficiency testing?

A proficiency testing scheme is defined as a system for objectively checking laboratory results by an external agency. It includes comparison of a laboratory's results at intervals with those of other laboratories, the main object being the establishment of trueness.

Proficiency testing schemes are based on the regular circulation of homogeneous samples by a co-ordinator, analysis of samples (normally by the laboratory's method of choice) and an assessment of the results. However, although many organisations carry out such schemes, it is only recently that the International Protocol has been agreed and being used by many proficiency testing scheme organisers.

4.2. Why proficiency testing is important ?

Participation in proficiency testing schemes provides laboratories with a means of objectively assessing, and demonstrating, the reliability of the data they produce. Although there are several types of schemes, they all share a common feature of comparing test results obtained by one testing laboratory with those obtained by other testing laboratories. Schemes may be "open" to any laboratory or participation may be invited. Schemes may set out to assess the competence of laboratories undertaking a very specific analysis (e.g. lead in blood) or more general analysis (e.g. food analysis). Although accreditation and proficiency testing are separate exercises, accreditation assessments will increasingly use proficiency testing data. It is now recommended by ISO Standard 17025 [ISO, 2005], the prime standard to which accreditation agencies operate, that such agencies require laboratories seeking accreditation to participate in an appropriate proficiency testing scheme before accreditation is gained.

4.3. ISO/IUPAC/AOAC INTERNATIONAL harmonised protocol for proficiency testing of (chemical) analytical laboratories

The International Standardising Organisations, AOAC INTERNATIONAL, ISO and IUPAC have co-operated to produce an agreed "International Harmonised Protocol For Proficiency Testing Of (Chemical) Analytical Laboratories" [Thompson & Wood, 1993]. That protocol is recognised within the food sector of the European Union and also by the Codex Alimentarius Commission. The protocol makes the following recommendations about proficiency testing, all of which are importance to laboratories carrying out analysis for toxicants:

4.3.1. Organisation of proficiency testing schemes

Framework

Samples must be distributed regularly to participants who are to return results within a given time. The results will be statistically analysed by the organiser and participants will be notified of their performance. Advice will be available to poor performers and participants will be kept fully informed of the scheme's progress. Participants will be identified by code only, to preserve confidentiality.

The scheme's structure for any one analyte or round in a series should be:

- Samples prepared;
- Samples distributed regularly;
- Participants analyse samples and report results;
- Results analysed and performance assessed;
- Participants notified of their performance;
- Advice available for poor performers, on request;

- Co-ordinator reviews performance of scheme;
- Next round commences.

Organisation

The running of the scheme will be the responsibility of a co-ordinating laboratory/organisation. Sample preparation will either be contracted out or undertaken in house. The co-ordinating laboratory must be of high reputation in the type of analysis being tested. Overall management of the scheme should be in the hands of a small steering committee (Advisory Panel) having representatives from the co-ordinating laboratory (who should be practising laboratory scientists), contract laboratories (if any), appropriate professional bodies and ordinary participants.

Samples

The samples to be distributed must be generally similar in matrix to the unknown samples that are routinely analysed (in respect of matrix composition and analyte concentration range). It is essential they are of acceptable homogeneity and stability. The bulk material prepared must be effectively homogeneous so that all laboratories will receive samples that do not differ significantly in analyte concentration.

The procedure to be followed to test for sample heterogeneity is:

- Prepare whole of bulk material in a satisfactory form.
- Divide the material into the containers.
- Select between 10-20 containers randomly.
- Homogenise contents of each selected container.
- From each take two test portions.
- Analyse portions in random order as one batch.
- Estimate sampling variance (one-way variance analysis, without exclusion of outliers).
- Assign σ as a precision standard at mean analyte value. (i.e. the target value for the standard deviation for the proficiency test at the analyte concentration of interest).
- For effective homogeneity $\sqrt{}$ of sampling variance / $\sigma < 0.3$.

The co-ordinating laboratory should also show that the bulk sample is sufficiently stable throughout the duration of the proficiency test. Thus, prior to sample distribution, the stability of the sample matrix and of the analyte must be assessed after appropriate storage. Ideally the quality checks on prepared samples should be performed by a second laboratory although it is recognised that this would probably cause considerable difficulty to the co-ordinating laboratory. The number of samples to be distributed per round for each analyte should not be more than five.

Frequency of sample distribution

Ideally sample distribution frequency in any one series should not be more than every 2 weeks and not less then every 4 months. A frequency greater than once every 2 weeks could lead to problems in turn-round of samples and results. If the period between distributions extends much beyond 4 months, there will be unacceptable delays in identifying analytical problems and the impact of the scheme on participants will be small. The frequency also relates to the field of application and amount of internal quality control that is required for that field. Thus, although the frequency range stated above should be adhered to, there may be circumstances where it is acceptable for a longer time scale between sample distribution, e.g. if sample throughput per annum is very low. Advice on this respect would be a function of the Advisory Panel.

Estimating the assigned value (the "true" result)

There are a number of possible approaches to determining the nominally "true" result for a sample but only three are normally considered. The result may be established from the amount of analyte added to the samples by the laboratory preparing the sample; alternatively, a "reference" laboratory (or group of such expert laboratories) may be asked to measure the concentration of the analyte using definitive methods or thirdly, the results obtained by the participating laboratories (or a substantial sub-group of these) may be used as the basis for the nominal "true" result. The organisers of the scheme should provide the participants with a clear statement giving the basis for the assignment of reference values which should take into account the views of the Advisory Panel.

Choice of analytical method

Participants can use the analytical method of their choice except when otherwise instructed to adopt a specified method. It is recommended that all methods should be properly validated before use. In situations where the analytical result is method-dependent the true value will be assessed using those results obtained using a defined procedure. If participants use a method which is not "equivalent" to the defining method, then an automatic bias in result will occur when their performance is assessed.

Performance criteria

For each analyte in a round a criterion for the performance score may be set, against which the score obtained by a laboratory can be judged. A "running score" could be calculated to give an assessment of performance spread over a longer period of time.

Reporting results

Reports issued to participants should include data on the results from all laboratories together with participant's own performance score. The original results should be presented to

enable participants to check correct data entry. Reports should be made available before the next sample distribution.

Although all results should be reported, it may not be possible to do this in very extensive schemes (e.g. 700 participants determining 20 analytes in a round). Participants should, therefore, receive at least a clear report with the results of all laboratories in histogram form.

Liaison with participants

Participants should be provided with a detailed information pack on joining the scheme. Communication with participants should be by newsletter or annual report together with a periodic open meeting; participants should be advised of changes in scheme design. Advice should be available to poor performers. Feedback from laboratories should be encouraged so participants contribute to the scheme's development. Participants should view it as their scheme rather than one imposed by a distant bureaucracy.

Collusion and falsification of results

Collusion might take place between laboratories so that independent data are not submitted. Proficiency testing schemes should be designed to ensure that there is as little collusion and falsification as possible. For example, alternative samples could be distributed within a round. Also instructions should make it clear that collusion is contrary to professional scientific conduct and serves only to nullify the benefits of proficiency testing.

Statistical Procedure for the analysis of results

The first stage in producing a score from a result x (a single measurement of analyte concentration in a test material) is to obtain an estimate of the bias, thus:

$$\text{bias} = x - X$$

where X is the true concentration or amount of analyte.

The efficacy of any proficiency test depends on using a reliable value for X. Several methods are available for establishing a working estimate of χ (i.e. the assigned value, outlined above).

Formation of a z-score

Most proficiency testing schemes compare bias with a standard error. An obvious approach is to form the z-score given by:

$$z = (x - \chi)/\sigma$$

where σ is a standard deviation. σ could be either an estimate of the actual variation encountered in a particular round (š) estimated from the laboratories' results after outlier elimination or a target representing the maximum allowed variation consistent with valid data. This is the procedure recommended in the International Protocol and hence that to be followed in the European Union.

A fixed target value for σ is preferable and can be arrived at in several ways. It could be fixed arbitrarily, with a value based on a perception of how laboratories should perform. It could be an estimate of the precision required for a specific task of data interpretation. σ could be derived from a model of precision, such as the "Horwitz Curve" [Horwitz, 1982]. However, while this model provides a general picture of reproducibility, substantial deviation from it may be experienced for particular methods. In the case of toxicants data on the target deviations will have been drawn from both collaborative trials and from predictions using the Horwitz Curve.

Interpretation of z-scores

If and σ are good estimates of the population mean and standard deviation then z will be approximately normally distributed with a mean of zero and unit standard deviation. An anlytical result is described as "well behaved" when it complies with this condition.

An absolute value of z ($|z|$) greater than three suggests poor performance in terms of accuracy. This judgement depends on the assumption of the normal distribution, which, outliers apart, seems to be justified in practice.

As z is standardised, it is comparable for all analytes and methods. Thus values of z can be combined to give a composite score for a laboratory in one round of a proficiency test.

The z-scores can therefore be interpreted as follows:

$|z| < 2$ "Satisfactory": will occur in 95% cases produced by "well behaved results"

$2 < |z| < 3$ "Questionable": but will occur in ≈ 5% of cases produced by "well behaved results"

$|z| > 3$ "Unsatisfactory": will only occur in ≈ 0.1% of cases produced by "well behaved results"

National proficiency schemes

Because of the European Union requirements a number of Member States have introduced proficiency testing schemes in the food area. Most operate according to the Harmonised Protocol and thus conform to the requirements internationally accepted for the operation of proficiency testing schemes in the food sector.

The International Protocol has now been revised and the re-draft will be published in early 2006 [Thompson, Ellison and Wood, 2006]. The essential principles of the revised protocol remain as given above.

5. Methods of analysis

Methods of analysis are a vital part of the quality assurance measures that a laboratory carrying out the analysis of toxicants must consider. The aspects that a laboratory must consider is best achieved by reference to the statutory requirements for methods of analysis in

the food sector in both the EU and Codex. In addition, the requirements of the AOAC INTERNATIONAL and the European Committee for Standardization (CEN) are described as those organisations are important sources of validated methods of analysis in the toxicants area. Indeed, CEN is specifically referenced in the OFFC Regulation.

5.1. AOAC International (AOACI)

This organisation has required for many years that all of its methods, which are quoted world-wide, be collaboratively tested before being accepted for publication. The procedure for carrying out the necessary collaborative trials is well defined and has formed part of the internal requirements of the organisation for many years. In addition, methods are adopted by the organisation on a preliminary/tentative basis (as "Official First Action Methods") before being adopted as "Official Final Action Methods" by the organisation after at least two years in use by analysts on a routine basis. This procedure ensures that the methods are satisfactory on a day-to-day basis before being finally accepted by the AOACI.

The AOAC INTERNATIONAL has also developed two other procedures for "method validation" which, though not dealing directly with the production of methods of analysis which have been validated by collaborative trial, do result in some validation. These are:

- Peer validated methods - in which a method of analysis is assessed in a very limited number of laboratories (usually three) and an assessment made of its performance characteristics, and
- Methods assessed by the AOAC Research Institute - such methods are normally proprietary products (e.g. "test kits") for which a "certificate" of conformance with specification is issued by the Research Institute.

5.2. The European Union

The Union attempted to harmonise sampling and analysis procedures in an attempt to meet the current demands of the national and inter-national enforcement agencies and the likely increased problems that the open market will bring. To aid this the Union issued a Directive on Sampling and Methods of Analysis [EEC, 1985]. The Directive contained a technical annex, in which the need to carry out a collaborative trial before a method in the food sector can be adopted by the Union is emphasised but which important information for all food analysis laboratories.

The requirements of that Directive have been incorporated into the specifications for methods of analysis given in the OFFC Regulation. The text from Article 11 and the associated Annex III of the Regulation is given above.

5.3. The Codex Alimentarius Commission

This was the first International Organisation working at the government level in the food sector which laid down principles for the establishment of its methods. That it was necessary

for such guidelines and principles to be laid down reflects the confused and unsatisfactory situation in the development of legislative methods of analysis that existed until the early 1980's in the food sector.

The "Principles For The Establishment Of Codex Methods Of Analysis" [FAO, 1995] are given below; other organisations which subsequently laid down procedures for the development of methods of analysis in their particular sector followed these principles to a significant degree.

5.3.1. Principles for the establishment of Codex methods of analysis

Purpose of Codex methods of analysis

The methods are primarily intended as international methods for the verification of provisions in Codex standards. They should be used for reference, in calibration of methods in use or introduced for routine examination and control purposes.

Methods of analysis

(A) Definition of types of methods of analysis

(a) Defining Methods (Type I)

Definition: A method which determines a value that can only be arrived at in terms of the method per se and serves by definition as the only method for establishing the accepted value of the item measure.

Examples: Howard Mould Count, Reichert-Meissl value, loss on drying, salt in brine by density.

(b) Reference Methods (Type II)

Definition: A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.

Example: Potentiometric method for halides.

(c) Alternative Approved Methods (Type III)

Definition: A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

Example: Volhard Method or Mohr Method for chlorides.

(d) Tentative Method (Type IV)

Definition: A Type IV Method is a method which has been used traditionally or else has been recently introduced but for which the criteria required for acceptance by the Codex Committee on Methods of Analysis and Sampling have not yet been determined.

Examples: chlorine by X-ray fluorescence, estimation of synthetic colours in foods.

(B) General Criteria for the Selection of Methods of Analysis

(a) Official methods of analysis elaborated by international organisations occupying themselves with a food or group of foods should be preferred.

(b) Preference should be given to methods of analysis the reliability of which have been established in respect of the following criteria, selected as appropriate:

- specificity
- accuracy
- precision; repeatability intra-laboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories)
- limit of detection
- sensitivity
- practicability and applicability under normal laboratory conditions
- other criteria which may be selected as required.

(c) The method selected should be chosen on the basis of practicability and preference should be given to methods which have applicability for routine use.

(d) All proposed methods of analysis must have direct pertinence to the Codex Standard to which they are directed.

(e) Methods of analysis which are applicable uniformly to various groups of commodities should be given preference over methods which apply only to individual commodities.

5.4. European Committee for Standardization (CEN)

In Europe the International Organisation which is now developing most standardised methods of analysis in the food additive and contaminant area is the European Committee for Standardization (CEN). Although CEN methods are not prescribed by legislation, the European Commission does place considerable importance on the work that CEN carries out in the development of specific methods in the food sector, as indicated by its reference in the OFFC Regulation. In addition it should be noted that CEN has been given direct mandates by the Commission to publish particular methods, e.g. those for the detection of food irradiation. Because of this some of the methods in the food sector being developed or which have been published by CEN in areas of interest to this book are outlined in Annex I to this Chapter.

CEN, like the other organisations described above, has adopted a set of guidelines to which its Methods Technical Committees should conform when developing a method of analysis. The guidelines are:

"Details of the interlaboratory test on the precision of the method are to be summarised in an annex to the method. It is to be stated that the values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than given in annex.

The precision clauses shall be worded as follows:

Repeatability: "The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability value r in not more than 5% of the cases.

The value(s) is (are): ...."

Reproducibility: "The absolute difference between two single test results on identical test material reported by two laboratories will exceed the reproducibility value R in not more than 5% of the cases.

The value(s) is (are): ...."

There shall be minimum requirements regarding the information to be given in an Informative Annex, this being:

- Year of interlaboratory test and reference to the test report (if available)
- Number of samples
- Number of laboratories retained after eliminating outliers
- Number of outliers (laboratories)
- Number of accepted results
- Mean value (with the respective unit)
- Repeatability standard deviation (sr) (with the respective unit)
- Repeatability relative standard deviation (RSDr) (%)
- Repeatability limit (r) w(with the respective units)
- Reproducibility relative standard deviation (sR) (with the respective unit)
- Reproducibility relative standard deviation (RSDR) (%)
- Reproducibility limit (R) (with the respective unit)
- Sample types clearly described
- Notes if further information is to be given"

In addition CEN publishes its methods as either finalised Standards or as Preliminary Standards, which are required to be reviewed after two years and then either withdrawn or converted to a full Standard. Thus, CEN follows the same procedure as the AOACI in this regard.

5.5. Requirements of official bodies for methods of analysis

Consideration of the above requirements means that all methods of analysis used in the food, and hence toxicants, area must be fully validated - i.e. have been subjected to a collaborative trial conforming to an internationally recognised protocol. It is important that this is fully recognised as it sets the quality standard for the methods of analysis aspects of quality assurance considerations. Because of this users of analytical methods must be fully appreciative of the requirements being imposed and have good reasons for not following them, e.g. the non-availability of suitably validated procedures in a particular determination.

The concept of the valid analytical method in the food sector, and some its requirements, are described below:

Accuracy is defined as the closeness of the agreement between the result of a measurement and a true value of the measurand [ISO, 1993]. It may be assessed by the use of reference materials.

However, in food analysis, there is a particular problem. In many instances, though not normally for food additives and contaminants, the numerical value of a characteristic (or criterion) in a Standard is dependent on the procedures used to ascertain its value. This illustrates the need for the [sampling and] analysis provisions in a Standard to be developed at the same time as the numerical value of the characteristics in the Standard are negotiated to ensure that the characteristics are related to the methodological procedures prescribed.

Precision is defined as the closeness of agreement between independent test results obtained under prescribed conditions [ISO, 1992].

In a standard method the precision characteristics are obtained from a properly organised collaborative trial, i.e. a trial conforming to the requirements an International Standard (the AOAC/ISO/IUPAC Harmonised Protocol or the ISO 5725 Standard). Because of the importance of collaborative trials, the fact that European Union methods must have been subjected to a collaborative trial before acceptance, and the resource that is now being devoted to the assessment of precision characteristics of analytical methods, they are described in detail below:

5.6. Collaborative trials

As seen above, all "official" methods of analysis are required to include precision data; such data can only be obtained through a collaborative trial and hence the stress that is given to collaboratively tested and validated methods in the food sector.

5.6.1. What is a collaborative trial?

A collaborative trial is a procedure whereby the precision of a method of analysis may be assessed and quantified. The precision of a method is usually expressed in terms of repeatability and reproducibility values. Accuracy is not the objective.

5.6.2. IUPAC/ISO/AOAC INTERNATIONAL harmonisation protocol

Recently there has been progress towards a universal acceptance of collaboratively tested methods and collaborative trial results and methods, no matter by whom these trials are organised. This has been aided by the publication of the IUPAC/ISO/AOAC INTERNATIONAL Harmonisation Protocol on Collaborative Studies [Horwitz, 1988]. That Protocol was developed under the auspices of the International Union of Pure and Applied Chemists (IUPAC) aided by representatives from the major organisations interested in conducting collaborative studies. In particular, from the food sector, the AOAC INTERNATIONAL, the International Organisation for Standardisation (ISO), the International Dairy Federation (IDF), the Collaborative International Analytical Council for

Pesticides (CIPAC), the Nordic Analytical Committee (NMKL), the Codex Committee on Methods of Analysis and Sampling and the International Office of Cocoa and Chocolate were involved.

The Protocol gives a series of recommendations, the most important of which are described below:

5.6.3. The components that make up a collaborative trial

Participants

It is of paramount importance that all the participants taking part in the trial are competent i.e. they are fully conversant and experienced in the techniques used in the particular trial, and they can be relied upon to act responsibly in following the method/protocol. It is the precision of the method that is being assessed, not the performance of the trial participants.

The number of participants must be at least 8. However to use only laboratories who are "experts" in the use of the method in question but will not ultimately be involved in the routine use of the method could give an exaggerated precision performance for the method.

Sample type

These should, if possible be normal materials containing the required levels of analyte. If this is not possible, then alternative laboratory prepared samples must be used. These have the obvious disadvantage of a different analyte/sample matrix from that of the normal materials. The types of samples used should cover as wide an area as possible of sample types for which the method is to be used. Ideally for collaborative trial purposes the individual sample/sample types should be identical in appearance so as to preserve sample anonymity.

Sample homogeneity

The bulk sample, from which all sub-samples which form the collaborative trial samples are taken, should be homogenous and the sub-sampling procedure such that the resulting subsamples have an equivalent composition to that of the original bulk sample. Tests should also be carried out to determine the stability of the sample over the intended time period of the trial. The precision of the method can only be as good as the homogeneity of the samples allows it to be; there must be little or no variability due to sample heterogeneity.

Sample plan

For the study of method performance at least five samples covering a range of analyte concentration representative of the commodity should be used. These samples should be duplicated, making a minimum of ten samples in all. The samples should be visually identical and given individual sample codes to preserve sample anonymity as far as possible.

However, in the case of blind duplicates it may be possible to link the pairs together. This possibility may be reduced by the use of:

Split level Samples, where the samples differ only slightly in concentration of analyte and now any artificial attempt to draw these "duplicate" samples closer together will in fact give

decreased precision as it is the difference between the split level samples that is involved in the calculation of the precision parameter, and this will be a quantifiable amount.

Sometimes it is not feasible to have blind duplicates or split level samples and laboratories are requested to analyse samples as known duplicates. This is the least favoured option.

The method(s) to be tested

The method(s) to be tested should have been tested for robustness (i.e. how susceptible the procedure is to small variations in method protocol/instructions) and optimised before circulation to participants. A thorough evaluation of the method at this stage can often eliminate the need for a retrial at a later stage.

Pilot study/Pre-trial

These are invaluable exercises if resources permit. A pilot study involves at least three laboratories testing the method, checking the written version, highlighting any hidden problems that may be encountered during the study and giving an initial estimation of the precision of the method. A pre-trial can be used in place or to supplement the pilot study, it is particularly useful where a new method or technique is to be used. Participants are sent one or two reference samples with which to familiarise themselves with the method before starting the trial proper. A successful pre-trial is usually a prerequisite to starting the trial proper and can be used to rectify any individual problems that the laboratories are having with the method.

The trial proper

Participants should be given clear instructions on the protocol of the trial, the time limit for return of results, the number of determinations to be carried out per sample, how to report results; to how many decimal places, as received or on a dry matter basis, corrected or uncorrected for recovery etc.

Statistical analysis

It is important to appreciate that the statistical significance of the results is wholly dependent on the quality of the data obtained from the trial. Data which contains obvious gross errors should be removed prior to statistical analysis. It is essential that participants inform the trial co-ordinator of any gross error that they know has occurred during the analysis and also if any deviation from the method as written has taken place. The statistical parameters calculated, and the outlier tests performed are those used in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies [Horwitz, 1988].

Analysis of data for outliers

More time has been devoted to the procedures to be used for the removal of outliers than is warranted. However, the procedure now prescribed in the Harmonised Protocol is now accepted within the food sector.

Precision parameters

The most commonly quoted precision parameters are the repeatability and reproducibility.

Typical definitions of these are:

r = Repeatability, the value below which the absolute difference between 2 single test results obtained under repeatability conditions (i.e., same sample, same operator, same apparatus, same laboratory, and short interval of time) may be expected to lie within a specific probability (typically 95%) and hence r = 2.8 x sr.

sr = Standard deviation, calculated from results generated under repeatability conditions.

RSDr = Relative standard deviation, calculated from results generated under repeatability conditions [(sr /) x 100], where is the average of results over all laboratories and samples.

R = Reproducibility, the value below which the absolute difference between single test results obtained under reproducibility conditions (i.e., on identical material obtained by operators in different laboratories, using the standardised test method), may be expected to lie within a certain probability (typically 95%); R = 2.8 x sR.

sR = Standard deviation, calculated from results under reproducibility conditions.

RSDR = Relative standard deviation calculated from results generated under reproducibility conditions [(sR /) x 100]

5.7. Assessment of the acceptability of the precision characteristics of a method of analysis

The calculated repeatability and reproducibility values can be compared with existing methods and a comparison made. If these are satisfactory then the method can used as a validated method. If there is no method with which to compare the precision parameters then theoretical repeatability and reproducibility values can be calculated from the Horwitz equation [Horwitz, 1982].

Horwitz trumpet and equation: $RSDR = 2^{(1-0.5\log C)}$

Values are:

concentration ratio		RSD_R
1	(100%)	2
10^{-1}		2.8
10^{-2}	(1%)	4
10^{-3}		5.6
10^{-4}		8
10^{-5}		11
10^{-6}	(ppm)	16
10^{-7}		23
10^{-8}		32
10^{-9}	(ppb)	45

Horwitz has derived the equation after studying the results from many (~3,000) collaborative trials. Although it represents the average RSDR values and is an approximation of the possible precision that can be achieved, the data points from "acceptable" collaborative trials lie within a range plus and minus two times the values derived from the equation. This idealised smoothed curve is found to be independent of the nature of the analyte or of the analytical technique that was used to make the measurement. In general the values taken from this curve are indicative of the precision that is achievable and acceptable of an analytical method by different laboratories. Its use provides a satisfactory and simple means of assessing method precision acceptability.

5.8. Summary requirements for a collaborative trial

For a method to be considered fully validated it must have been subjected to a collaborative trial which conforms to the following critical characteristics:

- the minimum number of laboratories should be 8 [i.e. there should be eight sets of valid data available for the calculation of precision characteristics]
- the minimum number of samples should be 5
- samples should not be sent to participants for analysis as known duplicates, except as a last resort
- all the original data obtained in the trial should be reproduced in the final report on the trial. A number of outlier identification procedures are given in the recommendations; although it is desirable that they should be used in the statistical analysis of the trial results it is not essential provided the raw collaborative trial data are available thus enabling other organisations to re-calculate if they so desire.

Precision Parameters

The most commonly quoted precision parameters are the repeatability and reproducibility.

The CEN definitions are given above. The AOACI has adopted the following definitions:

r = Repeatability, the value below which the absolute difference between 2 single test results obtained under repeatability conditions (i.e., same sample, same operator, same apparatus, same laboratory, and short interval of time) may be expected to lie within a specific probability (typically 95%) and hence r = 2.8 x sr.

sr = Standard deviation, calculated from results generated under repeatability conditions.

RSDr = Relative standard deviation, calculated from results generated under repeatability conditions [(sr /) x 100], where is the average of results over all laboratories and samples.

R = Reproducibility, the value below which the absolute difference between single test results obtained under reproducibility conditions (i.e., on identical material obtained by operators in different laboratories, using the standardised test method), may be expected to lie within a certain probability (typically 95%); R = 2.8 x sR.

sR = Standard deviation, calculated from results under reproducibility conditions.

RSDR = Relative standard deviation calculated from results generated under reproducibility conditions [(sR /) x 100]

6. Harmonised guidelines for single-laboratory validation of methods of analysis

The OFFC Regulation recognises that in some situations methods of analysis can only be validated within a single laboratory. In such situations they should be validated in accordance with internationally accepted Guidelines, e.g. IUPAC Harmonised Guidelines [Thompson, Ellison and Wood, 2002] or where performance criteria for analytical methods have been established, be based on criteria compliance tests.

The following recommendations are made regarding the use of single-laboratory method validation in those Guidelines

- Wherever possible and practical a laboratory should use a method of analysis that has had its performance characteristics evaluated through a collaborative trial conforming to an international protocol.
- Where such methods are not available, a method must be validated in-house before being used to generate analytical data for a customer.
- Single-laboratory validation requires the laboratory to select appropriate characteristics for evaluation from the following: applicability, selectivity, calibration, accuracy, precision, range, limit of quantification, limit of detection, sensitivity, ruggedness and practicability. The laboratory must take account of customer requirements in choosing which characteristics are to be determined.

Evidence that these characteristics have been assessed must be made available to customers of the laboratory if required by the customer.

7. Recovery factors: development of an internationally agreed protocol for the use of recovery factors

The estimation and use of recovery is an area where practice differs among analytical chemists. The variations in practice are most obvious in the determination of analytes such as veterinary drug residues and pesticide residues in complex matrices, such as foodstuffs. The same considerations apply in the determination of toxicants. Typically, methods of analysis for the determination of these analytes rely on transferring the analyte from the complex matrix into a much simpler solution that is used to present the analyte for instrumental determination. However, the transfer procedure results in loss of analyte. Quite commonly in such procedures a substantial proportion of the analyte remain in the matrix after extraction, so that the transfer is incomplete, and the subsequent measurement is lower than the true concentration in the original test material. If no compensation for these losses is made, then

markedly discrepant results may be obtained by different laboratories. Even greater discrepancies arise if some laboratories compensate for losses and others do not.

Recovery studies are clearly an essential component of the validation and use of analytical methods. It is important that all concerned with the production and interpretation of analytical results are aware of the problems and the basis on which the result is being reported. At present, however, there is no single well-defined approach to estimating, expressing and applying recovery information.

The most important inconsistency in analytical practice concerns the correction of a raw measurement, which can (in principle) eliminate the low bias due to loss of analyte. The difficulties involved in reliably estimating the correction factor deter practitioners in some sectors of analysis from applying such corrections.

In the absence of consistent strategies for the estimation and use of recovery, it is difficult to make valid comparisons between results produced in different laboratories or to verify the suitability of that data for the intended purpose. This lack of transparency can have important consequences in the interpretation of data. For example in the context of enforcement analysis, the difference between applying or not applying a correction factor to analytical data can mean respectively that a legislative limit is exceeded or that a result is in compliance with the limit. Thus, where an estimate of the true concentration is required, there is a compelling case for compensation for losses in the calculation of reported analytical result.

An illustration of the difference in approach towards the use of recovery factors is the UK, where, for all surveillance data in the food sector, it is required that laboratories adhere to the following instructions:

"Correcting" for analytical recovery

The Steering Group has promulgated advice on correcting for analytical recovery. It concluded that corrected values give a more accurate reflection of the true concentration of the analyte and facilitate interpretation of data and made the following recommendations:

1. unless there are overriding reasons for not doing so, results should be corrected for recovery;
2. recovery values should always be reported, whether or not results are corrected, so that measured values can be converted to corrected values and vice versa;
3. where there is a wide variation around the mean recovery value, correcting for recovery can give misleading results and therefore the standard deviation should also be reported;
4. each Working Party should establish upper and lower limits for acceptable recovery and for variability of recovery. Samples giving recovery values outside the range should be re-analysed or results reported as semi-quantitative; and
5. it is important to report analytical data in a way which ensures that their significance is fully understood. It is particularly important, especially where results are close to legal or advisory limits, to explain those factors, such as sampling and analytical errors and

corrections for recovery, which are sources of uncertainties. Both enforcement authorities and working parties need to consider the most appropriate way to present results."

and the US FDA, where the adjustment of data in the food sector for recovery factors is not encouraged.

As a result of such discrepancies in approach guidelines are being prepared which will provide a conceptual framework for consistent decisions on the estimation and use of recovery information in various sectors of analytical science.

7.1. Sources of error in analytical chemistry

The Guidelines are being developed in the light of the sources of error which may occur in an analytical process, as given in the list below:

1. Incomplete definition of the measurand (for example, failing to specify the exact form of the analyte being determined).
2. Sampling - the sample measured may not represent the defined measurand.
3. Incomplete extraction and/or pre-concentration of the measurand, contamination of the measurement sample, interferences and matrix effects.
4. Inadequate knowledge of the effects of environmental conditions on the measurement procedure or imperfect measurement of environmental conditions.
5. Cross contamination or contamination of reagents or blanks.
6. Personal bias in reading analogue instruments.
7. Uncertainty of weights and volumetric equipment.
8. Instrument resolution or discrimination threshold.
9. Values assigned to measurement standards and reference materials.
10. Values of constants and other parameters obtained from external sources and used in the data reduction algorithm.
11. Approximations and assumptions incorporated in the measurement method and procedure.
12. Variations in repeated observations of the measurand under apparently identical conditions.

and the sources of uncertainty in recovery estimation, which are also listed below:

1. Repeatability of the recovery experiment
2. Uncertainties in reference material values
3. Uncertainties in added spike quantity
4. Poor representation of native analyte by the added spike
5. Poor or restricted match between experimental matrix and the full range of sample matrices encountered
6. Effect of analyte/spike level on recovery and imperfect match of spike or reference material analyte level and analyte level in samples.

7.2. International guidelines

ISO, IUPAC and AOAC INTERNATIONAL which have co-operated to produce agreed protocols or guidelines on the "Design, Conduct and Interpretation of Collaborative Studies" on the "Proficiency Testing of (Chemical) Analytical Laboratories" and on "Internal Quality Control for Analytical Laboratories", have prepared guidelines on the use of recovery information in analytical measurement [Thompson, Ellison, Fajgelj, Willetts and Wood, 1999]

7.3. Recommendations

The following recommendations are made regarding the use of recovery information in the Guidelines.

1. Quantitative analytical results should be corrected for recovery unless there are specific reasons for not doing so. Reasons for not estimating or using correction factors include the situations where (a) the analytical method is regarded as empirical, (b) a contractual or statutory limit has been established using uncorrected data, or (c) recoveries are known to be close to unity. However, it is of over-riding importance that all data, when reported, should (a) be clearly identified as to whether or not a recovery correction has been applied and (b) if a recovery correction has been applied, the amount of the correction and the method by which it was derived should be included with the report. This will promote direct comparability of data sets. Correction functions should be established on the basis of appropriate statistical considerations, documented, archived and available to the client.
2. Recovery values should always be established as part of method validation, whether or not recoveries are reported or results are corrected, so that measured values can be converted to corrected values and vice versa.
3. When the use of a recovery factor is justified, the method of its estimation should be specified in the method protocol.
4. IQC control charts for recovery should be established during method validation and used in all routine analysis. Runs giving recovery values outside the control range should be considered for re-analysis in the context of acceptable variation, or the results reported as semi-quantitative.
5. It is important for the determination of toxicants that due consideration is given to these guidelines as they provide information and justification for the selection of the approach to be taken towards the use of recovery factors, an important aspect of the quality assurance of the laboratory.

8. Measurement uncertainty

There has been much discussion on measurement uncertainty by analytical chemists. Possibly the best "simple" Guidelines on Measurement Uncertainty in the food sector are

those adopted by the Codex Alimentarius Commission [CAC, 2004]. In view of their importance they are given in Annex II.

Although there has been discussion on how Measurement Uncertainty should be evaluated and expressed, it is only recently that there has been discussion in the food sector as to how measurement uncertainty should be used when assessing compliance. Most analysts and regulatory authorities now accept that the measurement uncertainty should be deducted from an analytical result before assessing compliance to a maximum limit. Only if the result after that deduction is made can it be confidently stated that the sample is non-compliant beyond reasonable doubt.

9. Conclusions

The need for a laboratory to demonstrate that is producing reliable and acceptable results has become of major importance. The quality assurance measures adopted by the laboratory must be such that this is achieved. This demonstration is aided by the introduction of:

1. The achievement of formal laboratory accreditation by the laboratory. However, even if this is not deemed to be necessary in the light of the individual circumstance of the laboratory it is important that the requirements of accreditation are recognised and followed by the laboratory in its internal procedures,
2. Participation in proficiency testing schemes,
3. Using validated methods and
4. Introducing appropriate internal quality control procedures.

All the above are essential aspects of ensuring that data released from a laboratory are fit for purpose. The last three aspects have now been described in appropriate International Protocols and Guidelines and due note should be taken of these if appropriate quality assurance measures are to be followed.

References

CAC, 2004, Guidelines on Measurement Uncertainty, CAC/GL 54-2004, FAO, Rome (downloadable directly from the Codex Alimentarius Commission Website).

EC, 2004, “Council Regulation 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules”, O.J. L165 of 30.4.2004.

EEC, 1985, “Council Directive 85/591/EEC Concerning the Introduction of Community Methods of Sampling and Analysis for the Monitoring of Foodstuffs Intended for Human Consumption”, O.J. L372 of 31.12.1985.

EEC, 1989, “Council Directive 89/397/EEC on the Official Control of Foodstuffs”, O.J. L186 of 30.6.1989.

EEC, 1993, “Council Directive 93/99/EEC on the Subject of Additional Measures Concerning the Official Control of Foodstuffs”, O.J. L290 of 24.11.1993.

FAO, 1993, “Procedural Manual of the Codex Alimentarius Commission”, Eighth Edition, FAO/WHO Food Standards Programme, Food and Agriculture Organisation, Rome.

FAO, 1997, Report of the twenty-first Session of the Codex Committee on Methods of Analysis and Sampling, ALINORM 97/23A, FAO, Rome. (now published as Codex CAC/GL 27-1997)

Horwitz, 1982, “Evaluation of Analytical Methods for Regulation of Foods and Drugs”, Anal. Chem., 54, 67A - 76A

Horwitz, 1988, “Protocol for the Design, Conduct and Interpretation of Method Performance Studies”, Pure Appl. Chem., 60, 855 - 864.

Horwitz, 1995, “Protocol for the Design, Conduct and Interpretation of Method Performance Studies”, Pure Appl. Chem., 67, 331 - 343.

ISO, 1981, “Precision of Test Methods”, Geneva [revised 1986 with further revision in preparation]

ISO, 1992, “Terms and Definitions used in Connections with Reference Materials”, ISO Guide 30, Geneva.

ISO, 1993, “General requirements for the competence of calibration and testing laboratories”, ISO Guide 25, 2nd Edition, Geneva.

ISO, 1993, “International Vocabulary for Basic and General Terms in Metrology”, 2nd Edition, Geneva.

ISO, 1993, “Calibration and testing laboratory accreditation systems - General requirements for operation and recognition”, ISO/IEC Guide 58 , Geneva

ISO, 2005, EN ISO/IEC 17025, 2005 on “General requirements for the competence of testing and calibration laboratories”, Geneva

Thompson, M and Wood, R, 1993, “The International Harmonised Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories”, Pure Appl. Chem., 65, 2123 - 2144 (Also published in J. AOAC International, 1993, 76, 926.)

Thompson, M and Wood, R, 1995, “Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories”, Pure Appl. Chem., 67, 649 – 666

Thompson, M, Ellison, SLR, Fajgelj, A, Willetts, P and Wood, R, 1999, “Harmonised Guidelines for the Use of Recovery Information in Analytical Measurement”, Pure Appl. Chem., 71, 337 – 348

Thompson, M, Ellison, SLR and Wood, R, 2002, “Harmonised Guidelines for Single-Laboratory Validation of Methods of Analysis”, Pure Appl. Chem., 74, 835 – 855.

ANNEX I: METHODS OF ANALYSIS BEING DEVELOPED OR PUBLISHED BY THE EUROPEAN COMMITTEE FOR STANDARDISATION (CEN) IN AREAS OF RELEVANCE TO THIS TEXT

A list of the published standards and the work programme for natural toxicants, trace elements and GMOs is given below. Further information may be given from each country's National Standards Institutes (the CEN and ISO Member Body). The list given below may not be exhaustive given that texts are adopted by CEN on a regular basis.

A: CEN PUBLISHED STANDARDS

GMOs

EN ISO 21571:2005	Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic acid extraction
EN ISO 21572:2004	Foodstuffs – Methods for the detection of genetically modified organisms and derived products – Protein based methods

Natural Toxicants

DD ENV 14194:2002	Determination of saxitoxin and dc-saxitoxin in mussels – HPLC method using post column derivatisation
EN 12955: 1999	Determination of aflatoxin B1, and the sum of aflatoxins B1, B2, G1 and G2 in cereals, shell-fruits and derived products – High performance liquid chromatographic method with post column derivatization and immunoaffinity column clean up
EN 13585:2002	Foodstuffs – Determination of fumonisins B1 and B2 in maize – HPLC method with solid phase extraction clean-up
EN 14123:2003	Foodstuffs – Determination of aflatoxin B1 and the sum of aflatoxin B1, B2, G1, and G2 in peanuts, pistachios, figs and paprika powder – High performance liquid chromatographic method with postcolumn derivatization and immunoaffinity column clean-up
EN 14132:2003	Foodstuffs – Determination of ochratoxin A in barley and roasted coffee – HPLC method with immunoaffinity column clean-up
EN 14133:2003	Foodstuffs – Determination of ochratoxin A in wine and beer – HPLC method with a clean-up on a immunoaffinity column clean-up
EN 14176:2003	Foodstuffs – Determination of domoic acid in mussels by HPLC
EN 14332:2004	Foodstuffs – Determination of trace elements – Determination of arsenic in seafood by graphite furnace atomic absorption (GFAAS) after microwave digestion
EN 14352:2004	Foodstuffs – Determination of fumonisin B1 and B2 in maize based foods – HPLC method with immunoaffinity column clean up
EN 14524:2004	Foodstuffs – Determination of okadaic acid in mussels – HPLC method with solid phase extraction clean-up, derivatisation and fluorimetric detection

EN 14526:2004	Foodstuffs – Determination of saxitoxin and dc-saxitoxin in mussels – HPLC method using pre-column derivatisation with peroxide or periodate oxidation
EN ISO 14675:2003	Milk and milk products – Guidelines for a standardized description of competitive enzyme immunoassays – Determination of aflatoxin M1 content
EN ISO 15141-1:1998	Foodstuffs – Determination of Ochratoxin A in cereals and cereal products: Part 1: High performance liquid chromatographic method with silica gel clean up
EN ISO 15141-2:1998	Foodstuffs – Determination of Ochratoxin A in cereals and cereal products: Part 2: High performance liquid chromatographic method with bicarbonate clean up

Trace Elements

EN 13804:2002	Foodstuffs – Determination of trace elements – Performance criteria, general considerations and sample preparation
EN 13805:2002	Determination of trace elements – Pressure digestion
EN 13806:2002	Food stuffs-Determination of trace elements – Determination of mercury by cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion.
EN 14082: 2003	Foodstuffs – Determination of Trace Elements – Determination of lead, cadmium, zinc, copper, iron and chromium by atomic absorption spectrometry (AAS) after ash drying
EN 14083: 2003	Foodstuffs – Determination of Trace Elements – Determination of lead, cadmium, chromium and molybdenum by graphite furnace atomic absorption spectrometry (GFAAS) after pressure digestion
EN 14084:2003	Foodstuffs – Determination of trace elements – Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after microwave digestion
EN 14546:2005	Foodstuffs – Determination of trace elements – Determination of total arsenic by hydride generation atomic absorption spectrometry (HGAAS) after dry ashing
EN 1456:2005	Foodstuffs – Determination of trace elements – Determination of total arsenic by hydride generation atomic absorption spectrometry (HGAAS) after dry ashing.
EN 14627:2005	Foodstuffs – Determination of trace elements – Determination of total arsenic and selenium by hydride generation atomic absorption spectrometry (HGAAS) after pressure digestion

B: CEN WORK PROGRAMME

GMOs

Methods for the detection of genetically modified organisms and derived products - Protein based methods (ISO 21572:2004)

Methods for the detection of genetically modified organisms and derived products - Protein based methods (ISO 21572:2004)

Methods of analysis for the detection of genetically modified organisms and derived products - Sampling (ISO/DIS 21568:2005)

Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods (ISO 21569:2005)

Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (ISO/DIS 21570:2003)

Methods of analysis for the detection of genetically modified organisms and derived products - Nucleic acid extraction (ISO 21571:2005)

Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions

Natural Toxicants

Criteria of analytical methods of mycotoxins

Determination of aflatoxin B1 and the sum of aflatoxin B1, B2, G1 and G2 in peanuts, pistachios, figs, and paprika powder - High performance liquid chromatographic method with postcolumn derivatization and immunoaffinity column clean-up

Determination of aflatoxin B1, and the sum of aflatoxins B1, B2, G1 and G2 in cereals, shell-fruits and derived products - High performance liquid chromatographic method with post column derivatization and immunoaffinity column clean up

Determination of domoic acid in mussels by HPLC

Determination of fumonisin B1 and B2 in maize based foods - HPLC method with immunoaffinity column clean up

Determination of fumonisins B1 and B2 in maize - HPLC method with solid phase extraction clean-up

Determination of ochratoxin A in barley and roasted coffee - HPLC method with immunoaffinity column clean-up

Determination of ochratoxin A in cereals and cereal products - Part 1: High performance liquid chromatographic method with silica gel clean up (ISO 15141-1:1998)

Determination of ochratoxin A in cereals and cereal products - Part 2: High performance liquid chromatographic method with bicarbonate clean up (ISO 15141-2:1998)

Determination of ochratoxin A in wine and beer - HPLC method with immunoaffinity column clean-up

Determination of okadaic acid in mussels - HPLC method with solid phase extraction clean-up, derivatization and fluorimetric detection

Determination of patulin in clear and cloudy apple juice and puree - HPLC method with liquid/liquid partition clean-up
Determination of saxitoxin and dc-saxitoxin in mussels - HPLC method using post column derivatisation
Determination of saxitoxin and dc-saxitoxin in mussels - HPLC method using pre-column derivatization with peroxide or periodate oxidation
Sample comminution for mycotoxin analysis - Comparison between dry milling and slurry mixing

Heavy metals/trace elements

Determination of arsenic in seafood by graphite furnace atomic absorption spectrometry (GFAAS) after microwave digestion
Determination of iodine in dietetic foods by ICP-MS (inductively coupled plasma mass spectrometry)
Determination of lead, cadmium, chromium and molybdenum by graphite furnace atomic absorption spectrometry (GFAAS) after pressure digestion
Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after microwave digestion
Determination of lead, cadmium, zinc, copper, iron and chromium by atomic absorption spectrometry (AAS) after dry ashing
Determination of mercury by cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion
Determination of total arsenic and selenium by hydride generation atomic absorption spectrometry (HGAAS) after pressure digestion
Determination of total arsenic by hydride generation atomic absorption spectrometry (HGAAS) after dry ashing
Performance criteria, general considerations and sample preparation
Pressure digestion sample preparation method.

ANNEX II: CODEX GUIDELINES ON MEASUREMENT UNCERTAINTY

It is important and required by ISO/IEC 17025:1999 that analysts are aware of the uncertainty associated with each analytical result and estimates that uncertainty. The measurement uncertainty may be derived by a number of procedures. Food analysis laboratories are required, for Codex purposes, to be in control (as outlined in Codex GL 27-1997 "Guidelines for the Assessment of the Competence of Testing Laboratories Involved in the Import and Export of Food"), use collaboratively tested or validated methods when available, and verify their application before taking them into routine use. Such laboratories therefore have available to them a range of analytical data which can be used to estimate their measurement uncertainty.

These guidelines only apply to quantitative analysis.

Most quantitative analytical results take the form of "a ± 2u or a ± U" where "a" is the best estimate of the true value of the concentration of the measurand (the analytical result) and "u" is the standard uncertainty and "U" (equal to 2u) is the expanded uncertainty. The range "a ± 2u" represents a 95% level of confidence where the true value would be found. The value of "U" or "2u" is the value which is normally used and reported by analysts and is hereafter referred to as "measurement uncertainty" and may be estimated in a number of different ways.

Terminology

The international definition for Measurement Uncertainty is:

"Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand" (see International vocabulary of basic and general terms in metrology, ISO 1993, 2nd Edition).

NOTES:

6. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.
7. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.
8. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion."

Recommendations

The measurement uncertainty associated with all analytical results is to be estimated.

The measurement uncertainty of an analytical result may be estimated by a number of procedures, notably those described by ISO [1] and EURACHEM [2]. These documents recommend procedures based on a component-by-component approach, method validation

data, internal quality control data and proficiency test data. The need to undertake an estimation of the measurement uncertainty using the ISO component-by-component approach is not necessary if the other forms of data are available and used to estimate the uncertainty. In many cases the overall uncertainty may be determined by an inter-laboratory (collaborative) study by a number of laboratories and a number of matrices by the IUPAC/ISO/AOAC INTERNATIONAL [3] or by the ISO 5725 Protocols [4].
The measurement uncertainty and its level of confidence must, on request, be made available to the user (customer) of the results.

References

[1] "Guide to the Expression of Uncertainty in Measurement", ISO, Geneva, 1993.
[2] EURACHEM/CITAC Guide Quantifying Uncertainty In Analytical Measurement (Second Edition), EURACHEM Secretariat, BAM, Berlin, 2000. This is available as a free download from http://www.eurachem.ul.pt/
[3] "Protocol for the Design, Conduct and Interpretation of Method Performance Studies", ed. W. Horwitz, Pure Appl. Chem., 1995, 67, 331-343.
[4] "Precision of Test Methods", Geneva, 1994, ISO 5725, Previous editions were issued in 1981 and 1986.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 5

Immunoassays

N. Alice Lee[a] and Ivan R. Kennedy[b]

[a]University of New South Wales, School of Chemical Engineering and Industrial Chem., Food Science and Technology, Sydney, NSW 2052, Australia

[b]University of Sydney, Faculty of Agriculture, Food and Natural Resources, Sydney, NSW 2006, Australia

1. Overview of immunoassays

The investigation on the antigenicity of small molecules by Landsteiner in 1917 has provided the framework for the development of immunological assays for detection of low molecular weight compounds. Landsteiner found that the specificity of the original carrier protein was changed by the newly introduced groups, which by themselves were not immunogenic [1]. His major finding was related to the exquisite specificity of antisera, demonstrated by his classical studies with *L*-, *D*- and meso-tartaric acid, and *m*-aminobenzoate [1-3]. The first competitive binding assays using radiolabelled ligands were developed by Berson and Yallow in 1959 [4]. Since then, immunoassays have been applied to a wide range of scientific disciplines including endocrinology, biomedical and clinical chemistry, facilitated by the simplification of test formats, the availability of electronic instrumentation and the development of monoclonal technology by Kohler and Milstein in 1975 [5]. Its application in analytical chemistry, especially in the food contaminant analysis, only commenced in the last three decades and the method has increasingly become recognized as a powerful analytical tool.

1.1. Advantages of immunoassays

Immunoassays offer a number of advantages in food contaminant analysis over the conventional methods, such as HPLC and GC. Immunoassays can provide a fast, simple and a cost-effective method of detection, with sensitivity and specificity comparable or better (in some cases) than the conventional methods. More important, on-site analysis is possible with immunoassays [6]. The ease of automation and the versatility in the applications of immunoassay have also made this method more popular in recent years.

One of the key advantages of immunoassay is the speed of analysis. In instrumental methods, extensive sample clean-up may be required to remove interferences and this can often be the rate determining step in residue analysis. The laborious sample preparation required for instrumental analysis limits its applications in circumstances where a large

number of analyses are needed, even though the actual time for each individual analysis on the instrument may be comparably short. Immunoassays have capacity for high throughput and numerous samples can be analysed simultaneously, significantly reducing the average analytical time. As an example, a comparison of instrumental methods and immunoassays for aflatoxins is given in Table 1.

Table 1. Comparison of various techniques for food contaminant analysis

Mycotoxin	Minicolumn	TLC	HPLC	IAC	ELISA
Limit of Detection (ng/g)	5	5	<1	method dependant	1-5
Clean up per sample (min)	60-120	60-120	60-120	10-30	10-30
Detection per sample (min)	30	120-360	120	15-30	10
Quantification per sample (min)	1-5	10	30	5-30	1-5
Total time (min)	151 - 155	490	270	45 – 90	15 - 50

TLC = Thin Layer Chromatography
HPLC = High Performance Liquid Chromatography
IAC = Immunoaffinity Chromatography
ELISA = Enzyme-Linked ImmunoSorbent Assay

Major costs involved in the food contaminant analysis by instrumental methods include chemical reagents, solvents, the labor of technical personnel and the capital cost of instruments. As a result, with limited funding and infrastructure, immunoassays, if available, are likely to be more cost-effective. The capital cost of setting up an immunoassay laboratory is comparably many times lower; thus the major cost in immunoassay is largely the time of the analyst. The estimated cost per sample is frequently as much as ten times lower with immunoassays than with instrumental analyses. Hence more samples can be analyzed with immunoassays, and this can be an imperative factor for reliable results.

The sensitivity of immunoassay is often as good as or better than those of the instrumental methods. Immunoassay requires less concentration of analytes in solid matrices such as foods, while water samples are generally analyzed without any pre-concentration. Sensitive immunoassays for those pesticides and mycotoxins that otherwise need derivatization for instrumental analysis have been developed and effectively eliminate the derivatization step. Some examples of immunoassays for pesticides are 2,4-D [7-9], diuron

[10-12] and permethrin [13,14]. Examples for mycotoxins are aflatoxins [15,16] and fumonisins [17].

Immunoassays developed for food contaminants may be either compound-specific or group-specific, or specific to a particular chemical configuration. Whereas screening of a class of compounds is possible with a group-specific immunoassay of broad specificity directed at a common chemical moiety, the ability of an antibody to bind specifically to the compound enables the immunoassay to be used for quantitative analysis of a specific analyte. It is even possible to develop an immunoassay that is specific for a single stereoisomer, enabling the detection of only the insecticidal active isomer. Immunoassays developed for *S*-bioallethrin [18,19], bioresmethrin [20] and deltamethrin [21] are examples of such high stereospecificity. An antibody specific to one congener is also possible and good examples are aflatoxin B_1 [16] and ochratoxin A [22].

On-site analysis of possible contaminants has become more important in agricultural and food industries in recent years. For example, the losses of pesticide either by volatilization or degradation have been observed during storage and transport to the analytical laboratory [23]. Testing for veterinary residues at abattoirs or segregating mycotoxin contaminated produce at receival bays [24] are now an integral part of contamination management. User-friendly simple field immunoassays have been developed and some commercialized for food contaminants. Examples of these are atrazine [25], 2,4-D [26], cyclodiene [27], aflatoxin B_1 [28,29] and fumonisins [30]. The recent widespread adoption of the immunochromatography technique in contaminant analysis is a result of increasing demands for rapid decision-support tools involving minimal preparation of samples. Obviously, rapid antibody-based tests such as these or modified forms would be of great benefits to farmers, consultants and regulators in their decision making for contaminant management.

1.2. Disadvantages of immunoassays

Although immunoassay offers many advantages as discussed above, it also is limited also by a number of shortcomings. These are:

[1] the lengthy development time required to prepare immunoreagents for new analytes

[2] unsuitability for multiresidue analysis as immunoassays are usually only capable of detecting about two to five related compounds

[3] the limited amount of information delivered in assays

[4] very few immunoassays have official status

A new analytical method based upon GC or HPLC can usually be developed and validated in several weeks. The time taken to develop a validated immunoassay, however, ranges from several months to more than a year. The procedures involved in immunoassay development are more complicated than for GC or HPLC methods. The cost involved in this development can also be expensive compared to the development of GC or HPLC methods where equipment is already available. Thus, careful consideration is required before proceeding with the immunoassay development.

Multiresidue analysis in immunoassays does not hold the same meaning as for instrumental methods. They are usually capable of detecting two to five compounds with similar structures of the same class of pesticide, but do not detect structurally different compounds - for instance, immunoassays developed for organochlorine insecticides do not detect organophosphate insecticides [27]. The GC or HPLC methods depend on the response of the instrument to produce an analyte signal as a peak at a certain retention time, allowing quantitative analysis of each compound. By contrast, the detection system of immunoassay does not always provide a quantitative analysis, except where the detection of each compound in a group is of equal sensitivity, a very rare situation. For instance, immunoassays for cyclodiene insecticides [27], sulfonylurea herbicides [31] and sulfonamide antibiotics [32] are only useful as screening tools due to their varying affinity to individual compounds. Detection of a single compound of interest may also be difficult from a pool of unknown contaminants and the presence of compounds with similar structure in the sample can result in a significant number of false positives. Nevertheless, with additional information such as knowledge of which compounds have recently been applied, useful conclusions from the data can usually be obtained.

Only a few immunoassay methods have official status at present and these are predominantly mycotoxin ELISAs. As immunoassays depend on the use of biological reagents such as antibodies and enzyme conjugates, ELISA is often falsely described as a biological method by many analysts who are accustomed to chemical methods. In addition, the lack of extensive validation of immunoassays with data from incurred and naturally contaminated samples has slowed its potential widespread in many laboratories, even though immunoassays for most of the major food contaminants have now been developed (Table 2) and readily applied in various situations.

Table 2. ELISAs developed for pesticides, mycotoxins and veterinary drugs.

Compound	Limit of Detection	Sample	Ref.
Insecticides			
Aldicarb	300 μg/L	water, citrus juices	[33]
Aldicarb sulfone/ sulfoxide	0.25 μg/L	food	[34]
Aldrin/dieldrin	700 pg/150pg	water	[35]
Azinphos-methyl	0.05 μg/L	water	[36]
BAY SIR 8514	9.6 μg/L	water, milk	[37]
S-Bioallethrin	0.05 nmol/tube	water	[18,19]
Bioresmethrin	2 μg/L	grain	[20]
Carbaryl	3.6 μg/L, 2 μg/L	Water, soil, urine, food	[38,39]
Carbofuran	10 mg/L, 0.06 μg/L	water, soil, meat, liver	[40,41]
Chlordane	2.5 μg/L	soil	[42]
Chlorpyrifos	0.1 μg/L	soil	[43]
Chlorpyrifos-methyl	0.2 μg/mL	grain	[44]
Cyclodienes	3.8 μg/L, 20 μg/L	water, soil, meat	[45,46]
Cypermethrin	0.05 μg/mL	water, soil	[47]
DDT	0.0035 μg/L	water	[48,49]
Diflubenzuron/ Penfluron	5 μg/L, 6.8 μg/L	water, milk	[37]
Endosulfan	0.2 μg/L, 0.003 μg/L, 3 μg/L	buffer, water, vegetables, soil	[27,50,51]
Fenitrothion	100 μg/L	grain	[52,53]
Methoprene	1, 0.25 and 50 μg/L	water, tobacco, grain	[54,55]
1-Naphthol	10 μg/L	soil, urine	[56]
Paraoxon	275 μg/L, 1mg/mL	water	[44,57]
Parathion	4 ng, 7.8 μg/L, 1 μg/L	plasma, plants, water, milk	[58-60]
Permethrin	15 μg/L, 1.5 μg/L	Meat, grain	[61,14]
Phenothrin	1.5 μg/L	grain	[14]
Pirimiphos-methyl	30 μg/L	grain	[44]
Fungicides			
2-Benzimidazolyurea	0.62 μg/L	water, soil, orange juice	[62]

Compound	Limit of Detection	Sample	Ref.
Benomyl	350 μg/L, 1.25 μg/L	food, fruits, vegetables	[10,63]
Bentazon	2 μg/L	water	[64]
Captan	5 μg/L	fruits, vegetables	[10]
Carbendazim	0.1 μg/L	water, soil, orange juice	[62]
Fenpropimorph	13 μg/L	water	[68]
Iprodione	30 μg/L	food	[65]
Metalaxyl	0.06 μg/L	food	[65]
Methyl-2-benzimidazole	125 μg/L, 0.5 μg/L	food, soil, orange juice, wine	[63,66,67]
Norflurazon	1 μg/L	water	[68]
Penconazole/ Tetraconazole	12 μg/L	water	[69]
Thiabendazole	20 μg/L, 5 μg/L	liver, fruits, vegetables	[70,71]
Triadimefon	1 μg/L	food	[72]
Herbicides			
2,4-D	1 μg/L, 0.08 μg/L, 100 μg/L	water, food, water, urine	[8,9,73]
2,4-D/ 2,4,5-T	13 pg	water	[7]
Alachlor/ Butachlor	0.25 μg/L/0.7 μg/L, 1 μg/L	water	[74, 158]
Amidochlor	0.2μg/L, 1.1 μg/L, 1 ng/L	water	[76-78]
	0.5 μg/L, 0.05 μg/L	water	[79-81]
Bromacil	0.02 μg/L	water, soil	[82,83]
Chlorsulfuron	0.1 μg/L	soil	[84]
Clomazone	0.5 μg/L, 5 μg/L	soil	[85,86]
Cyanazine	0.035 μg/L	water, soil	[87]
Dichlorprop	10 μg/L		[88]
Diclofop-methyl	23 μg/L	water	[89]
Diuron	0.2μg/L, 40 ng/L, 10 ng/L	water, orange juice	[11,12,31]
Hydroxy atrazine	0.05 μg/L	water, soil	[90]
Hydroxy simazine	~0.1μg/L	water	[91]
Imazaquin	0.4 μg/L	water	[92]
Imazethapyr	0.3 μg/L	soil	[93]
Isoproturon	1 μg/L	soil	[94]
Linuron/ Monuron	0.08 μg/L/0.05 μg/L	water, orange juice	[12]

Compound	Limit of Detection	Sample	Ref.
Metolachlor	0.05 μg/L, 0.1 μg/L, 1 μg/L	water, soil	[87,95,96]
	0.6 μg/L, 10 μg/L	water	[76,97]
Molinate	20 μg/L	water	[98]
Monolinuron / Diuron	0.1 μg/L	food	[10]
Paraquat	0.3μg/L, 20 μg/L, 0.1 μg/L	plasma, water, food	[99-102]
Picloram	100 μg/L	urine, water, soil, plant	[73,103]
Propazine	0.1 ng/L	water	[78]
Terbytryn	4.8 μg/L	water	[105]
Triasulfuron	0.01 μg/L	soil	[106]
Triazine	0.3 μg/L, 0.1-1 μg/L	water	[107-109]
Mycotoxins			
Aflatoxin B_1	5μg/L	oil nuts, grains	[15,16]
Aflatoxin B_2	50pg/assay	Water	[67]
Aflatoxin G_1	15-25 pg/assay	water	[110]
Aflatoxin M_1	1-10 ng/assay, 10pg/mL	water	[111,112]
Aflatoxin Q_1	2μg/L	urine	[113]
Total aflatoxin	0.25, 3.34, 0.32 and 4.0 ng/assay for B_1, B_2, G_1 and G_2	buffer	[114,115]
Ochratoxin A	5 μg/L,3 μg/L	barley	[116, 117]
Ochratoxin A	5pg/assay	chicken meat, wheat flour, plasma, serum	[22]
Fumonisin B_1	0.05 μg/L	starch	[118]
Fumonisin B_1, B_2, B_3, B_4	1.9-7.6μg/L	corn	[119]
Fumonisin B_1, B_2, B_3	~ 43μg/L	water	[120]
Deoxynivalenol	1ng/L, 0.2 μg/L, 18μg/L	water, wheat	[121-124]
Citrinin	1 μg/L	Wheat	[125]
Zearalenone	0.3 μg/L, 1μg/L, 0.2 μg/L	water, barley, Job's-tears	[126-128]
T2 toxin	0.2 μg/L, 25 pg/assay	serum, urine, milk	[129,130]
AAL toxins	~5μg/L	water	[131]
Veterinary Drugs			
Sulfonamides	2 nM/assay,10μg/L,100 μg/L	water, milk	[32,132,133]

Compound	Limit of Detection	Sample	Ref.
Sulfathiazole	0.12µg/L, 6µg./L, 10µg/L, 20µg/L	honey, liver, milk	[134-137]
Streptomycin	40 µg/mL, 10µg/L, 6 µg/L	plasma, milk, kidney	[138-140]
Ractopamine	0.2 µg/L (racemic mix)	water	[141]
Melengestrol acetate	0.05 and 0.4 µg/L	Muscle, fat	[142]
Chloramphenicol	0.08 µg/L	Meat, egg	[143]
Ampicillin	10 µg/L	milk	[144]
Sulfadimethoxine	0.2 µg/L	animal tissue	[145]
Sarafloxacin	~ 1 µg/L	water	[146]
Halofuginone	0.5 µg/L	animal liver	[147]

2. Principles of immunoassay

Immunoassay is based on the ability of antibodies to specifically bind particular molecules possessing a structure complementary to a specific region of the antibody molecule. Formation of the antibody-antigen complex involves the interaction of the antigen binding site with the antigenic determinant, a single, minor structure on the surface of the antigen that is the specific antibody target, in response to the law of mass action [148]. Selectivity is a factor dependent on the complementary nature of the antigen surface and the antibody binding site and governs the specificity of the immunoassay. The affinity constant (*Keq*), which is a ratio of bound and unbound antigen and antibody at equilibrium as shown in Eq. (1), is a measure of how well an antibody can function in an immunoassay. For food contaminant immunoassays, K_{eq} is typically of an order of 10^{-4} – 10^{-12} L/mol, governing the sensitivity of the detection.

$$Ab + Ag \leftrightarrow Ab - Ag\ complex$$

$$K_{eq}\left(Lmol^{-1}\right) = \frac{[Ab - Ag]}{[Ab][Ag]} \qquad (1)$$

Ab = antibody
Ag = antigen
Keq = equilibrium constant or affinity constant
[Ab-Ag] = concentration of bound antigen
[Ab] = concentration of free antibody
[Ag] = concentration of free antigen

3. Development of immunoassay for food contaminants

Regardless of whether the target analyte is pesticide, veterinary drug or mycotoxin, there are a number of steps involved in the development of immunoassays. These are:

- Hapten synthesis
- Antibody production
- Assay optimization and characterization
- Assay validation
- Stability testing
- Prototype kit production (optional)
- Technology transfer to end users
- Follow-up on assay performance

Unlike instrumentation methods, the development of a new immunoassay can be time consuming and costly as mentioned previously. Therefore, it is important that the objectives are clearly defined before commencing with the development or applying commercial test kits. Objectives can be defined by carefully evaluating the potential contaminants (target analytes) present within the test sample, their potential risks, degradation pathways of the target analyte and the extent of the contamination problem. The appropriate threshold for the sensitivity and specificity of the assay to be developed also should be considered at this early stage. Knowledge of the maximum residue limits (MRLs), current limit(s) of detection, potential application, availability of official methods and their advantages and disadvantages could also be useful guides for the purposes of the new development.

3.1. Hapten synthesis

Molecules with molecular weights of less than 5000-10,000 do not elicit immune responses in animals. In order to produce antibodies against these molecules, they must be attached to a larger molecule that stimulates the cellular immune system. Such small molecules, which only become immunogenic after attachment to a larger carrier molecule, are called haptens. The design of an appropriate hapten is a critical step in immunoassay development. The following section is intended to provide a general guideline to hapten design and synthesis which is just as applicable to any food contaminants, even though most of the examples given are of pesticide origin.

An appropriate hapten is one that preserves most of the steric and electronic characteristics of the pesticide and structurally mimics the target molecule as closely as possible. In most immunoassays, antisera with high affinity and specificity were raised

against protein conjugates with haptens retaining most of the functionalities of the target pesticide. Despite this, useful antibodies had been obtained with protein-conjugated haptens only partially mimicking the target analyte. Haptens have also been prepared using a known metabolite of the analyte with desirable functional groups, such as a hydroxyl group on a metabolite formed by hydrolysis of the parent/original compound. This approach may only be desirable when the assay is designed to detect metabolites as well as the parent molecule. For example, the endosulfan hydrolysis product, endosulfan diol, was used to synthesise endosulfan hemisuccinate for coupling to a carrier protein [27,50,51] and hydroxyatrazine [90,91], a hydrolytic product of atrazine, was used in a hapten synthesis for atrazine.

The hapten is virtually always attached through covalent bonding to a carrier protein (as an immunogen) or an enzyme (as a detector). A linker or a spacer arm is often used for attachment to offset the hapten from the surface of the protein to enable greater antibody recognition. However, useful antibodies have also been raised against haptens directly coupled to a carrier protein without a linker. For example, Ercegovich *et al.* [57] prepared a parathion hapten by reducing the nitro- group of parathion to an amino group that was directly coupled to bovine serum albumin (BSA) via diazotization, producing useful antibodies for development of a radioimmunoassay with a limit of detection of 4 ng parathion. Jung *et al.* [149] also obtained antibodies with a good titre and sensitivity for fenpropimorph when rabbits were immunized with an immunogen prepared by a direct coupling of an acid derivative of fenpropimorph to BSA. Highly sensitive ochratoxin A specific antibody was raised against ochratoxin A directly conjugated to a carrier protein [22]. An immunoassay was developed for detection of diclofop-methyl (with a detection range of 0.023-4.6 mg/L) by generating antibodies with high titre against diclofop acid directly coupled to BSA [89]. 2,4-D immunoassay with a limit of detection at 0.08 μg/L was also developed using direct coupling of the acid moiety of 2,4-D to protein [9].

Antibody specificity is directed to sites on the hapten that are distal to the point of linker attachment [1,89,150]. A good displacement of hapten by the spacer arm generally produce antibodies with high titre, but sometimes fails to provide the required sensitivity in an immunoassay. Antibodies have been raised against parathion haptens with bulky functionalities in the linker, such as succinyl, propenoic acid, glutarimine and diazobenzoic acid [151]. These antibodies, however, were more specific to free haptens than parathion, showing such bulky functionalities were strong determinants (i.e., functionalities that recruit a specific immune response).

3.2. Selection of spacer arms and the point of attachment

A spacer arm is preferably an alkyl chain with three to six carbons. The use of bulky functionalities, such as aromatic, cyclic rings, or conjugated double bonds in a spacer arm should be avoided to minimize the recognition of this region by the antibodies [89,152]. Propionic (3-carbon chain), succinic (4-carbon chain) and caproic acids (6-carbon chain) have been used most commonly in hapten synthesis for contaminant immunoassays. A spacer arm longer than eight carbons may not be appropriate as folding of the hapten may occur, resulting in a production of antibodies with low affinity for the target analyte. Also, flexibility should be taken into consideration when designing a spacer arm; a more rigid spacer arm, such as those consist of a double bond, enables a better offset of the hapten than flexible alkyl type spacer arms. However, it could also reduce the solubility of a hapten in a conjugating reaction, resulting in lower than the ideal hapten to protein ratio if the hapten is already highly hydrophobic. Hydrophobicity of bulky spacer arm also increases the level of non-specific binding in an immunoassay, causing a greater deviation at the lower part of dose-response curve. Hence, the bulky spacer arm approach would be less likely to yield useful haptens in hydrophobic environments.

Spacer arm recognition has been observed in many cases. For example, antibodies produced against aminophenyl linkage to molinate showed strong spacer arm recognition, and were unable to show inhibition with molinate even in a heterologous system (i.e., coating antigen containing a heterologous hapten). Antibodies raised against a molinate hapten with a propionic acid linker also showed spacer recognition, but sufficiently weak to show inhibition by molinate in a heterologous system [153]. Vallejo *et al.* [151] also observed strong spacer arm recognition, especially those with “bulky” spacer arms, in parathion immunoassays, as discussed above.

The most sensitive competitive immunoassays for pesticides and veterinary drugs have been developed employing heterologous systems. That is where the hapten used for recognition in the immunoassay is different to that used to raise antibodies. There are three kinds of heterologous systems; hapten heterology, bridge heterology and linker attachment site heterology. The most common or easily applied approach is to use the hapten with different spacer arm length. In the *s*-triazine immunoassay developed by Harrison and coworkers [153], the use of a hapten with shorter spacer arm as a coating antigen with antibody produced against a hapten with longer spacer arm had improved the sensitivity of the immunoassay by 100-fold. Paxton *et al.* [154] examined the effects of two bridging groups, acetyl and pentanoyl, on the sensitivity of the assay for diphenylhydrantoin. They found that the acetyl linker failed to elicit antibodies that recognize free diphenylhydrantoin,

whereas the pentanoyl linker, the longer of the two, produced antibodies specific for free diphenylhydrantoin. Sensitive immunoassays, however, have also been developed using homologous system, for example, immunoassays for diazinon [155], chlorpyrifos [43], 2,4-D [10], aflatoxin B_1 [15,16] and ochratoxin A [22].

For mycotoxins, however, hapten heterology does not seem to yield a greater sensitivity. Most of sensitive mycotoxin immunoassays developed utilized homologous system [16,156], in which the same hapten (but a different carrier protein) was used for both immunogen and coating antigen. The complex structure of mycotoxins certainly is one of the factors that limits the creativity in hapten design and synthesis, preventing full utility from the concepts of heterology. However, their greater molecular weight and complex chemical structures naturally provide them with immunogenic advantages over synthetic chemicals such as simpler pesticides and veterinary drugs. Thus most antibodies generated for mycotoxin, even if homologous systems are employed, generally exhibit an adequate sensitivity. For instance, citrinin immunoassay with a limit of detection at 2 µg/L utilized a polyclonal antibody and a hapten-enzyme conjugate prepared using citrinin directly conjugated to respective protein and an enzyme [126]. Thus it appears that applying general rules for hapten design may be useful for research planning, but there always remains a degree of uncertainty in the response of the immune system, which could only be determined through experiment.

The use of bulky functionalities such as aromatic or cyclic groups in the spacer arm for the enzyme conjugate has also improved assay sensitivity by reducing the affinity of the antibody for the spacer arm. Brady *et al.* [157] used this approach in the development of an immunoassay for aldicarb. The immunoassay developed for bioresmethrin [14] provided a limit of detection of 100 fold more sensitive when a bulky functionality linker was used in the enzyme conjugate. Bulky functionalities have also been exploited for other purposes, such as coating antigen or immunogen. For example, a phenyl acetic acid linker was used in preparation of coating antigen for a diflubenzuron immunoassay [37,158]. The "bulky" spacer arm strategy employed by Vallejo *et al*. [151] was mainly to raise antibodies against a number of haptens with different linker groups. However, the use of bulky spacer arms does not always warrant greater sensitivity. For example, the use of chlorendic *trans*-4-aminomethyl cyclohexane carboxylic acid as a hapten for an enzyme conjugate in endosulfan immunoassay did not prove to yield a greater sensitivity (unpublished data). This was mainly due to the reduced solubility of such a hapten in an aqueous environment.

The actual point of the spacer arm attachment to the hapten has greatly influenced the selectivity and sensitivity of the assay. It was thought that the shielding effect of the carrier

protein in the immunizing antigen was the reason [152,159]. The spacer arm attachment should be distal to the functional groups of the target molecule. The specificity of the immunoassay determined by the site of coupling can be exemplified using the immunoassays developed for triazine herbicides as shown in Table 3. Haptens prepared by coupling at three different sites on atrazine generated antibodies with quite different specificity.

The coupling site at the 2-position of triazine via the formation of ametryn sulfoxide generated antibodies with broad specificity, detecting atrazine, propazine, simazine, prometryn, ametryn and simetryn [77]. This hapten retained the two alkylamino- side chains of atrazine and the cross-reactivity of this antibody was greatest for compounds with these substituents (i.e., 4-ethylamino- and 6-isopropylamino-) such as atrazine and ametryn. The cross-reactivities for compounds with identical side chains, either with 4,6-bis(ethylamino) or 4,6-bis(isopropylamino), were about 50%, suggesting that the antibody detected either the 4-ethylamino group or 6-isopropylamino group on these compounds. Thus, protein conjugated to the hapten retaining 4-ethylamino- and 6-isopropylamino- substituents had elicited the production of antibodies detecting a wide range of compounds with similar structures.

The cyanazine analogue was also covalently coupled to a carrier protein via 3-mercaptopropionic acid group at the 2- position [87]. The resulting antibodies were specific for cyanazine, indicating that cyano- functionality was important for antibody recognition. Cyanazine differs from atrazine and other related compounds by the presence of a cyano group in the alkylamino- side chain at 4-position, instead of a methyl group.

Antibodies produced against the haptens coupled at the 4-ethylamino side chain of atrazine via a 6-carbon chain linker generated more specific antibodies [48,78,109]. These antibodies were predominantly specific for atrazine and propazine, although a significant cross-reaction for azidoatrazine was observed with antibody produced by Dunbar *et al*. [109]. Azidoatrazine differs from atrazine only by the functional group on the 2- position; azo- ($-N_3$) rather than chloro- group. This shows that the 2-chloro- group on the hapten was essential for the discrimination of methylthiol atrazine compounds.

Antibodies specific for the hydroxy metabolite of triazine were produced by deriving the hapten from hydroxyatrazine. The hydroxyatrazine analogues were coupled to the carrier proteins either via 5-carbon chain linker at the 2-ethylamino- group [90] or via 3-carbon chain linker at 4-isopropylamino- group of triazine [91]. Importantly, these haptens retained the hydroxyl group at the 2-position and the antibodies generated indeed detected the hydroxyl metabolites of triazine herbicides. The former detected hydroxyatrazine and

hydroxypropazine, and some significant cross-reactions were observed with hydroxysimazine and hydroxydesmetryn. The later was specific for hydroxysimazine, and hydroxyatrazine was detected less sensitively. The difference between the specificities of the two antibodies is a result of the two different amino- side chains of the haptens. The hapten prepared by Schlaeppi *et al*. [90] consisted of an isopropylamino- side chain and the cross-reactions of resulting antibody with hydroxyatrazine and hydroxypropazine would be expected. On the other hand, the ethylamino side chain of a hydroxyatrazine hapten synthesised by Lucas and coworkers would produce antibodies specific for hydroxysimazine and hydroxyatrazine. The cross-reactions of the antibodies generated by Huber [77], Dunbar *et al.* [109], Lawruk *et al.* [87] and Schlaeppi *et al.* [90] are shown in Table 3.

3.3. Coupling procedures to carrier protein, enzyme and antibodies

Regardless of the protein carrier used, the same functional groups are usually available for attachment to the hapten. Functional groups such as the carboxylic groups of the C-terminal end of the aspartic and glutamic acid residues, the amino groups of the N terminal and lysine residues, the imidazole and phenolic functions of the histidine and tyrosine residues, respectively and the sulfhydryl group of the cysteine residues have been used for the preparation of immunogenic hapten-protein conjugates [160]. The coupling procedure involves either a cross-linking agent reacting with the active groups of protein or the spacer arm is activated instead. The coupling via functional groups which are a part of or contribute to the active site of enzyme or antibodies, can abrogate biological activity. Loss of biological activity can also result if a severe modification of inter- or intra-molecular binding occurs through coupling. Furthermore, over-substitution can lead to either inaccessibility of the active site or to an increase in the nonspecific adherence of the conjugated macromolecules and reduce immune response in an animal [161].

The coupling method is selected according to the functional groups of the hapten. The most common of all in the food contaminant immunoassays are linkages through a carboxylic acid and amine. But other methods of coupling have also been employed.

3.3.1. Carboxylic groups - Mixed function anhydride

Carboxylic acids may either be present as an inherent part of the hapten or as an added moiety to terminate the spacer arm. The coupling of carboxylic acid via the mixed anhydride procedure is a simple one-step reaction which does not require isolation of an active derivative. The haptenic group is converted in *situ* to an acid anhydride at low temperature, which can react in aqueous-water miscible solvent mixture with the amino

groups of carrier protein. The most common reagents used in this type of reaction are isobutylchloroformate with tributyl or triethylamine. The chemical reaction of the mixed function anhydride is shown in Figure 1. The chemical yields are usually low (20-30%) and can vary significantly from 1 to 50%.

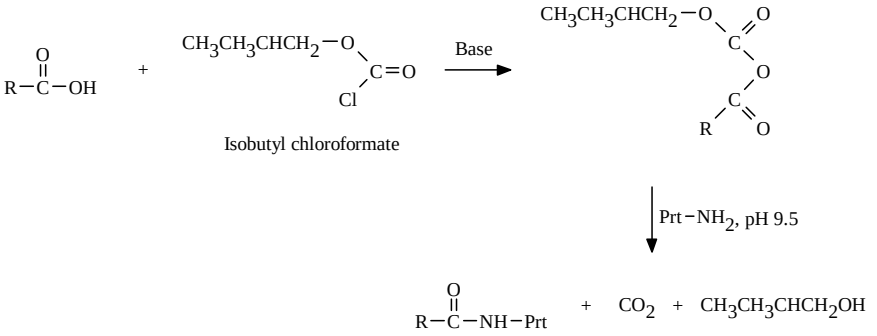


Figure 1. Mechanism of mixed function anhydride reaction

3.3.2. Carboxylic groups - Carbodiimide

Carbodiimide-mediated reaction is another coupling method via carboxylic acid. There are two possible mechanisms for this reaction, the desirable one being catalysed with H^+, as shown in Figure 2. The lysine residues of the carrier protein, however, are reactive at high pH where dissociation of the amino group to ammonium occurs; thus it is necessary to operate at a pH near to neutrality during conjugation. This method is useful when coupling unstable haptens as the reaction is carried out without isolating an active ester. Because the reaction is usually carried out by stirring the carrier protein, an excess of hapten and the reagent together in a solution, some cross-linking of the carrier protein may occur, which may aid antigenicity but will reduce the solubility of the protein conjugate [162]. The residual carbodiimide can deactivate protein, or react directly with protein, resulting in antibodies being raised against guanidino- or acyl derivative [89]. Some researchers experienced extensive alteration of the conformational structure of the carrier with little or no substitution by haptenic groups and the resulting enzyme conjugate provided significantly lower immunoreactivity. The average yield of the type of reaction is about 20%. The carbodiimides commonly used for coupling are 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)

carbodiimide methyl *p*-toluene sulfonate (CMC), 1-cyclohexyl-3-92-morpholioethyl)carbodiimide metho-4-toluenesulfonate (CDI) and dicyclohexylcarbodiimide (DCC).

A comparison of the carbodiimide coupling method using DCC, CMC and EDC in the development of an *s*-triazine immunoassay was examined by Wittmann and Hock [78]. The EDC and CMC procedures were conducted in an acidic environment (pH 5.5), where the DCC procedure was carried out in a neutral solution (pH 7.2). The CMC procedure yielded the highest epitope density (30-35 haptens per BSA molecule) and the lowest was observed with the DCC procedure (2-4 haptens per BSA molecule). The EDC procedure tended to result in insoluble products at higher hapten/BSA ratio in the reaction. A comparison between the CMC and DCC procedures for enzyme-conjugate preparation also indicated that the CMC procedure provided better coupling reaction than the DCC procedure.

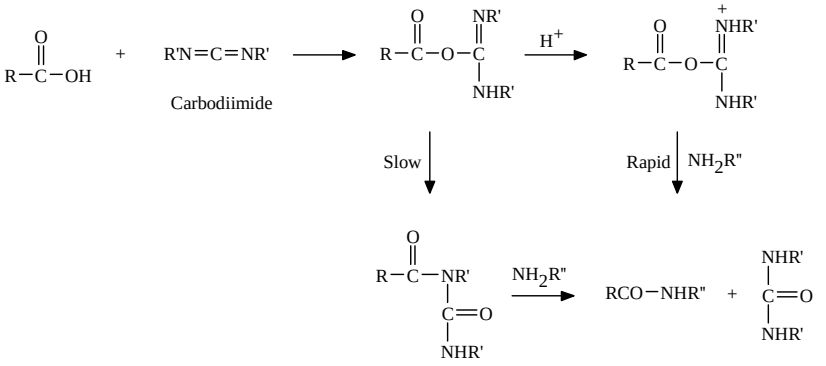


Figure 2. Mechanism of carbodiimide-mediated preparation of amides.

3.3.3. Carboxylic groups - N-hydroxysuccinimide

The formation of an *N*-hydroxysuccinimide ester by reaction of the carboxylic acid group with *N*-hydroxysuccinimide (NHS), frequently in the presence of dicyclohexylcarbodiimide, is another carboxylic acid mediated reaction, and one that is frequently used in preparing conjugates for contaminant immunoassays. The chemical reaction of NHS is shown in Figure 3. The advantage of this reaction is that the active ester is reasonably stable under acidic conditions and so purification and storage of the active ester is possible. The active ester of NHS reacts quickly with amino groups of protein resulting in good yields (i.e., higher epitope density). Although some loss of enzymatic activity during coupling has been reported, this reaction is less severe on the structure of the protein than the carbodiimide

reaction. In a comparison of carbodiimide reaction and NHS reaction for coupling of atrazine derivative to the enzymes, alkaline phosphatase and horseradish peroxidase, it was evident that the NHS reaction resulted in an enzyme-conjugate with a higher epitope density and immunoreactivity than the enzyme-conjugate prepared by the carbodiimide reaction.

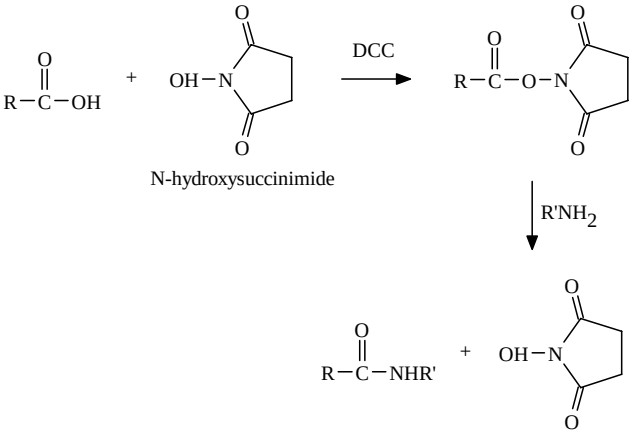


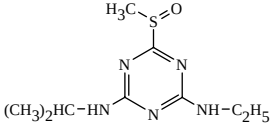
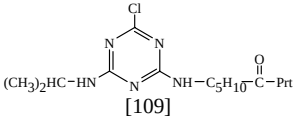
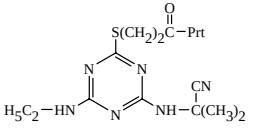
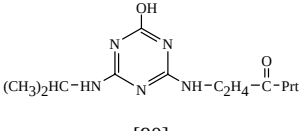
Figure 3. Reaction of N-hydroxysuccinimide. DCC, dicyclohexylcarbodiimide.

3.3.4. Miscellaneous carboxylic methods

Coupling of carboxylic acid to a protein is also possible via a formation of an acid chloride. This type of the coupling procedure was noted with haptens for picloram [103], malathion and DDA [163]. The picloram acid chloride was prepared by treating picloram with thionyl chloride followed by direct coupling of the resulting acid chloride to rabbit serum albumin in basic solution. The acid chloride of malathion half ester was also prepared in the similar manner. The use of carbonyldiimidazole (CDI) as a cross-linking reagent in preparation of enzyme-conjugate for endosulfan immunoassays has also been reported [50,51], even though this reagent is more commonly used for coupling of hydroxyl group containing haptens. The chemical reaction of CDI for endosulfan diol is shown in Figure 4.

Table 3

The specificities of the triazine immunoassays developed using different haptens.

Compounds	 [77]	 [109]	 [87]	 [90]
Atrazine	100	100	-	-
Propazine	57	87	0.1	-
Simazine	55	10	0.2	-
Ametryn	106	7	0.5	-
Prometryn	56	-	< 0.1	-
Simetryn	75	-	-	-
Terbutryn	14	-	2.9	-
Azidoatrazine	-	58	-	-
Terbuthyazine	12	-	3.6	-
Cyanizine	-	-	100	-
De-ethylatrazine	-	6	-	-
Hydroxyatrazine	-	-	-	100
Hydroxydesmetryn	-	-	-	71
Hydroxysimazine	-	-	-	42
Hydroxyterbutylazine	-	-	-	15
Hyroxypropazine	-	-	-	83

Dash - No inhibition

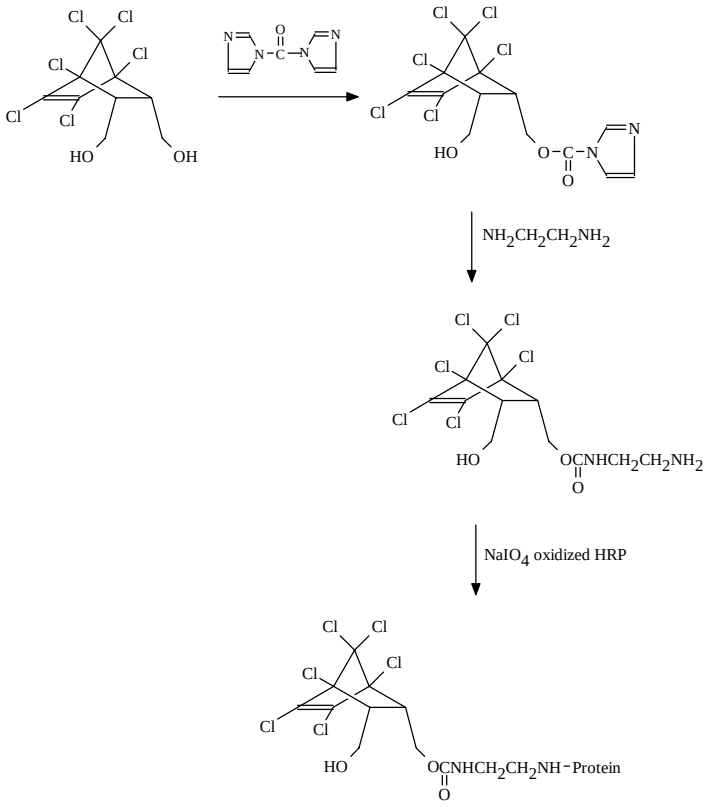


Figure 4. Synthetic route of endosulfan-enzyme conjugate using 1,1-carbonyldiimidazole and ethylenediamine [50].

3.3.5. Hydroxyl groups

The conversion of alcohol to hemisuccinate with succinic anhydride in pyridine has been one of the most commonly used reactions to introduce the carboxylic acid group onto the hapten. The coupling of a hydroxy derivative of allethrin was achieved by conversion to a hemisuccinate followed by the formation of active ester using NHS [18]. The coupling of the carboxylic acid to a carrier protein (or enzyme) is achieved via mixed function anhydride, carbodiimide or NHS. A triadimefon hapten was synthesised by the formation of succinyl triadimenol to introduce the carboxylic acid functionality onto the compound for coupling as illustrated in Figure 5 [72]. DTP, a triazole fungicide, was converted to a hemisuccinate derivative and coupled to protein via mixed function anhydride [69].

The hydroxyl group can react with phosgene to form chlorocarbonate, which can react directly with an amino group of protein. This approach was used by Abad and Montoya [38] to prepare a carbaryl hapten, as illustrated in Figure 6. The hydroxyl group can be converted to carboxymethylether by reacting with ethyldiazoacetate followed by hydrolysis [89,164]. Carbonyldiimidazole has also been used to link the hydroxyl group of hapten to protein. Treatment of the hydroxyl group with *p*-toluenesulfonyl chloride results in a *p*-toluenesulfonate derivative, which provides a means for protein coupling.

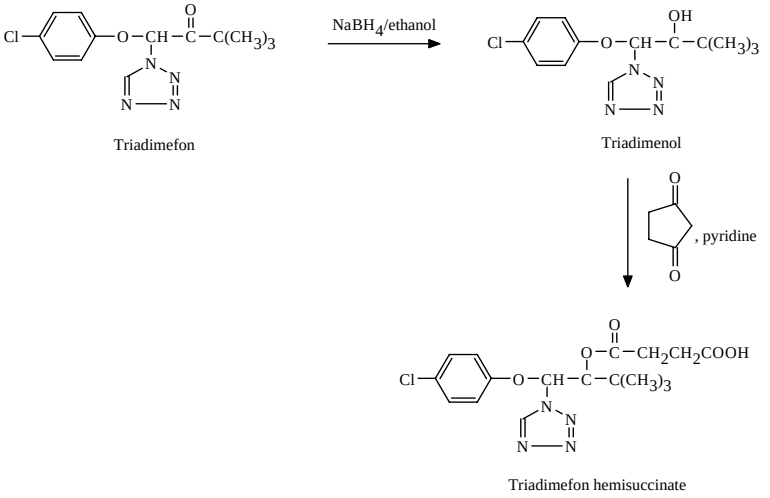


Figure 5. The reaction scheme of a triadimefon hapten [**72**].

3.3.6. Amines

Aromatic amines can be converted to diazonium salts and allowed to react with proteins at alkaline pH [52,57,58]. This reaction occurs primarily with histidine, tyrosine, and tryptophan residues of the protein. Coupling with diazonium salts very often leads to a considerable loss of enzyme activity, since histidine/tyrosine residues are present near or in the active site of most enzymes [161]. While the bonds produced by diazonium coupling are easily dissociated, successful application of this coupling method has been reported in pesticide immunoassay. For example, the conversion of parathion to a diazonium salt was employed in the coupling of amino parathion to bovine serum albumin [58], producing useful antibodies. The coupling of (*p*-aminophenyl)-paraoxon to KLH was also via the formation of diazonium salt [57] and the monoclonal antibodies obtained from this immunogen, although of very low affinity (detection range was 10-100 mg/L of paraoxon), enabled detection of paraoxon from parathion (3% cross-reaction relative to paraoxon). The

coupling of fenitrothion hapten (reduced fenitrothion) to a carrier protein was also achieved by conversion of amide derivative to a diazonium salt; the resulting antibody was useful in developing the fenitrothion immunoassay for grain (Figure 7) [165].

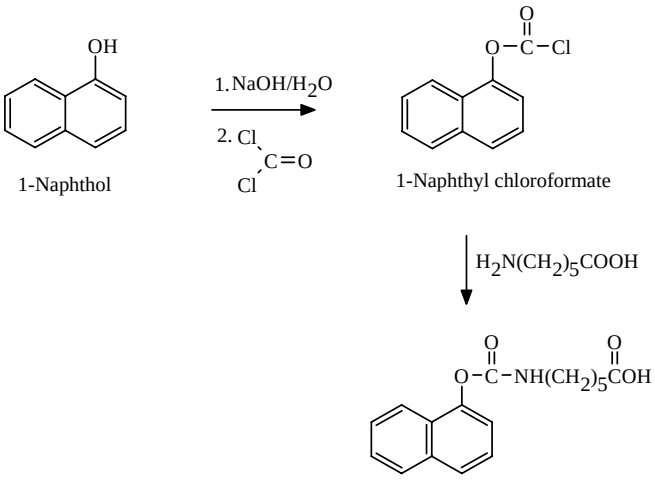


Figure 6. The reaction scheme for a carbaryl hapten using phosgene [38]

The aliphatic amines are often reacted with succinic anhydride to provide carboxylic functionality for coupling. For example, the amino derivative of benzimidazole was converted to 2-succinamidobenzimidazole [63] or 2-succinamidothiabenzimidazole [70] with succinic anhydride, and then coupled to a protein using the carbodiimide reaction. Coupling of aliphatic amides can also be achieved using 4,4'-difluoro-3,3'-dinitrophenyl sulfone, cyanuric chloride or toluene 2,4-diisocyanate [161]. Coupling using these reagents, however, yielded low conjugation as they are hydrophobic and must be dissolved in solvent before addition to protein solution. The active groups of these reagents can undergo hydrolysis, resulting in less available active groups for conjugation. Under basic conditions, 4,4'-difluoro-3,3'dinitrophenyl sulfone and cyanuric chloride react not only with amino groups of lysine but also with other groups containing hydrogen such as the sulfhydryl group of cysteine, phenolic group of tyrosine and imidazole group of histidine [161]. A triazine hapten, 4,6-dichloro-*N*-ethyl-1,3,5-triaz-2-amine, was prepared by reacting ethylamine, *N*,*N*-diisopropylethylamine and cyanuric chloride for directly coupled to BSA and thyroglobulin (THY) [107]. Jung *et al*. [166] used toluene-2,4-diisocyanate to couple 2-nitrophenylsulfenyl derivative of 5-(3-hydroxypropyl)-triazole to protein. Glutaraldehyde also has been used for conjugation of amines, for example, Vallejo *et al.* [151] used glutaraldehyde as a cross-linker to couple 2-aminoparathion and reduced parathion to BSA.

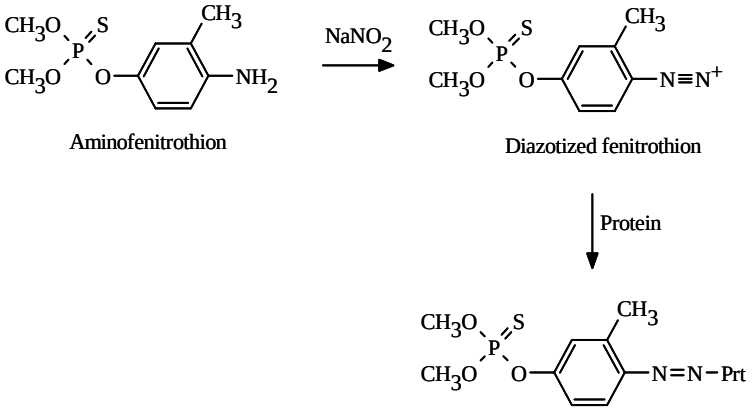


Figure 7. The coupling of fenitrothion hapten using diazotization [165].

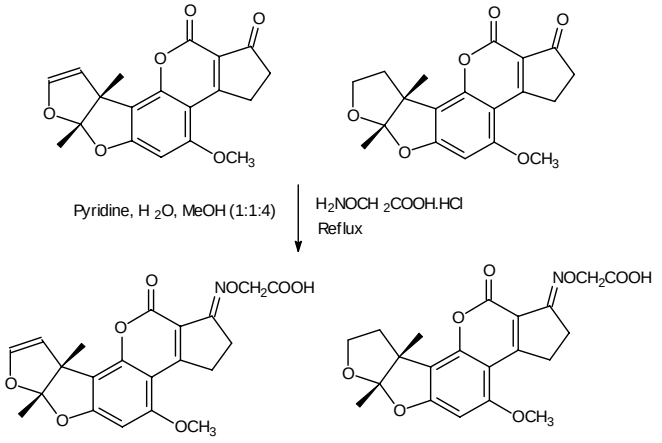


Figure 8. The synthetic schemes for aflatoxin B_1 (left) and B_2 (right) haptens using carboxymethyloxime [15,168].

The use of thiophosgene or phosphogene in the coupling of aliphatic amines has also been successful, via the formation of an isothiocyanate or isocyanate derivative. 2-

Aminobenzimidazole was coupled to a protein by reacting the amine with thiophosgene followed by the addition of protein [167]. This procedure was also utilised by Wong and Ahmed [92] for coupling an imazaquin derivative to an enzyme. The amino derivative of imazaquin was reacted with thiophosgene to form isothiocyanatoimazaquin, then coupling was achieved by reacting with an enzyme (Figure 9). The conjugation of 6-aminobenzopyrene to BSA was achieved using phosgene to form an isocyanate derivative [168].

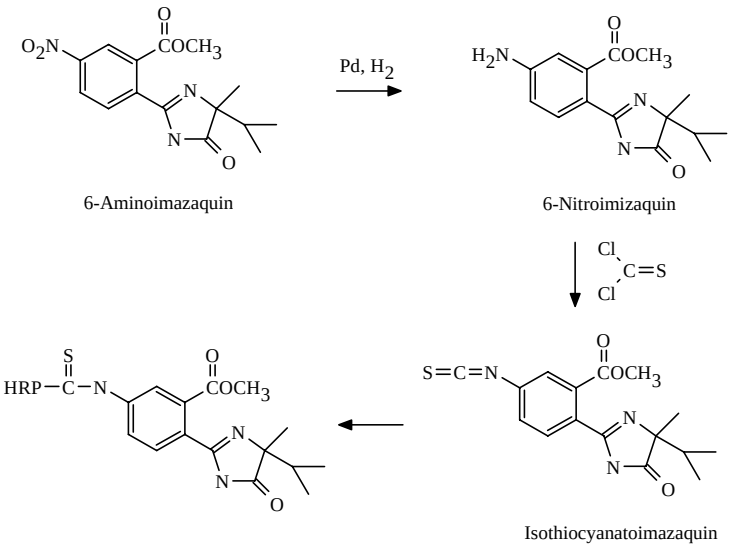


Figure 9. The synthetic scheme for imazaquin hapten using thiophosgene [65]

3.3.7. Carbonyl, phenols and thiol groups

Ketones and aldehydes can be converted to *o*-(carboxylmethyl) oximes by reacting with *o*-(carboxylmethyl)hydroxylamine (CMA). This serves to introduce a carboxyl group and the coupling can be achieved by using one of the procedures described previously for carboxylic acid. The hapten for *S*-bioallethrin was prepared using this strategy, by formatting an allethrin carbomethoxyoxime derivative using CMA [19]. A similar approach was adapted in the synthesis of aflatoxin B_1 and B_2 haptens (Figure 8) [15,16,169]. The aldehyde can be reduced to alcohol as in the hapten synthesis for triamefon [72] and the esterification of the resulting alcohol with succinic anhydride can provided a succinyl spacer with terminal carboxylic group for coupling to protein. Wong and Ahmed [92] have

employed a coupling procedure for the aldehyde derivative of imazaquin by first preparing hydrazine groups on protein, then reacting with the aldehyde derivative of imazaquin. Aldehyde can also be conjugated directly to protein by Schiff base formation followed by stabilization of the bond by reduction with sodium borohydride (Figure 9).

The phenolic metabolite of diflubenfuron was derivatized by the reaction with either ethylbromoacetate or ethylbromobutyrate depending on the intended spacer arm length. The resulting ethyl esters were hydrolysed to the corresponding free acids [37]. Phenols can also be coupled using bis diazotized benzidine [161,164]. Phenols can also be coupled using toluene 2,4-diisocyanate or cyanuric chloride [89].

Coupling of sulfhydryl functionality is achieved via the formation of a disulfide bond, usually with 2-mercaptoethanol. Most proteins do not have many free sulfhydryl groups and one way to achieve the coupling of this group is to introduce sulfhydryl groups onto the protein using thiolating reagents; the most common one is 2-iminothiolane (Traut's reagent). Feng *et al.,* [77] used thiolating reagents, *N*-acetylhomocystein thiololactone (AHT) and *S*-acetylmercaptosuccinic anhydride (AMSA), to provide sulfhydryl groups onto the lysine residues of proteins for coupling of alachlor hapten to protein (Figure 10).

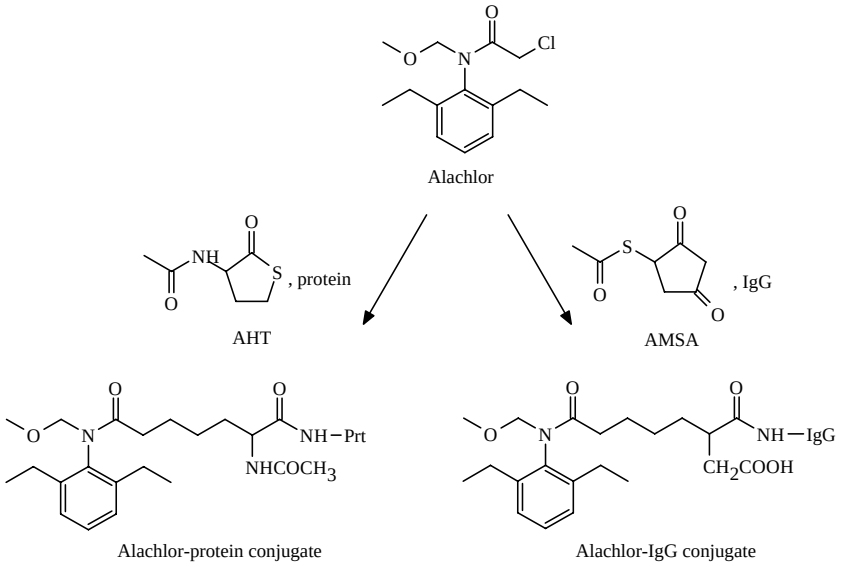


Figure 10. The reaction scheme for synthesis of an alachlor hapten using thiolating reagents, *N*-acetylhomocystein thiololactone (AHT) and *S*-acetylmercaptosuccinic anhydride (AMSA) [74].

3.3.8. Bifunctional reagents

Several bifunctional reagents are commercially available for preparation of hapten-protein conjugation, but they are not readily used in contaminant immunoassay. These reagents provide more specific coupling methods for conjugating protein and reducing the unwanted side reactions such as excessive conjugation to the carrier protein. There are two types of bifunctional crosslinkers, homobifunctional and heterobifunctional. Homobifunctional crosslinkers have two identical reactive groups for cross linking, and usually are utilized in a one-step coupling reaction. Heterobifunctional cross-linkers have different reactive groups for crosslinking and usually utilized in a two-step reaction.

Advantages of heterobifunctional cross-linkers are greater control over the coupling reaction, greater efficiency of coupling between a hapten to a carrier protein and minimize self coupling. Most commonly used reactive groups in bifunctional crosslinkers are amine reactive NHS ester or sulfo-NHS ester, sulfhydryl reactive maleimide and non-specific photoreactive hydroxyphenyl azide with various spacer lengths. Factors such as solubility of haptens as well as cross linkers, intended use (i.e., immunogen or competing spices), available functional groups on haptens and protein carriers and hapten to protein ratio are a few issues that need to be taken into consideration. However, similar guidelines applied to hapten design, as discussed previously, could be utilized when choosing a bifunctional crosslinker. The heterobifunctional reagents containing "bulky" functionalities include succinimyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (MCC) [170] *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) [171] and sulfo derivatives of these reagents. *N*-Succinidyl 3-(2-pyridyldithio)propionate (SPDP) is a heterobifunctional reagent containing a linear chain linkage and also used as a protein thiolating reagent. Jung *et al.* [74] used SPDP and MBS to couple a 2-nitrophenylsulfenyl derivative of thiotriazole to carrier proteins. Two bifunctional derivatives were synthesized by McAdam and Skerritt [165] in preparation of organothiophosphate antigens. They were methoxy and ethoxy derivatives of *t*-butyl 3-chlorophosphorothionylaminopropanoate.

3.4. Antibody production

The immune response in higher vertebrate animals consists of a series of events which enable the body to recognize foreign organisms or substances and eliminate them from the body system. Antibodies are proteins produced by the immune system to destroy or neutralize foreign substances by specifically binding to them. The antibodies are globular proteins which function as antibodies are called immunoglobulins. Immunoglobulins can be divided into various classes on the basis of structural differences. The predominant immunoglobulin in serum is IgG, with a molecular weight of about 150 kilodaltons.

Antibody specific to an analyte is produced by immunizing an animal with an immunogen of a hapten-protein conjugate. Most of the sensitive immunoassays developed for detection of food contaminants have been based on polyclonal antibodies. However, sensitive immunoassays have also been developed using monoclonal antibodies, for example, the immunoassay for 2,4-D [172] has a detection limit of 0.2 μg/L. The atrazine immunoassay using monoclonal antibody has a limit of detection of 0.5 μg/L [79], which is comparable to other immunoassays based on polyclonal antibodies. A sensitive immunoassay for the herbicide, metolachlor has been developed using monoclonal antibodies (the limit of detection is 0.1 μg/L) [95]. Another sensitive immunoassay using monoclonal antibody was developed for triasulfuron, having a limit of detection of 0.01 μg/L [106]. Sensitive immunoassays for paraquat (the limit of detection is 0.1 μg/L) and picloram (the limit of detection is 5 μg/L) were developed also using monoclonal antibodies [103,173].

3.4.1. Polyclonal antibodies

Polyclonal antibodies are a heterogeneous mixture of immunoglobulins, produced by many different B-lymphocytes. Antiserum is the term used to describe serum formed in animals containing specific antibodies, usually produced by repeated immunization. Rabbits are usually used for polyclonal antibody production, simply due to the ease of handling. The process has been scaled up to larger animals such as goats, sheep, and more recently chicken. Numerous animals are immunized to produce a specific antibody as each animal's immune system will respond differently.

There is no standardized protocol for immunization. However, the common approach begins by an initial injection of the antigen, which is followed by subsequent boosting injections in strict schedule. The first bleed is taken 7-10 days after the first boosting injection The subsequent bleeds are taken at regular intervals, usually monthly. The immunogen is usually given as an emulsified solution with either complete or incomplete adjuvant. The immunogen for the first injection is prepared with complete adjuvant and the immunogen for the subsequent injections is prepared with incomplete adjuvant. Freund’s adjuvant is an oil emulsion containing nonionic detergent, such as Tween 80, with (for complete Freund's adjuvant) or without killed mycobacteria (for incomplete adjuvant) which will form a stable emulsion with buffer. Addition of killed mycobacteria in the first immunization aims to attract macrophages and other appropriate cells to the injection site. Injection of immunogen prepared in this manner will provide low-level stimulations of the animal immune system and minimize breakdown of the immunogen by metabolic enzymes, to ensure maximal exposure of the immune system to a foreign protein, thus generating a strong response.

The main routes of immunization are different for polyclonal and monoclonal antibody production. That is, rabbits for polyclonal antibody production are generally immunized either by subcutaneous, intradermal or intramuscular routes or a combination of these. Mice for monoclonal antibody production are usually immunized by a combination of subcutaneous and intraperitoneal injections. Most routes have produced high titre antibodies, showing that this step is not as critical as other steps in the development of a sensitive immunoassay. The multiple-site injection has been preferred to spread injection volume and ensure at least one site elicits the animal immune response.

It is not generally possible to reproduce in different animals antibodies with the exact titre and specificity for the same immunogen, as the immune response of individual animals differ even though they may be physiologically identical. However, once the useful antibody is raised, up to millions of assays can be prepared from one animal. Assays can be prepared even after the animal is no longer available, as antisera can be stored frozen for many years without loss of activity.

3.4.2. Monoclonal antibodies

Monoclonal antibodies are homogeneous antibodies produced by a clone of a hybridoma cell (i.e., a monoclonal antibody is derived from a single cell). Hybridomas are cell lines derived by fusion of an immunized B-lymphocyte and myeloma (tumor) cell. Monoclonal antibody is produced by fusion of a non-secreting myeloma tumor cell (with approximately 65 chromosomes) and an antibody-producing B-cell (with a normal complement of 40 chromosomes). Mice are commonly used to generate antibody-producing B-cells.

The fused cells retain properties of both cell lines, that is, “immortality” of myeloma cells and antibody secretion of the B-cell. Selection of the fused cells is achieved by culturing in a medium containing hypoxanthine, aminopterin and thymidine (HAT). B-cell myelomas, which lack the purine salvage enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT), are unable to grow in this medium as are unfused B-cells, leaving hybridomas to survive. In the presence of aminopterin, *de novo* nucleic acid synthesis is blocked, and the hybridoma cells can survive by switching to the salvage pathway using nucleotides in the HAT medium.

3.4.3. Recombinant antibodies

Antibodies can be generated *in vitro* using antibody engineering technology. Recombinant antibody (rAb) technology can deliver specific antibodies with greater speed and at low costs. More importantly, inherent binding properties of antibody or binding fragment (scFv, Fv and Fab) can be manipulated *in vitro*, thus able to fine tuning to the desired specification. However, to date only a small number of recombinant antibodies have been generated for food contaminants and some of these has been applied to biosensors

[174,175]. The recombinant antibodies for food contaminants are diuron [173,176], dioxin [176], picloram [177], parathion [178], chlorpyrifos [179], 2,4-dichlorophenoxyacetic acid [180], atrazine [181], aflatoxin B_1 [182], deoxynivalenol [183] and fumonisin B_1 [184]. Two approaches have been generally pursued for the recombinant antibody production for food contaminants. One approach typically used at the early stage of is 1) isolation of mRNA from hybridoma cell lines, 2) synthesis of first strand cDNA from the mRNA, 3) amplification of Ig heavy chain and light chain genes, 4) ligation of genes into an Ig expression vector, 5) expression and purification of recombinant fragments, and 6) characterization of rAb in an immunoassay. The second approach is to construct a phage display Fab or scFv library either from immunized or naïve lymphocytes. The purpose of the library is to increase the diversity of potential antibody specificity of the order of 10^7-10^9 for selection. The common approach to the phage display library construct is 1) isolation of heavy and light chain genes from either immunized or naïve lymphocytes, 2) cloning of these genes in random combinations into phage display vectors, 4) screening for desired rAb fragments using a panning process. More recently, an innovative approach to isolate peptides that mimic analytes from the phage display peptide library to substitute with competing species in a competitive immunoassay has been proven possible [187]. It is envisaged that recombinant antibody technology will play a much greater role in immunodiagnostic and affinity sensor fields in the near future.

3.5. Immunoassay formats

Immunoassays can be classified into two groups: (1) the homogeneous assay, in which the detection system is measured without a separation of free and antibody bound components, and (2) the heterogeneous immunoassay, in which the detection system is measured after a separation of free and antibody bound components. Most of the immunoassays developed for food contaminants have employed the heterogeneous system. Heterogeneous immunoassays require at least one separation step that allows the differentiation of the antibody-antigen complex from free reactants. In contrast to homogeneous immunoassays, the activity of the enzyme on the labelled antigen or antibody is not affected by the presence of analytes. Labels such as isotopes, fluorophores and enzymes have been used for detection in immunoassays. Problems associated with radioimmunoassays are health hazard, cost (expensive reagents and equipment), stability (limited for some radioisotopes), disposal of radioactive material and license is required to handle these compounds. In contrast, enzyme-labelled materials have longer shelf lives and the reagents are less expensive. Most of the sensitive immunoassays for food contaminant analysis, however, utilize enzyme as a detection system. The enzyme property of substrate turnover provides an amplification system in an immunoassay, and the resulting color is

measured spectrophotometrically, which is cheaper to set up compared with the instrumentation for radioimmunoassay.

Heterogeneous immunoassays can be classified as either competitive or noncompetitive. The competitive immunoassay is a format where free analyte competes with labelled antigen or antibody for antigen binding sites on a limited amount of antibody. The non-competitive immunoassay is a system in which test antigen is reacted with excess antibody. Immunoassays for food contaminants are based on direct (conjugation of enzyme) or indirect (via enzyme labelled anti-species antibody) competition formats.

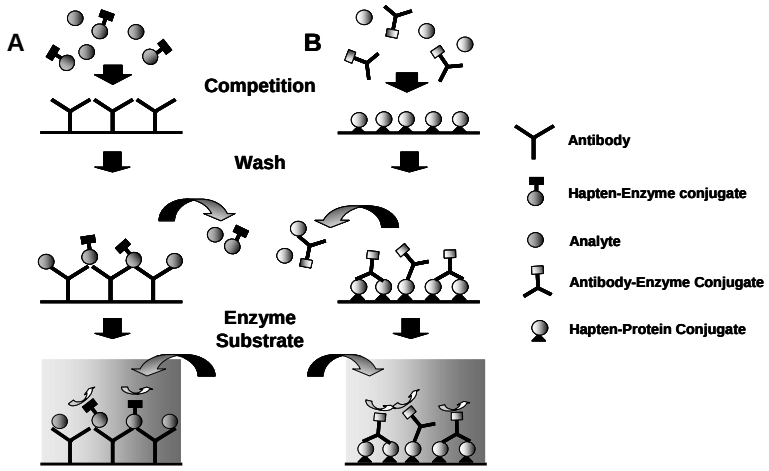


Figure 11. Competitive immunoassay formats. A. Immobilized antibody and B. immobilized antigen.

In a direct immunoassay based on a solid-phase antibody format, incubation of a sample containing pesticide and labelled antigen allows free pesticide in a test sample to compete with labelled antigen for binding sites on limited amount of coated antibody until the antigen-antibody reaction attains equilibrium. After the removal of unreacted materials by washing, the bound antigen-antibody complex is incubated with a solution of substrate/chromogen. Subsequently the enzyme reaction is stopped and the concentration is determined using a photometer or fluorometer (Figure 11A). In a solid-phase antigen format, incubation of test sample and labelled antibody results in a competition between free pesticide and immobilized antigen for antigen binding sites. The concentration is inversely proportional to the color developed by antibody-antigen interaction (Figure 11B). Purified antibodies are typically required in this assay format for immobilization and preparation of enzyme-antibody conjugate. Enzyme-antibody conjugate is usually the least stable

component in the direct immunoassay and the conjugation may modify the properties of antibody, resulting in the changes in the specificity of immunoassay. Matrix interferences have been observed in this type of immunoassay as enzyme is in direct contact with sample matrix or matrix extract, for example, the benomyl and thiabendazole immunoassay for fruits and vegetables [9] and endosulfan immunoassay [23].

In an indirect competitive immunoassay, binding of the antibody to immobilized antigen is inhibited by the presence of free analyte. After removal of the unbound materials, an enzyme-labelled secondary antibody (specific for IgG of the animal species in which the primary antibody is raised) binds to the primary antibody-antigen complex. The color is developed by addition of the substrate/chromogen, after removal of the unbound secondary antibody. The sensitivity of this assay format has been improved: 1) through preincubation of the antibody and pesticide containing samples prior to exposure to the immobilized pesticide-protein conjugate [23,188] or 2) by use of commercial amplification systems such as the biotinyltyramide-based ELAST kit [189].

In general, it is easier to obtain greater sensitivity with a direct format than with an indirect format. This is mainly attributed to a greater non-specific binding inherently occur in an indirect format, reducing detectable sensitivity. However there are exceptions to this rule, for example, an immunoassay for endosulfan [23]. The greater sensitivity of the direct assay format is attributed to the lower substitution of pesticide hapten to enzyme (0.5-2.5 haptens per molecule) as compared to hapten substitution on protein in an indirect assay format. The direct immunoassay is simpler and faster than the indirect format and produces lower background as the nonspecific adsorption of commercially labelled anti-species antibody often increase the background color in an indirect immunoassay.

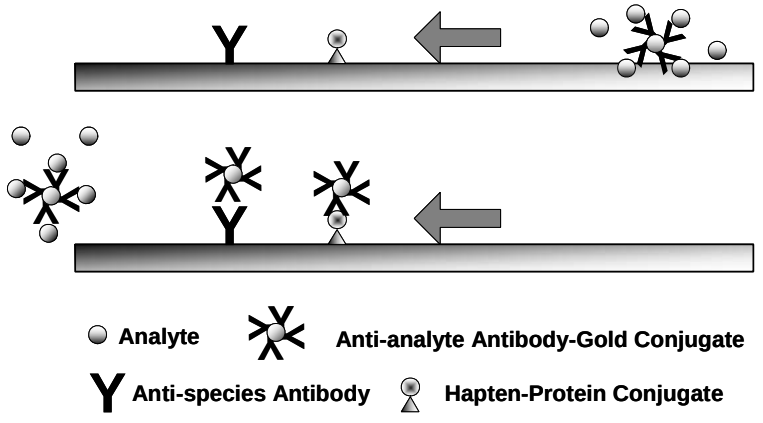


Figure 12. Lateral flow test for a competitive immunoassay.

Immunochromatography is a technique that combines immunochemistry and chromatography.

It is initially developed for clinical and medical diagnostics, but recently has been adapted to food contaminant analysis. The technique provides a simple yet very fast, cost-effective, user-friendly decision-support tool designed to be portable. These features are ideal for on-site decision making situation such as at receival bays and on the processing lines. It represents an extension to dipstick tests. There are two versions of immunochromatography that are currently utilized, the lateral flow format and the flow-through format. As illustrated in Figure 12, a lateral flow format for food contaminants relies on an antigen immobilized on a membrane, usually as a strip. An antibody conjugated to visible particles such as gold colloid, latex beads and dyed liposome mixed with an analyte is moved by capillary action towards the immobilized antibody strip. Here only free conjugated antibody will bind onto the immobilized antigen. The degree of binding between the conjugated antibody and immobilized antigen will depend on the amount of analyte interacted with conjugated antibody. Excess free conjugated antibody will bind to an anti-species antibody that is immobilized on a membrane as a reference line. Thus, two visible strips indicate negative and one strip indicates positive. Flow-through format works on the same principle except that a conjugated antibody and an analyte mixture is vertically "flowed-through" the layers of filters which are placed on top of the immobilized antigen strip. Upon reaching the immobilized antibody, the interaction between free conjugated antibody and immobilized antigen will occur. Some of the dipstick test, lateral flow test and flow-through tests for food contaminants are listed in Table 4.

3.5.1. Immobilization

The immobilization of antigen or antibody on a solid-phase permits the complete separation of free and bound reactants without disturbing the equilibrium of the reaction or impairing the label. The immobilization of antibody or antigen generally involves passive adsorption (noncovalent binding) onto microwell plates/tubes consisting of polystyrene, polyvinylchloride and polyethylene with a binding capacity of 300-400 ng/cm^2. Passive adsorption of antibody and antigen may change the conformational structure of proteins. In the case of immobilized antibody, this can result in 1) loss of binding sites on the antibodies, 2) reduction of affinity constant of antibody and 3) alternation of specificity of antibodies, and in the case of immobilized antigen 1) alteration of antigenicity, 2) conformational changes and 3) formation of aggregates. Heterogeneity of the solid phase can provide low consistency of binding and partial denaturation of the immobilized reactants during storage may also occur. Non specific binding of reactants is also possible [202,203].

Table 4. Dipstick, lateral flow, flow through and immunofiltration tests reported for food contaminants

Compound	Ref.
Dipstick tests	
Clenbuterol	[190]
β-agonists	[191]
Tetracycline antibiotics	[192]
Carbaryl	[22,193]
Atrazine, terbuthylazine, ametryn	[25,194,195]
Fenthion	[196]
2,4-dichlorophenoxyacetic acid	[28,197]
Sporidesmin A	[198]
Aflatoxin B_1, T-2 toxin, 3-acetyldeoxynivalenol, roridin A, and zearalenone	[105]
Lateral flow tests	
2,4-dichlorophenoxyacetic acid	[28]
Alachlor	[26]
Carbaryl	[199]
Avermectin A2a	[20]
Aflatoxin B_1	[29,201]
Flow through tests	
Carbaryl	[20]
Fumonisins	[30]

While immobilization provides less freedom to improve the assay sensitivity, several methods have been advocated to improve the capacity of immobilization. Passive adsorption results in adsorbed antibodies in different orientation - that is adsorption occurring on any part of the antibody. Staphylococcal protein A binds specifically to the Fc region of immunoglobulin [204-206]. Use of protein A can improve the orientation of adsorbed antibody and subsequently improve the assay sensitivity [189,207,208] Use of glutaraldehyde treated polystyrene has also been used to improve the capacity of immobilization (the binding capacity is 4-5 $\mu g/cm^2$) [202,203].

3.5.2. Antibody characterization

Antibody characterization includes the determination of sensitivity, limit of detection, dynamic behavior, specificity, matrix interferences, data precision and accuracy and reagent stability. Unlike instrumental methods where there is a direct relationship between the pesticide concentration and the response as appearance of chromatographic peak, in a competitive immunoassay the presence of pesticide is manifested as inhibition of color development; an inversely proportional log-linear relationship between pesticide concentration and the color development. Thus, a dose response curve is typically of sigmoidal shape, obtained by plotting either percentage of control absorbance, percentage of inhibition of antibody binding or absorbance on Y axis in linear scale and the concentration of pesticide on X axis in log scale.

The percentage of control absorbance is determined by Eq. (2) where the control absorbance is determined by adding a sample matrix with no pesticide with enzyme conjugate. The percentage inhibition is the reverse of the percentage of control and calculated as Eq. (3). The blank wells are usually included in the assay for determination of background. The blank absorbance is determined by either adding to a well a water/diluted matrix extract containing no pesticide or excess pesticide and enzyme conjugate. The background is slightly higher when single wavelength is used for reading and usually in the order of 0.01-0.05 absorbance unit. However, the background can be reduced by the use of dual wavelength mode, for example, 450 nm and 650 nm for 3,3'4,4'-tetramethylbenzidine (TMB) substrate.

$$\%\frac{B}{B_0} = \left(\frac{A - A_{blank}}{A_{control} - A_{blank}}\right) \times 100 \tag{2}$$

$$\%\frac{B}{B_0} = \left(\frac{A - A_{blank}}{A_{control} - A_{blank}}\right) \times 100 \tag{3}$$

A = absorbance

$A_{control}$ = absorbance of analyte at zero concentration

A_{blank} = absorbance of blank wells

Point-to-point plotting or linear interpolation can be carried out with a reasonable precision and low bias. However, the quality of results depends on the skill and care of the person performing the calculation. The point-to-point plot is most precise where the two mean responses between neighbouring calibrators are nearly linear. Where curvature is greatest, the point-to-point plot has significant bias. Thus, the distance between neighbouring calibrators is an important factor for assay precision. Usually, a 2-3 fold difference in calibrator concentrations is adequate when this method of curve fitting is

chosen. It is, however, time consuming and impractical when high throughput routine use is desired.

Computerized logit-log and four-parameter log-logistic curve fitting programs have been extensively employed nowadays for routine application. Logit-log function transforms a sigmoidal curve with a single inflection point into a linear relationship and plot concentration in log scale against logit of signal response, as shown in Eq. (4). Four-parameter log-logistic curve fitting is an extension of log-logistic fitting and it plots point of inflection, the slope of linear section of curve, upper and lower asymptotes of sigmoidal curve (Eq. (5)).

$$where \quad Y = \frac{Bound - NSB\,(\%\ bound)}{B_0 - NSB\,(\%\ bound)} \tag{4}$$

NSB = non specific binding (background absorbance)
Bo = binding at zero concentration

$$y = \left[\frac{a-d}{1+\left(\frac{x}{c}\right)^b}\right] + d \tag{5}$$

y = signal response
x = analyte concentration
a = response at zero concentration
b = slope
c = IC_{50}
d = response at infinite concentration

The advantages of these types of curve fittings are that they are widely available, easy to use and provide certain quality control over poor replicates or outliers, particularly at lower concentrations where deviations are typically greater. These types of curve fittings rely on accurate estimates of zero and maximum binding and curve symmetry to be accurately plotted the dose-response of an immunoassay. Hence in cases where dose-response does not follow an ideal immunoassay behavior, for example, due to polyclonal antibody–antigen interaction or enzyme-substrate reaction, significant bias can occur as a result. Thus, these factors must be taken into consideration during assay characterization and validation. As illustrated in Figure 13, both the point-to-point plot and four-parameter log-logistic curve match best at the linear part of the curve that includes IC_{50}. Around parts of the curve where curvature is greater, there is some difference between these two plots and hence the

differences in estimates are due to interpolation at these parts. The degree of difference whether it is upper or lower regions of the curve will depends on the degree of curvature and deviation of absorbance and curve symmetry.

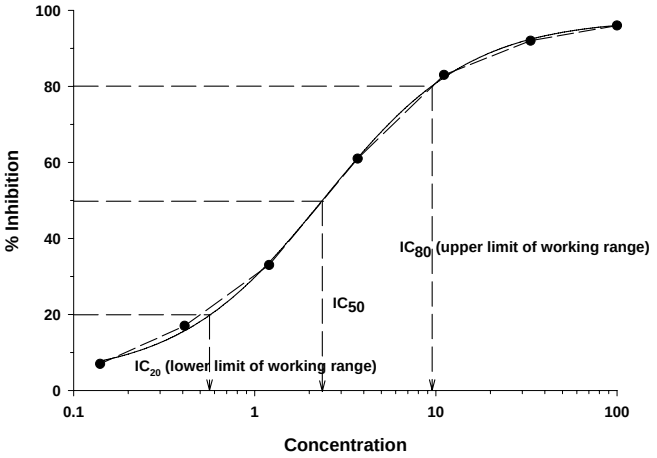


Figure 13. Typical dose-response curve of an immunoassay. The dotted line represents point-to-point curve and the solid line represents 4-parameter logistic curve fitting.

3.5.3. Sensitivity and limit of detection

Sensitivity of an assay is commonly determined by the dynamic behavior of the standard curve and the IC_{50} (concentration of the pesticide required to reduce the color development by 50%). The precision of the assay is greatest at the IC_{50}. In practical terms, it is more difficult to define the range and limit of detection in immunoassays than with instrumental methods. Most immunoassay users and test kit manufacturers, however, define the range of detection as the most linear part of the curve, usually between 15-20% and 80-85% inhibition. The limit of detection of the assay is determined by the concentration of pesticide that reproducibly provides either 15% or 20% inhibition of color development (IC_{15} or IC_{20}).

The dynamic behavior of the assay is determined by the slope of the standard curve. The steepness of the slope in turn determines the precision and accuracy of the assay and hence whether the assay is suitable for quantitative or semi-quantitative analyses. That is, if the slope of the standard curve is relatively flat, the assay is usually only useful for qualitative analysis as precision and accuracy will be lower. In general, a slope of 35% to 50%

difference in absorbance over a 10-fold difference in concentration of the analyte is considered acceptable for quantitative analysis. Greater than 50% slope results in narrow detection range, making analysis tedious and sometimes impractical. The dynamic behavior of an immunoassay can be affected by the assay format. In general, the immobilized antibody format provides a steeper standard curve than the immobilized antigen format, but exceptions to this observation have been observed.

Generally it is not difficult to obtain adequate sensitivity in water and unconcentrated extracts of soil or food with immunoassays. Sensitive immunoassays have been described for most major food contaminants, including pesticides, veterinary drug residues and mycotoxins, with the limits of detection ranging from 0.01-5 μg/L. The demand for immunoassays with greater sensitivity, however, is becoming more critical to meet the stringent legal limits, for example, the maximum residue level of individual pesticides in drinking water is set below 0.1 μg/L by the European Community. New legislation regarding the lower maximum permissible limits for mycotoxins (aflatoxin and ochratoxin) in certain commodities has been established in recent years. To increase the assay sensitivity through sample preparation is less demanding than for instrumental methods, as less extract cleanup is required. Use of reverse phase columns for trace enrichment of pesticide residues in water can be used without a need for extract concentration and resolution in an appropriate solvent. The overall assay is much simpler, however, if such manipulation can be avoided. The required assay sensitivity and limit of detection vary with intended use. That is, some immunoassays are designed to detect higher concentration, for example, the maximum residue levels of pesticide in foods, commodities and soil are 0.1-2 mg/L in the actual matrix, thus the important detection range is 0.01-0.2 mg/L. While the detection limits in water need to be at very low part-per-billion or high parts-per-trillion to meet the legal requirements. Excess dilution of sample/sample extract to fit in the detection range of the assay can add to potential errors. Therefore in designing an immunoassay, the intended use of the assay must be considered.

3.5.4. Specificity

The performance of immunoassays can be affected by two factors, matrix effects and cross reactivity. Cross reactivity is a measure of antibody's ability to bind compounds other than the analyte and is directly related to the specificity of immunoassay. The specificity of the immunoassay is determined by the cross-reactivity of the antibody for compounds with related structures. It is normally expressed as a percentage and calculated according to Eq. (6). The IC_{20} has also been used to determine the cross-reactivity of the antibody at the lower limit of detection.

$$\%\, CR = \left(\frac{IC_{50}\ reference\ compound}{IC_{50}\ test\ compound} \right) \times 100 \qquad (6)$$

Specificity directs the potential use of the immunoassay. A broad specificity assay is useful for screening a group of related food contaminants and a single compound specific assay provides a quantitative analysis of that analyte from a pool of many different contaminants. The specificity of an immunoassay is influenced by the chemistry of analyte-protein conjugation, which effect on assay specificity has been discussed substantially in the hapten synthesis section. The importance of assay specificity is defined by the use of the cross-reacted analytes in the intended matrix. For instance, the immunoassay for chlorpyrifos-methyl in grain cross-reacted with iodophos, bromophos and fenchlorphos, which are not used on grains and, thus, these cross-reactions are insignificant in practical terms [209].

Some pesticide metabolites are either equally toxic (such as the sulfate metabolite of endosulfan) or more toxic (such as dieldrin which is the oxidative metabolite of aldrin) than their parent compounds, and detection of both the parent compound and the toxic metabolites are environmentally important. Many of the contaminant immunoassays intended to measure the parent analyte show some degree of cross-reaction with its metabolites. Some immunoassays are developed for detection of the metabolites, such as the hydroxy metabolites of triazine herbicides [90,91] and paraoxon, a toxic metabolite of parathion [57,120]. However, breakdown residues are of environmental as well as health significance and metabolite specific immunoassays will play an important role in this area in future.

3.5.5. Matrix effects

Immunoassay is generally less affected by matrix effects than instrumental methods, but these must still be carefully managed if present. In instrumental methods, the matrix effects are usually overcome by extensive sample preparation involving extraction of residues and cleanup of the matrix extract prior to analysis, which can be tedious and laborious. The matrix effects encountered by immunoassay are different to those of instrumental methods and are usually handled differently. Generally, water samples are analyzed unconcentrated and other matrices such as soil and food are extracted with a water miscible solvent and the extract is analyzed after dilution with either buffer or purified water. There are cases, however, where extra sample preparation steps are required to overcome matrix effects in immunoassays and even in such cases the procedure is a lot simpler than that of instrumental methods. More recently, integration of supercritical fluid extraction with immunoassay has been reported for detection of alachlor, carbofuran, atrazine, benomyl and 2,4-D in meat products [210,211]. Use of solid-phase extraction in immunoassays for matrices other than water has also been reported [212,213].

The effects of the matrices are determined by comparing the dose-response curve prepared in a particular matrix, and the dose-response curve prepared in matrix free

solution; purified water as in the case of liquid samples such as juices, wine and beer, and diluted blank extract if the matrices are solid foods. If the two curves are superimposable, the effect of the matrix is considered not significant, then the samples can be analyzed using the standard curve prepared in the matrix free solution. It is, however, best practice to treat the dose-response curve in the same manner as the field samples to minimize any variations in sample constituents that might interfere with analysis, thus preparation of the dose-response curve should be done in the anlayte-free matrices if they are obtainable.

Assuming that the sample matrix used is free of contamination, matrix effects are apparent when one of the following occurs:

- reduction in assay sensitivity, as evidenced by the standard curve being shifted to the right, but no changes in maximum color development
- reduction in maximum color development (in absence of competing analyte enzyme-conjugate is less able to bind to antibody), but no changes in % inhibition
- combination of these two phenomena

Fewer matrix effects are usually observed for an indirect immunoassay than with a direct immunoassay. This is because the labelled anti-species antibody is incubated after the removal of sample matrix in an indirect immunoassay. Whereas enzyme-conjugate and sample are incubated together in a direct immunoassay. Matrix effects are generally different for different sample matrices and the degree of matrix to interfere with assay performance would depend on sample types, and the degree of processing (Figure 14). For example, the procymidone immunoassay suffered from the phenolic content of red wine [155], which was reduced when protein and detergent were added to the sample diluent. Red wine inhibited the enzyme activity of endosulfan ELISA, but not an antibody binding, hence reducing the maximum color development, but the percentage inhibition remained the same (unpublished data). The same ELISA was little affected by white wine matrix and the problem was corrected by a simple dilution. The method and solvent of extraction and degree of extraction can produce different effects on immunoassay. High levels of sodium sulfate were found in the neutralized paraquat extract using sulfuric acid [100]. To compensate for the reduced sensitivity, sodium sulfate was also added to the standard curve. The degree of grinding or particle size of ground samples affected the color development of aflatoxin B_1 ELISA [16]. The finer the particle size is, the greater color inhibition will be observed. Thus, the degree of grinding was adjusted to gain low interferences but retain the optimum recovery. Methanol as an extraction solvent did not affect the overall performance of fumonisin B_1 ELISA, but acetonitrile was a better choice when the assay was formatted to complete within 15 min (unpublished data). It probably results from different co-extractants formed in different solvents. A similar phenomenon was observed when rice was extracted with acetone, acetonitrile and methanol, where color development of endosulfan ELISA was inhibited by 50% [214]. Degree of processing can induce significant

interferences on immunoassays. Ground wheat samples did not interfere with immunoassays for grain protectants, fenitrothion, chlorphyrifos-methyl, pirimiphos-methyl, permethrin, biosementhrin and methoprene. However, the processed products such as flour and noodles affected the assay sensitivity [215]. To overcome matrix interferences, increased dilution of thmatrix extract can be beneficial [216] or filtration to remove the interfering particles can also improve the analysis [16,217]. Although dilution is typically used in situations where matrix effects can be diluted out, but assay sensitivity must be compromised. Some matrix effects cannot be diluted out due to their nature (e.g., tea, green or roasted coffee) or in a particular ELISA system. Use of simple solid-phase extraction or immunoaffinity chromatography with ELISAs that may suffer from severe matrix effects could gain benefits of still maintaining high sensitivity.

3.5.6. Assay accuracy

Although immunoassays for most major food contaminants have been developed, acceptance of immunoassays by many food analysts is still slow even after three decades of research. This is mainly attributed to the unavailability of published extensive validation data of the method. As with any analytical method, thorough validation of immunoassay performance and the availability of the validation data are essential.

There are two ways to measure the accuracy of an immunoassay; 1) immunoassay data are compared to the levels of analyte spiked into food matrix or 2) by comparing immunoassay data and instrumental analyses of samples containing incurred or naturally contaminated residues. The former is usually used when the performance of immunoassay is initially validated. The latter is, however, preferred as residues are adsorbed onto matrix components upon aging and extraction of such residues is usually less efficient. That is, the recoveries of the spiked samples are usually higher than the samples containing incurred or naturally contaminated residues. Sometimes the erroneous assumption is made here that the instrumental methods yield correct results. Correlation between immunoassay data and instrumental analyses are analyzed by three parameters [218]: 1) the correlation coefficients determined as either r or r^2, 2) the slope of the regression line and 3) how close the plot is from the origin. In most cases, immunoassay data correlate very well with those obtained by instrumental analyses and the percentage correlation coefficients are usually over 0.9, but the regression plots generally pass above the origin with the slope greater than 1, reflecting greater estimation by immunoassay.

In some cases, lower recoveries by instrumental method arise from the loss of pesticide during the cleanup procedure [218]. The differences between immunoassay and instrumental method can be due to different methods of extraction (i.e., two different extraction efficiencies for incurred residues), different standards of the same pesticides being used or degradation of pesticides occurring during storage for instrumental method

(because of the length of sample preparation procedure for instrumental method, samples are usually stored for a period of time, whereas immunoassay can provide immediate analyses). Instrumental methods pose greater risk of pesticide loss as extensive sample preparation is required. Immunoassay usually provides analysis of unconcentrated sample (for water), or of extract obtained from a simple extraction with a water-miscible solvent, so there is less risk of pesticide loss. In addition, adsorption of pesticide onto suspended particles in field water can result in differences in analyses by two different methods. Incubation of the sample in detergent containing buffer during immunoassay can aid in the release of adsorbed pesticides into solution, resulting in greater estimation. There is no evidence, however, that immunoassay only measures dissolved residues and it is possible that immunoassay is capable of measuring both dissolved and particle-bound residues as a result of re-equilibration; this ara will need some attention for future studies.

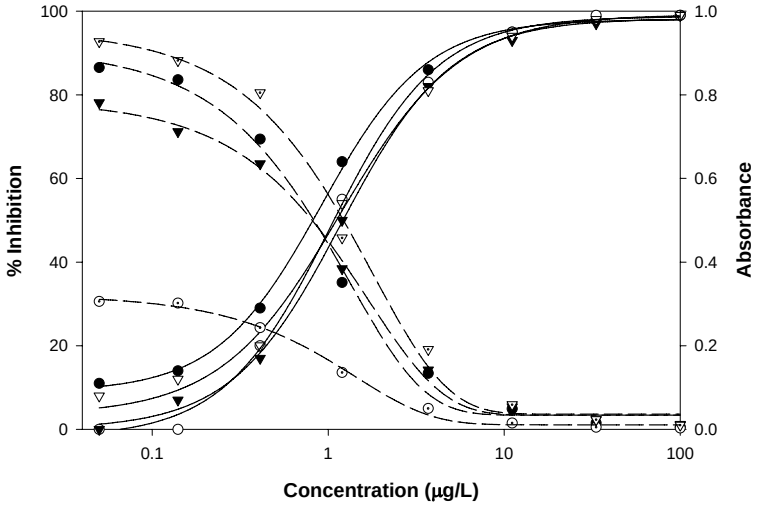


Figure 14. Matrix interference on immunoassay performance. Solid lines represent plots of % inhibition vs concentration and broken lines represent corresponding plots of absorbance vs concentration. ▽ is water, ▼ is 1:2 dilution of grape juice in water, ● is 1:10 dilution of grape juice in water and ○ is 1:50 dilution of grape juice in water.

3.5.7. Assay precision

Precision of immunoassay is determined in two ways; 1) determining the standard deviation of the standard curve and spiked samples and 2) determining assay precision within laboratories and between laboratories. The assay precision is initially determined by running the standard curve several times on the same day and on different days. The next step is to analyse spiked samples relative to the standard curve on different days. More thorough studies include within-laboratory and between-laboratory precision. A thorough inter-laboratory collaborative trial suggested by Skerritt [218] involved 6-15 blind samples containing various concentrations of analyte. These were analyzed twice by 6-12 laboratories after pretrial assay ++to e0nsure that the collaborative laboratory could perform the immunoassay. The precision was determined for three factors; 1) the standard deviation obtained for two analyses of each laboratory (intra-laboratory precision), 2) the standard deviation determined for mean results0 obtained by all collaborative laboratories (interlaboratory precision) and 3) the standard deviation between blind pairs in each laboratory.

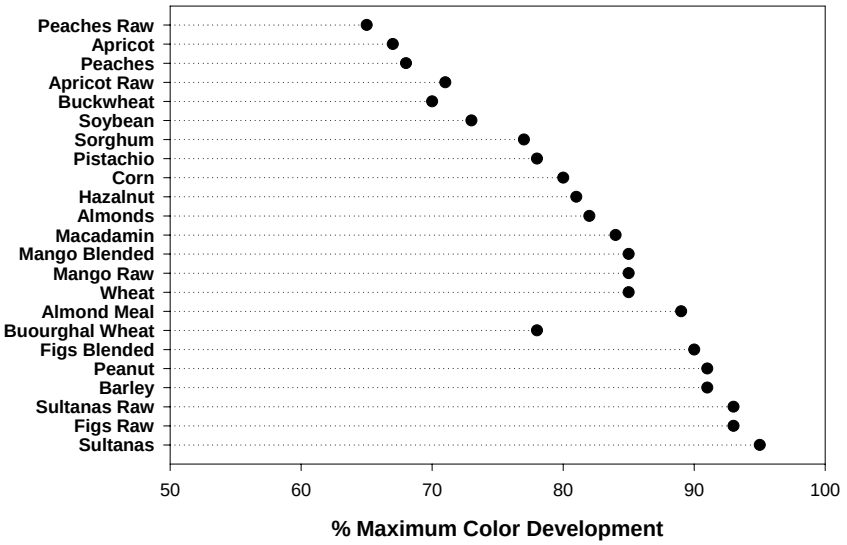


Figure 15. Effects of various food matrices on the maximum color development of aflatoxin B1 ELISA.

Precision of immunoassay is limited by several factors and the sources of error of immunoassay are quite different to those of instrumental method [208,218]. Many microwell plates and microreaders can show up to 0.05 absorbance unit difference between wells that are actually of equal absorbance. An added deviation is assay drift, resulting from timing of the immunochemical incubation between individual samples. Potential pipetting error can not be ignored. Hayes *et al.* [208] has demonstrated using *s*-triazine immunoassay that there is little difference in results when pipetting error of within ± 20% of prescribed volume was made for all samples, but if pipetting error was made in one sample the error in the final results will be one magnitude proportional to the pipetting error. Most samples are heterogeneous in nature and uneven distribution of pesticide in a matrix can produce additional error in the final results. The assay components prepared at different times can show significant difference in the immunoassay, thus reducing the assay precision. Thus some measure of quality control of immunoassay is needed.

3.5.8. Quality assurance and quality control

Immunochemical analysis should be subjected to the same stringent analytical quality control measures as any other instrumental methods. It is part of the development that assay performance is determined. Performance of immunoassay is initially validated for a number of samples. These include negative samples in simple matrices (pure water or buffer), negative samples in the matrices of interests, positive samples in simple matrices and positive samples in the matrices of interests. Positives samples in the matrices of interest can be divided into two types, one where known amounts of the analytes are spiked and another where they are incurred or contaminated naturally. Since immunoassay is typically validated against an instrumental method it is vital that the corresponding instrumental method be already validated and operating at optimum conditions. Use of contaminant free matrix during the characterization and validation of an immunoassay is thus vitally important to avoid ambiguous results. More so than instrumental methods, the immunoassay technique can be disadvantaged by plate-to-plate, day-to-day and batch-to-batch variations and it is important that each immunoassay run must be accompanied by a Quality Control (QC) or a reference sample. In most cases, immunoassays are performed in complement to instrumental methods and we normally recommend that 20% of the samples (both negative as well as positives) should be confirmed by an official instrumental method. For the purpose of QC, statistical monitoring of certain analytical parameters such as maximum color development, IC_{20} (as a lower limit do working range), IC_{50} and IC_{80} (as an upper limit of working range) using a control chart would provide additional assurance. For example, a cusum control chart, as illustrated in Figure 16, was used as a form of QC for monitoring the assay performance for aflatoxin B_1 analysis, where limits were set at 2 standard deviations above and below a mean derived under a specific situation. It was necessary to derive a

mean under a normal operating situation within a particular facility (e.g., analytical laboratories in developing countries), but within an adequately controlled environment. It should be obvious from the control chart in Figure 16 that the sensitivity has drifted from the acceptable range. Therefore, investigation on the cause of the drift and corrective measures must be performed. The same concept could be applied to the reference sample and other QC control parameters as part of QC.

3.5.9. Reagent stability

Although immunoassays are physical methods based on mass action, they utilize biological components. There is a need for quality control of the components, as "batch to batch" differences in these components is common (i.e. difference in immunoassay between components prepared at different time) and these components should be at their optimal conditions at all time during analysis. The stability of immunoassay components are evaluated by performing accelerated trials at elevated temperatures. The availability of stability data of the immunoassay components, though not critical for government agencies, is essential if immunoassays are to be commercialized or intended for routine analysis. Antibodies are usually stable, they can be stored up to ten years without loss of activity. Enzyme conjugate, however, is not stable for long storage and these are the most labile component in immunoassay, often requiring fresh preparation to maintain quality of results.

There are three components in an enzyme-conjugate that can undergo “degradation”: the enzyme, the pesticide hapten and the linker between the enzyme and the hapten [218]. The last is the most labile of the three and undergoes hydrolysis in the diluent. Several approaches reported to stabilize the enzyme-conjugate such as freeze-drying or coupling to a more stable analogue or hydrolytic metabolite [86,90,91]. The assay precision can be improved by use of components prepared in one batch; thus the use of stabilized components is important.

4. Conclusion

The enormous advance in the immunodiagnostic field in the last three decades has established an immunoassay as well-established food contaminant analytical technique. The technique has provided many analytical advantages that are otherwise not possible with instrumental methods. These advantages include simplicity, high throughput capability, time and cost-efficiency and field portability, which could potentially benefit the agri-food industry and their analytical needs. In an immunoassay, there are two main factors that clearly govern quantification and hence determine the usefulness as an analytical technique. These are the capturing antibody and the signal generating systems. In most cases, antibody is the limiting step in the development of sensitive immunoassay as the characteristic of the

final assay is very much dependent upon the antibody's binding property. So far polyclonal and monoclonal antibodies have dominated the immunoassay development for food contaminants and they have been relatively effective in term of final assays. A recent advance in the recombinant antibody technique may help solving this limiting step by providing technology to produce antibodies with binding properties that may not be attainable through the conventional approaches. It is envisaged that a greater number of recombinant antibodies will find their way into food contaminant analysis in a near future.

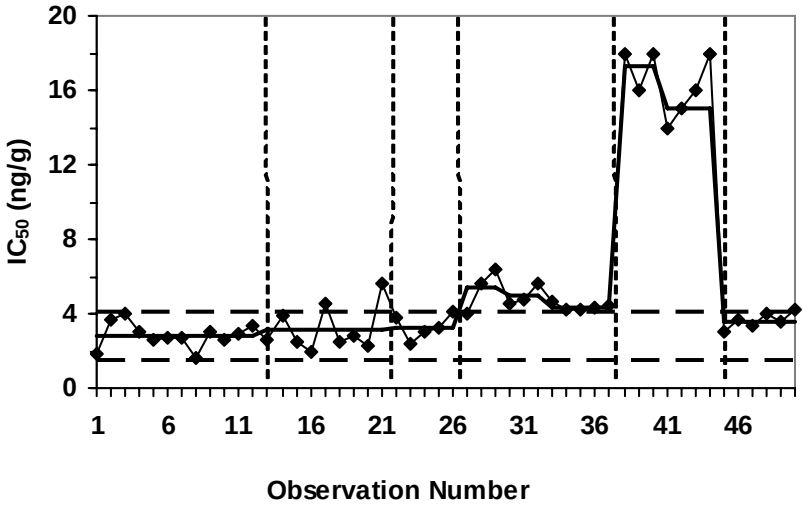


Figure 16. A control chart for monitoring IC50 values of aflatoxin B1 immunoassay. The vertical dash lines indicate separate analytical events, solid horizontal lines indicate steps (means of each analytical event), horizontal dash lines indicate upper and lower limits.

Sensitivity of an immunoassay can be improved many fold by employing more advanced signal generating systems. Various signal amplification systems such as avidin-biotin amplification system or use of more prevailing chemiluminescent or fluorescent substrates have been proven to be effective, but each of these applications compromises the costs, sometimes equipment necessary for measurement and may influence the portability. Recently, affinity sensor technology provides much of the excitement in the immunodiagnostics arena, proving their feasibility in food contaminant analysis. Apart from simplicity, ease of automation and miniaturization, rapidity, portability and ability for real-time measurement, one factor that is potentially important in food analysis is the ability of sensor transducers to potentially reduce the matrix interference, hence improving the

precision, accuracy and possibly sensitivity. Matrix effects and interference have been one of the major limiting steps in the wide spread of immunoassay technique. The basis of much of the matrix effects observed in our experience was from effects on enzyme activity rather than on the antibody-antigen interaction. The implication of this phenomenon would be a potential risk of unacceptably large number of false positives. It is believed that much of these problems could be solved by biosensor technology utilizing non-biological signal generating systems. One other area that needs urgent attention in immunodiagnostic technology is standardization of quality assurance or control procedures for food contaminant immunoassay. There is a much scope to improve and standardize Quality Assurance (QA)/QC protocols, and consequently enhance the quality of data generated by this technique as required by the agri-food industry in fulfilling their stringent analytical requirements. The implication of standardized QA/QC procedures would be very great in developing countries where immunoassays could be best utilized for food safety management. Establishing a standardized validation protocol for immunoassay technique is also a task requiring an urgent attention. A standardized validation protocol should provide a step-by-step guideline to ensure each immunoassay being developed meets all the analytical criteria for the intended purpose. Only fully validated immunoassays will prove their performance as good analytical technique and will subsequently provide a good ground for better acceptance. Finally, immunodiagnostic technology is a multi-disciplinary field that has an immense potential to grow and expand. We strongly believe that the future progress in immunodiagnostic technology will continue to play an important role for advances in food contaminant analysis.

References

[1] K. Landsteiner, The specificities of serological reactions, Dover Publishers, New York, 1945.
[2] K. Landsteiner and J. Van Der Scheer, J. Exp. Med., 48 (1928) 315.
[3] K. Landsteiner and J. Van Der Scheer, , J. Exp. Med, 59 (1934) 751.
[4] R.S. Yalow and S.A. Berson, Nature (London, United Kingdom), 184 (1959) 1648.
[5] G. Kohler and C. Milstein, Nature, 256 (1975) 495.
[6] N.A. Lee and I.R. Kennedy, J. AOAC Int., 84(5) (2001) 1393.
[7] D.F. Rinder and J.R. Fleeker, Bull. Environ. Contam. Toxicol., 26 (1981) 375.
[8] J.R. Fleeker, J. AOAC Int., 70 (1987) 874.
[9] W.H. Newsome and P.G. Collins, Food Agric. Immunol., 1 (1989) 203.
[10] Newsome, W. H. and Collins, P. G. (1990) Development of an ELISA for urea herbicides in foods. Food Agric. Immunol., 2 (1990) 75.
[11] A.E. Karu, M.H. Goodrow, D.J. Schmidt, B.D. Hammock and M.W. Bigelow, J. Agric. Food. Chem., 42 (1994a) 301.

[12] P. Schneider, M.H. Goodrow, S.J. Gee and B.D. Hammock, J. Agric. Food. Chem., 42 (1994) 431.
[13] L.H. Stanker, C. Bigbee, J. Van Emon, B. Watkins, R.H. Jensen, C. Morris and M.J. Vanderlaan, J. Agric. Food. Chem., 37 (1989) 834.
[14] J.H. Skerritt, A.S. Hill, D.P. McAdam and L.H. Stanker, J. Agric. Food. Chem., 40 (1992a) 1287.
[15] F.S. Chu, T.S.L. Fan, G.S. Zhang, Y.C. Xu, S. Faust and P.L. McMahon, J. AOAC Int., (1987) 70, 854.
[16] N.A. Lee, S. Wang, R.D. Allan and I.R. Kennedy, J. Agric. Food. Chem., 52(10) (2004) 2746.
[17] E. Schneider, E. Usleber and E. Maertlbauer, J. Agric. Food. Chem., 43(9) (1995) 2548.
[18] K.D. Wing, B.D. Hammock and D.A. Wunster, J. Agric. Food. Chem., 26 (1978) 1328.
[19] K.D. Wing and B.D. Hammock, Experientia, 35 (1979) 1619.
[20] A.S. Hill, D.P. McAdam, S.L. Edward and J.H. Skerritt, J. Agric. Food. Chem., 41 (1993) 2011.
[21] N. Lee, D.P. McAdam and J.H. Skerritt, J. Agric. Food. Chem., 46(2) (1998) 520.
[22] O. Kawamura, S. Sato, H. Kajii, S. Nagayama, K. Ohtani, J. Chiba and Y.Ueno, Toxicon:Official Journal of the International Society on Toxicology, 27 (1989) 887.
[23] N. Lee, H.L. Beasley, M. Silburn, N. Woods, S. Kimber, J.H. Skerritt and I.R. Kennedy, J. Agric. Food. Chem., 45 (1997) 4147.
[24] U.S. Department of Agriculture, Grain Inspection, Packers and Stockyards Administration, Grain fungal disease and mycotoxin reference, GIPSA Technical Services Division, Kensas City, MO, 1999.
[25] J.A. Gabaldon, J.M. Cascales, S. Morias, A. Maquieira and R. Puchades, Food Addit. Contam., 20 (2003) 707.
[26] S.T.A. Siebert, S.G. Reeves, M.A. Roberts and R.A. Durst, Anal. Chim. Acta, 311 (1995) 309.
[27] N. Lee, N, J.H. Skerritt and D.P. McAdam, J. Agric. Food. Chem., 43 (1995)1730.
[28] H.H. Weetall and K.R. Rogers, Anal. Lett., 35 (2002) 1341.
[29] X. Sun, X. Zhao, J. Tang, Z. Jun and F.S. Chu, Int. J. Food Microbiol., 99 (2005) 185.
[30] C. Paepens, S. De Saeger, L. Sibanda, I. Barna-Vetro, I. Leglise, F. Van Hove and C. Van Peteghem, Anal. Chim. Acta, 523 (2004) 229.
[31] N. Lee, J.H. Skerritt, M. Thomas, W. Korth, K.H. Bowmer, K.A. Larkin. and B.S. Ferguson, Bull. Environ. Contam. Toxicol., 55 (1995) 479.
[32] P.Cliquet, E. Cox, W. Haasnoot, E. Schacht and B.M. Goddeeris, J. Agric. Food Chem., 51 (2003) 5835.
[33] J.F. Brady, J.R. Fleeker, R.A. Wilson and R.O. Mumma, Enzyme immunoassay for aldicarb, Biological Monitoring for Pesticide Exposure, Eds. R.G.M. Wang, C.A. Franklin, R.C. Honeycutt and R.C. Reinert, ACS Symposium Series 382, Washington DC, 1989.
[34] J.A. Itak, M.Y. Selisker and D.P. Herzog, Chemosphere, 24 (1992) 11.

[35] J.J. Langone and H. Van Vunakis, Res. Commun. Chem. Pathol. Pharmacol., 10, (1975)163.
[36] W.T. Jones, D. Harvey, S. Jones, G.B. Ryan, H. Wynberg, W. Ten Hoeve and P.H.S. Reynolds, Food Agric. Immunol., 7 (1995) 9.
[37] S.I. Wie and B.D., J. Agric. Food. Chem., 30 (1982), 949.
[38] A. Abad and A. Montoya, J. Agric. Food. Chem., 42 (1992) 1818.
[39] M-P. Marco, S.J. Gee, H.M. Cheng, Z.Y. Liang and B.D. Hammock, J. Agric. Food Chem., 41 (1993) 423.
[40] S. Lehotay and R.J.Argauer, J. Agric. Food Chem., 41 (1993) 2006.
[41] S.W. Jourdan, A.M. Scutellaro, J.R. Fleeker, D.P. Herzog and F.M. Rubio, J. Agric. Food Chem., 43 (1995) 2784.
[42] R.J. Bushway, W.M. Pask, J. King, B. Perkins and B.S. Ferguson, Determination of chlordane in soil by enzyme immunoassay, Field Screening Methods for Hazardous Waste Site Investigation Proceedings, First International Symposium, Las Vegas, NV, 1988.
[43] A.S. Hill, J.H. Skerritt, R.J. Bushway, W. Pask, K.A.. Larkin, M. Thomas, W. Korth and K. Bowmer, J. Agric. Food. Chem., 42 (1994) 2051.
[44] K.W. Hunter Jr. and D.E. Lenz, Life Sci., 30 (1982) 355.
[45] A.E. Karu, J.E. Liliental, D.J. Schmidt, A.K. Lim, R.E. Carlson, T.A. Swanson, A.W. Buirge and M. Chamerlik, Monoclonal antibody-based immunoassay of cyclodienes. Proceedings of 6th Annual EPA Waste Testing and Quality Assurance Symposium, Washington DC, 1990.
[46] L.H. Stanker, B. Watkins, M. Vanderlaan, R. Ellis and J. Rajan, Analysis of heptachlor and related cyclodiene insecticides in food products, Immunoassays for Trace Chemical Analysis, Eds M. Vanderlaan, L.H. Stanker, B.E. Watkins and D.W. Roberts, ACS Symposium Series 451, American Chemical Sosiety, Washington DC, 1990.
[47] M.J. Wraith, E.J. Hitchings, E.R. Cole, D. Cole, A.P. Woodbridge and T.R. Roberts, Development of immunoassay methods for pyrethroid insecticides, The Sixth IUPAC Congress on Pesticide Chem., IUPAC, Ottawa, 1986.
[48] D. Bürgisser, S. Frey, B. Gutte and S. Klauser, Biochem. Biophys. Res. Commun., 166 (1990) 1228.
[49] A.E. Botchkareva, S.A. Eremin, A. Montoya, J.J. Manclus, B. Mickova, P. Rauch, F. Fini and S. Girotti, J. Immunol. Methods, 283 (2003) 45.
[50] R.M. Dreher and B. Podratzki, J. Agric. Food. Chem., 36 (1988) 1072.
[51] B. Reck and J. Frevert, Competitive- and inhibition-type immunoassay for determination of endosulfan: Immunochemical methods for environmental analysis, Eds J.V. Van Emon and R.O. Mamma, ACS Symposium Series 442, Washington DC, 1990.
[52] D.P. McAdam, A.S. Hill, H.L. Beasley and J.H. Skerritt, J. Agric. Food. Chem., 40 (1992) 1466.
[53] A.S. Hill, H.L. Beasley, D.P. McAdam and J.H. Skerritt, J. Agric. Food Chem., 40 (1992) 1471.

[54] J.V. Mei, C.M. Yin and L.A.Carpino, J. Agric. Food Chem., 39 (1991) 2083.
[55] A.S. Hill, J.V. Mei, C-M. Yin, B.S. Ferguson and J.H. Skerritt, J. Agric. Food Chem., 39 (1991) 1882.
[56] P.M. Krämer, M-P. Marco and B.D. Hammock, J. Agric. Food Chem., 42 (1994) 934.
[57] A.A. Brimfield, D.E. Lenz, C. Graham and K.W. Hunter Jr., J. Agric. Food. Chem., 33 (1985) 1237.
[58] C.D. Ercegovich, R.P. Vallejo, R.R. Gettig, L. Woods, E.R. Bogus and R.O. Mumma, J. Agric. Food. Chem. 29 (1981) 559.
[59] A.Y. Al-Rubae, The ELISA, a new method for the analysis of pesticide residue in water, Ph.D. Dissertation, Pennsylvania State University, 1978.
[60] A.M.A. Ibrahim, M.A. Morsy, M.M. Hewedi and C.J. Smith, Food Agric. Immunol., 6 (1994) 23.
[61] L.H. Stanker, C. Bigbee, J. Van Emon, B. Watkins, R.H. Jensen, C. Morris, M.J. Vanderlaan, J. Agric. Food Chem., 37 (1989) 834.
[62] J.A. Itak, M.Y. Selisker, S.W. Jourdan, J.R. Fleeker and D.P. Herzog, J. Agric. Food Chem., 41 (1993) 2329.
[63] W.H. Newsome and J.B. Shields, J. Agric. Food. Chem., 29 (1981) 220.
[64] Q.X. Li, S.J. Gee, M.M. McChesney, B.D. Hammock and J.N. Seiber (1989) Comparison of an enzyme-linked immunosorbent assay and a gas chromatography procedures for the analysis of molinate residues. Anal. Chem., 61 (1989) 819.
[65] W.H. Newsome, J. Agric. Food Chem., 33 (1985) 528.
[66] R.J. Bushway, B.E.S. Young, L.R. Paradis and L.B. Perkins, Abstract, 205th ACS National Meeting, 1993.
[67] R.J. Bushway, S.A. Savage and B.S. Ferguson, J. Agric. Food Chem., 35 (1990) 51.
[68] B. Riggle and B. Dunbar, J. Agric. Food Chem., 38 (1990) 1922.
[69] F. Forlani, A. Arnoldi and S. Pagani, J. Agric. Food. Chem., 40 (1992) 328.
[70] D.L. Brandon, R.G. Binder, A.H. Bates and W.C. Montague Jr., J. Agric. Food. Chem., 40 (1992) 1722.
[71] R.J. Bushway, B.E.S. Young, L.R. Paradis and L.B. Perkins, J. AOAC Int., 77 (1994) 1243.
[72] W.H. Newsome, Bull. Environ. Contam. Toxicol., 36 (1986) 9.
[73] J.C. Hall, R.J.A. Deschamps and K.K. Kreig, J. Agric. Food Chem., 37 (1989) 981.
[74] P.C.C. Feng, S.J. Wratten, S.R. Horton, C.R. Sharp, E.W. Logusch and Eugene W. J. Agric. Food Chem., 38 (1990), 159.
[75] J.H. Rittenburg, G.D. Grothaus, D.A. Fitzpatrick and R.K. Lankow, Rapid on-site immunoassay systems, agricultural and environmental applications, Immunoassays for Trace Chemical Analysis, Eds M. Vanderlaan, M., L.H. Stanker, B.E. Watkins and D.W. Roberts, ACS Symposium Series 451, Washington DC, 1990.
[76] P.C.C. Feng, S.R. Horton and C.R. Sharp, J. Agric. Food Chem., 40 (1992) 211.

[77] S.J. Huber, Chemosphere, 14 (1985) 1795.
[78] C. Wittmann and B. Hock, Food Agric. Immunol., 1 (1989) 211.
[79] T. Giersch and B. Hock, Food Agric. Immunol., 2 (1990) 85.
[80] T. Giersch, J. Agric. Food. Chem., 41 (1993) 1006.
[81] F.M. Rubio, J.A. Itak, A.E. Scutellaro, M.Y. Selisker and D.P. Herzog, Food Agric. Immunol., 3 (1991) 113.
[82] F. Szurdoki, H.K.M. Bekheit, M.P. Marco, M.H. Goodrow and B.D. Hammock, Food Agric. Immunol., 40 (1992) 1459.
[83] H.K.M. Bekheit, A.D. Lucas, F. Szurdoki, S.J. Gee and B.D. Hammock, J. Agric. Food Chem., 41 (1993) 2220.
[84] M.M. Kelley, E.W. Zahnow, W.C. Petersen and S.T. Toy, J. Agric. Food Chem., 33 (1985) 962.
[85] F.K. Koppatschek, R.A. Libel, A.L. Kriz and L.L. Melhado, J. Agric. Food Chem., 38 (1990) 1519.
[86] R.V. Dargar, J.M. Tymonoko and P. Van Der Werf, J. Agric. Food Chem., 39 (1991) 813.
[87] T.S. Lawruk, C.E. Lachman, S.W. Jourdan, J. Fleeker, D.P. Herzog and F.M. Rubio, J. Agric. Food. Chem., 41 (1993) 747.
[88] F. García-Sánchez, A. Navas, F. Alonso and J. Lovillo, J. Agric. Food Chem., 41 (1993) 3326.
[89] B.D. Hammock and R. O. Mumma, Potential of immunochemical technology for pesticide analysis, Pesticide Analytical Methodology, Eds J. Harvey Jr. and G. Zweig, ACS Symposium Series 136, Washington DC, 1980.
[90] J.M. Schlaeppi, W. Föry and K. Ramsteiner, J. Agric. Food. Chem., 37 (1989) 1532.
[91] A.D. Lucas, H.K.M. Bekheit, M.H. Goodrow, A.D. Jones, S. Kullman, F. Matsumura, J.E. Woodrow, J.N. Seiber and B.D. Hammock, J. Agric. Food. Chem. 41 (1993) 1523.
[92] R.B. Wong and Z.H. Ahmed, J. Agric. Food. Chem., 40 (1992) 811.
[93] A.N. Anis, M.E. Eldefrawi and R.B. Wong, J. Agric. Food Chem., 41 (1993) 843.
[94] E. Liegeois, Y. Dehon, B. Brabant, P. Perry, D. Portelle and A. Copin, Sci. Total Environ., 123/124 (1992) 17.
[95] J-M. Schlaeppi, H. Moser and K. Ramsteiner, J. Agric. Food Chem., 39 (1991) 1533.
[96] J.C. Hall, L.K. Wilson and R.A. Chapman, J. Environ. Sci. Health., Part B, B27 (1992) 523.
[97] H. Von Scholz and B. Hock, Z.Wasser-Abwasser. Forsch, 24 (1991) 29. (in German)
[98] S.J. Gee, T. Miyamoto, M.H. Goodrow, D. Buster and B.D. Hammock, J. Agric. Food Chem., 36 (1988) 863.
[99] Z. Niewola, S.T. Walsh and G.E. Davies, Int. J. Immunopharmacol., 5 (1983) 211.
[100] Z. Niewola, J.P. Benner and H. Swaine, Analyst, 111 (1986) 399.
[101] J. Van Emon, B.D. Hammock and J.N. Seiber, Anal. Chem., 58 (1986) 1866.
[102] J. Van Emon, J.N. Sieber and B.D. Hammock, Bull. Environ. Contam. Toxicol., 39 (1987) 490.

[103] R.J.A. Deschamps, J.C. Hall and M.R. McDermott, J. Agric. Food. Chem., 38 (1990) 1881.
[104] S.T. Huber and B. Hock, J. Plant Diseases Prot., 92 (1985) 147.
[105] E. Schneider,E. Usleber,E. Maertlbauer,R. Dietrich and G. Terplan, Food Addit. Contam., 12 (1995) 387.
[106] J.M.A. Schlaeppi, W. Meyer, K.A. Ramsteiner, J. Agric. Food Chem., 40 (1992), 1093.
[107] M.H. Goodrow, R.O. Harrison and B.D. Hammock, J. Agric. Food. Chem., 38 (1990) 159.
[108] R.O. Harrison, M.H. Goodrow and B.D. Hammock, J. Agric. Food Chem., 39 (1991) 122.
[109] B. Dunbar, B. Riggle and G. Niswender, J. Agric. Food. Chem., 38 (1990) 433.
[110] F.S. Chu, B.W. Steinert and P.K. Gaur, J. Food Safety, 7 (1985) 161.
[111] W.O. Harder and F.S. Chu, Experientia, 35 (1979) 1104.
[112] H. Okumura, J. Okimoto, S. Kishimoto, A. Hasegawa, O. Kawamura, M. Nakajima, M. Miyabe, Y. Ueno, Food Agric. Immunol., 5 (1993) 75.
[113] T.S. Fan, G.S. Zhang and F.S.Chu, Appl. Environ. Microbiol., 47 (1984) 526.
[114] G. Zhang and F.S. Chu, Experientia, 45 (1989) 182.
[115] S.L. Hefle and F.S. Chu, Food Agric. Immunol., 2 (1990) 181.
[116] W-B. Shim, A.Y. Kolosova, Y-J. Kim, Z-Y Yang, S-J. Park, S.A. Eremin, I-S Lee, D-H Chung, Int. J. Food Sci. Technol., 39 (2004) 829.
[117] J.R.Clarke, R.R. Marquardt, A.. Oosterveld, A.A. Frohlich, F.J. Madrid and M. Dawood, M.J. Agric. Food Chem., 41 (1993) 1784.
[118] F-Y.Yu and F.S. Chu, J. Food Prot., 59 (1996) 992.
[119] H.R. Christensen, F-Y. Yu and F.S. Chu, J. Agric. Food Chem., 48 (2000) 1977.
[120] M.E. Savard, R.C. Sinha, R. Lau, C. Seguin and S. Buffam, Food Agric. Immunol.,15 (2003) 127.
[121] E. Usleber, E. Maertlbauer, R. Dietrich and G. Terplan, J. Agric. Food Chem., 39 (1991) 2091.
[122] O. Kawamura, Kagawa Daigaku Nogakubu Gakujutsu Hokoku, 57 (2005) 27. (In Japanese)
[123] M.J. Nicol, D.R. Lauren, C.O. Miles, W.T. Jones, Food Agric. Immunol., 5 (1993) 199.
[124] C.M. Maragos and S.P. McCormick, Food Agric. Immunol., 12 (2000) 181.
[125] D. Abramson, E. Usleber and E. Maertlbauer, Appl. Environ. Microbiol., 61 (1995) 2007.
[126] R. Teshima, M. Kawase, T. Tanaka, K. Hirai, M. Sato, J. Sawada, H. Ikebuchi, M. Ichinoe and T. Terao, J. Agric. Food Chem., 38 (1990) 1618.
[127] M.T. Liu, B.P. Ram, L.P. Hart and J. Pestka, Appl. Environ. Microbiol., 50 (1985) 332.
[128] R. Teshima, M. Kawase, T. Tanaka, K. Hirai, M. Sato, J. Sawada, H. Ikebuchi, M. Ichinoe and T. Terao, J. Agric. Food Chem., 38 (1990) 1618.
[129] T.S.L. Fan, G.S. Zhang and F.S. Chu, J. Food Prot., 47 (1984) 964.
[130] K. Ohtani, O. Kawamura, H. Kajii, J. Chiba and Y. Ueno, Mycotoxins, 22 (1985) 31.
[131] F. Szurdoki, E. Trousdale, B. Ward, S.J. Gee, H.D. Hammock and D.G. Gilchrist, J. Agric. Food Chem., 44 (1996) 1796.

[132] H.B. Sheth and P. Sporns, J. Agric. Food Chem., 39 (1991) 1696.
[133] H. Zhang, Z. Duan, L. Wang, Y. Zhang and S.Wang, J. Agric. Food Chem., (2006), 54 (2006) 4499.
[134] N. Pastor-Navarro, C. Garcia-Bover, A. Maquieira and R. Puchades, Anal. Bioanal. Chem., 379 (2004) 1088.
[135] N. Lee, C.K. Holtzapple, M.T. Muldoon, S.S. Deshpande and L.H. Stanker, Food Agric. Immunol., 13 (2001) 5.
[136] W. Haasnoot, J. Du Pre, G. Cazemier, A. Kemmers-Voncken, R. Verheijen and B.J.M Jansen, Food Agric. Immunol., 12 (2000) 127.
[137] C.A. Thomson and P. Sporns, J. Food Sci., 60 (1995) 409.
[138] T. Tanaka, H. Ikebuchi, J-I Sawada, M. Okada and Y. Kido, J. AOAC Int., 79 (1996) 426.
[139] W. Haasnoot, P. Stouten, G. Cazemier, A. Lommen, J.F.M. Nouws and H.J. Keukens, Analyst, 124 (1999) 301.
[140] P. Schnappinger, E. Usleber, E. Maertlbauer and G. Terplan, Food Agric. Immunol., 5 (1993) 67.
[141] W.L. Shelver, D.J. Smith and E.S. Berry, J. Agric. Food Chem., 48 (2000) 4020.
[142] M. Hageleit, A. Daxenberger and H.H.D. Meyer, Food Agric. Immunol., 18 (2001) 285.
[143] E. Petruzzelli, A. Ius, S. Berta, M. Dovis and A. Albertini, Food Agric. Immunol., 8 (1996) 3.
[144] Z.V. Samsonova, O.S. Shchelokova, N.L. Ivanova, M.Y. Rubtsova and A.M. Egorov, Appl. Biochem. Microbiol., 41 (2005) 589.
[145] M.T. Muldoon, C.K. Holtzapple,S.S. Deshpande, R.C. Beier and L.H. Stanker, J. Agric. Food. Chem., 48 (2000) 537.
[146] C.K. Holtzapple, S.A.Buckley and L.H. Stanker, J. Agric. Food. Chem., 45 (1997) 1984.
[147] L.D. Rowe, R.C. Beier, M.H. Elissalde and L.H. Stanker, J. Agric. Food. Chem., 42 (1994) 1132.
[148] E. Harlow and D. Lane, Using antibodies: a laboratory manual, Cold Spring Habor Laboratory Press, 1998
[149] F. Jung, H.H.D. Meyer and R.T. Hamm, R. T, J. Agric. Food. Chem. 37 (1989) 1183-1187.
[150] J.H. Skerritt and N. Lee, Approaches to the synthesis of haptens for immunoassay of organophosphate and synthetic pyrethroid insecticides, Residue analysis in food safety: applications of immunoassay methods, Eds R.C. Beier and L.H. Stanker, ACS Symposium Series Book 621, 1996.
[151] E.P. Vallejo, E.R. Bogus and R.O. Mumma J. Agric. Food. Chem., 30 (1981) 572.
[152] R.O. Harrison, M.H. Goodrow, S.J. Gee and B.D. Hammock, Hapten synthesis for pesticide immunoassay development, Immunoassays for trace chemical analysis, Eds. M. Vanderlaan, L.H. Stanker, B.E. Watkins and D.W. Roberts, ACS Symposium Series 451, Washington, DC, 1990.

[153] S.J. Gee, R.O. Harrison, M.H. Goodrow, A.L. Braun and B.D. Hammock, Development of immunoassays for thiocarbarmate herbicides. , Immunoassays for trace chemical analysis, Eds. M. Vanderlaan, L.H. Stanker, B.E. Watkins and D.W. Roberts, ACS Symposium Series 451, Washington, DC, 1990

[154] J.X. Paxton, F.J. Rowell and J.G. Ratcliffe, J. Immunol. Methods, 10 (1976) 317

[155] B.S. Ferguson, K.A. Larkin, J.H. Skerritt and H.L. Beasley, Abstract 241 The detection of diazinon in lanolin using a rapid and simple enzyme-immunoassay. Associations of Official Analytical Chemists, 107th Annual Meeting, Washington DC, 1993.

[156] S. Wang, Y. Quan, N.A. Lee and I.R. Kennedy, J. Agric. Food. Chem., 54 (2006) 2491.

[157] J.F. Brady, J.R. Fleeker, R.A. Wilson and R.O. Mumma, Enzyme immunoassay for aldicarb, Biological Monitoring for Pesticide Exposure, Eds. R.G.M. Wang, C.A. Franklin, R.C. Honeycutt and R.C. Reinert, ACS Symposium Series 382, Washington DC, 1989.

[158] S.I Wie, A.P. Sylwester, K.D. Wing and B.D. Hammock, J. Agric. Food. Chem., 30 (1982) 943.

[159] F.B. Szurdoki, H.K.M. Bekheit, M-P. Marco, M.H. Goodrow and B.D. Hammock, Important factors in hapten design and enzyme-linked immunosorbent assay development, New frontiers in agrochemical immunoassay, Eds. D.A. Kurtz, J.H. Skerritt and L.H. Stanker, AOAC International, Arlington, VA, 1995.

[160] B.F. Erlanger, Methods in Enzymology, 70 (1980) 85.

[161] S. Avrameas, T. Ternynck and J-L. Guesdon, Scand. J. Immunol. Supplement, 8 (1978), 7.

[162] D.S. Kabakoff, Chemical aspects of enzyme-immunoassay, Enzyme-Immunoassay, Ed. E.T. Maggio, CRC Press, Boca Raton, Florida, 1983.

[163] G.J. Haas and E.J. Guardia, Production of antibodies against insecticide-protein conjugates. Proceedings of society for experimental biology and medicine, vol 129, 1968.

[164] B.F. Erlanger, Pharmacol. Rev., 25 (1973) 271.

[165] D.P. McAdam and J.H. Skerritt, Aust. J. Chem., 40 (1993) 959.

[166] F. Jung, A. Székás, Q.X. Li and B.D. Hammock, J. Agric. Food. Chem., 39 (1991) 129.

[167] H.R. Lukens, C.B. Williams, S.A. Levison, W.B. Danaliker, D. Murayama and R.L. Baron, Environ. Sci. Technol., 11 (1977), 292.

[168] N.Y. Kado and E.T. Wei, J. Natl. Cancer Inst., 61 (1978), 221.

[169] K.L. Hastings, W.M. Hagler Jr., T.M. Harris, R.D. Voyksner, J.H. Dean, J. Agric. Food Chem., 37 (1989), 393.

[170] S. Yoshitake, M. Imagawa and E. Ishikawa, Anal. Lett., 15 (1982) 147.

[171] T. Kitagawa and T. Aikawa, J. Biochem., 79 (1976) 233.

[172] M. Fránek, V. Kolár, M. Granátova and Z. Nevoránková, J. Agric. Food Chem., 42 (1994) 1369.

[173] A.F. Karu, K-B.G. Scholthof, G Zhang and C.W. Bell, Food Agric. Immunol., 6 (1994), 277.

[174] K. Grennan, G. Strachan, A. J. Porter, A.J. Killard, M.R. Smyth, Anal. Chim. Acta, 500 (2003), 287.
[175] L. Dunne, S. Daly, A. Baxter, S. Haughey and R. O'Kennedy, Spectrosc. Lett., 38 (2005), 229.
[176] K-B.G. Scholthof, G. Zhang and A.E. Karu, J. Agric. Food Chem., 45 (1997), 1509.
[177] N. Lee, C.K. Holtzapple and L.H. Stanker, J. Agric. Food Chem., 46 (1998) 3381.
[178] 80 K.Y.F. Yau, N.L. Tout, J.T. Trevors, H. Lee, J.C. Hall, J. Agric. Food Chem., 46 (1998) 4457.
[179] S.D. Garrett, D.J.A. Appleford, G.M. Wyatt, H.A. Lee and M.R.A. Morgan, J. Agric. Food Chem., 45 (1997) 4183.
[180] M.J. Alcocer, C. Doyen, H.A. Lee and M.R.A. Morgan, J. Agric. Food Chem., 48 (2000) 4053.
[181] J. Brichta, H. Vesela and M. Franek, Veterinarni Medicina (Prague, Czech Republic), 48 (2003) 237.
[182] W. Kusharyoto and R.D. Schmid, Annales Bogorienses, 9 (2004), 1.
[183] A. Moghaddam, I. Lobersli, K. Gebhardt, M. Braunagel and O.J. Marvik, J. Immunol. Methods, 254 (2001) 169.
[184] S.J. Daly, P.P. Dillon, B.M. Manning, L. Dunne, A. Killard, R. O'Kennedy, Food Agric. Immunol., 14 (2002) 255.
[185] G-H. Choi, D-H. Lee, W-K. Min, Y-J. Cho, D-H. Kweon, D-H. Son, K. Park, J-H. Seo, Protein Expression and Purification, 35 (2004), 84.
[186] B. Lauer, I. Ottleben, H-J. Jacobsen and T. Reinard, J. Agric. Food Chem., 53 (2005) 899.
[187] S. Cardozo, A. Gonzalez-Techera, J.A. Last, B.D. Hammock, K. Kramer and G.G. Gonzalez-Sapienza, Environ. Sci. Technol., 39 (2005) 4234.
[188] A.E. Karu, D.J. Schmidt, C.E. Clarkson, J.W. Jacobs, T.A. Swanson, M.L. Egger, R.E. Carlson and J.M. Van Emon, Avermectins, Detection with monoclonal antibodies. In Immunochemical methods for environmental analysis, Eds. J.V. Van Emon and R.O. Mamma, ACS Symposium Series 442, American Chemical Society, Washington DC, 1996.
[189] N.J. Lee, Development of Enzyme Immunoassays for Detection of Endosulfan, Diuron and Synthetic Pyrethroids in Soil and Water, Ph.D. Dissertation, University of Sydney, 1996.
[190] M.E. Ploum, W. Haasnoot, R.J.A. Paulussen,G.D. Van Bruchem, A.R.M. Hamers, R. Schilt, F.A. Huf, J. Chromatogr., 564 (1991) 413.
[191] K.E.I. Vanoosthuyze, C.J.M. Arts and C.H. Van Peteghem, J. Agric. Food Chem., 45 (1997) 3129.
[192] G. Alfredsson, C. Branzell, K. Granelli and A. Lundstroem, Anal. Chim. Acta, 529 (2005), 47.
[193] S. Wang, C. Zhang and Y. Zhang, Anal. Chim. Acta, 535 (2005) 219.
[194] C. Wittmann, U. Bilitewski, T. Giersch, U. Kettling and R.D. Schmid, Analyst, 121 (1996) 863.

[195] M.Y.U. Rubtsova, J.V. Samsonova, A.A. Ezhov, A.M. Egorov, Bioluminescence & Chemiluminescence: Progress & Current Applications, Proceedings of the Symposium on Bioluminescence and Chemiluminescence, 12th, Cambridge, United Kingdom, 2002.
[196] Y. A.Cho, Y.J. Kim, B.D. Hammock, Y.T. Lee and H-S. Lee, J. Agric. Food Chem., 51 (2003) 7854.
[197] N.V. Cuong, T.T. Bachmann and R.D. Schmid, Fresenius J. Anal. Chem., 364 (1999) 584.
[198] R. Collin, E. Schneider, L. Briggs and N. Towers, Food Agric. Immunol., 10 (1998) 91.
[199] S. Wang, C. Zhang, J. Wang and Y. Zhang, Anal. Chim. Acta, 546 (2005) 161.
[200] J.F. Brady, T.E. Wilson, J.H. Lawton, T.S. Fan and B.S. Ferguson, AGRO-023, Abstracts of Papers, 224th ACS National Meeting, Boston, MA, United States, 2002.
[201] B.S. Delmulle, S.M.D.G. De Saeger, L. Sibanda, I. Barna-Vetro and C.H. Van Peteghem, J. Agric. Food Chem., 53 (2005) 3364.
[202] A.E. Karu, Monoclonal antibodies and their use in measurement of environmental contaminants, Hazard Assessment of Chemicals, vol. 8, Ed. J. Saxena, Taylor and Francis International, Washington DC, 1993.
[203] J.W. Goding, J. Immunol. Methods, 20 (1978) 241.
[204] J.J. Langones, M.D. Boyle and T. Borsos, J. Immunol., 121 (1978) 327.
[205] D. Pain and A. Surolia, FEBS Lett., 107 (1979) 73.
[206] P.K.M. Ngai, F. Ackermann, H. Wendt, R. Savoca and R. Bosshard, J. Immunol. Methods, 158 (1993) 267.
[207] S.L. Guihot, J.H. Skerritt, M. Thomas and W. Korth, Food Agric. Immunol., 8 (1996) 169.
[208] M.C. Hayes, J.X. Dautlick and D.P. Herzog, Quality control of immunoassays for pesticide residues, New frontiers in agrochemical immunoassay, Eds. D.A. Kurtz, J.H. Skerritt and L.H. Stanker, AOAC international, Arlington, VA, 1995.
[209] J.H. Skerritt, A.S. Hill, H.L. Beasley, S.L. Edward and D.P. McAdam, J. AOAC Int.., 75 (1992) 519.
[210] J.E. France and J.W. King, J. AOAC Int., 74 (1991) 1013.
[211] K-S. Nam, and J.W. King, J. Agric. Food Chem., 42 (1994) 1469.
[212] D.S. Aga and E.M. Thurman, Application of solid-phase extraction for trace analysis of herbicides in water and soil by enzyme-linked immunosorbent assay, New Frontiers in Agrochemical Immunoassay, Eds. D.A. Kurtz, J.H. Skerritt and L.H. Stanker, AOAC International, Arlington, VA, 1995.
[213] J.F.Brady, J-L. Turner and D.H. Skinner, J. Agric. Food Chem., 43 (1995) 2542.
[214] J.H. Skerritt, A.S. Hill, R.R.B. Sashidhar, H.L. Beasley, B.E.A. Rani, C.G. Udaya Kumari, Y.N. Vijayashankar, N.B.R.K. Venugopal, and N.G.K. Karanth, Food Agric. Immunol., 15 (2003) 17.
[215] J.H. Skerritt, S.L. Guihot, A.S. Hill, J. Desmarchelier and P.J. Gore, Cereal Chem., 73 (1996) 605.

[216] K.S. Goh, J. Hernandez, S.J. Powell and C.D. Greene, Bull. Environ. Contam. Toxicol., 45 (1990) 208.

[217] J.H. Skerritt and B.E.A. Rani, (1996) Detection and removal of samples matrix effects in agrochemical immunoassays. New frontiers in agrochemical immunoassay, Eds. D.A. Kurtz, J.H. Skerritt and L.H. Stanker, AOAC International, Arlington, VA, 1995.

[218] J.H. Skerritt, Analytical aspects of immunoassays of agrochemicals. New frontiers in agrochemical immunoassay, Eds. D.A. Kurtz, J.H. Skerritt and L.H. Stanker, AOAC International, Arlington, VA, 1995.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 6

Polymerase chain reaction (PCR)

Nelson Marmiroli and Elena Maestri

University of Parma, Department of Environmental Sciences, Parco Area delle Scienze 11/A, 43100 Parma, Italy

1. Introduction

The purpose of detecting toxicants of food is to identify and quantify any toxic compounds present. This is necessary both to assure compliance with national and international regulations and to protect the consumer. A food contaminant is defined as any substance present as a consequence of the production processes, but not intentionally added. Many food toxicants - for instance, natural toxins produced *in vivo* by plants, fungi or microbes - do not fall into this category. However, the detection of these toxicants should ideally rely on a single set of analytical techniques, based mainly on chemical and/or physical measurements, which are designed to detect the presence of particular molecules and macromolecules. Current technology can assay contaminants of biological origin, via nucleic acid analysis, which represents a good alternative to conventional methods. In this case, the analytical target is a "marker" - a DNA sequence characteristic of the specific contaminating organism, which produces a toxin or an allergen. This chapter will illustrate some case examples of the application of DNA analysis to identify food toxicants.

Because the amount of contaminating DNA is typically small, the direct application of a nucleic acid assay without any prior amplification is generally ineffective. Nucleic acid amplification dramatically increases assay sensitivity whilst retaining a high level of specificity. The Polymerase Chain Reaction (PCR) is the method of choice for DNA amplification, both as analytical and as diagnostic technique, because it rapidly generates a large number of copies of the target DNA sequence. Refinement of the PCR technique has allowed the detection of DNA fragments in samples where the quantity and/or the quality of DNA present is too low to permit other types of molecular analysis. In particular, it is now possible to detect the presence of a target DNA sequence in small samples in which the DNA has been heavily degraded by ageing and/or processing treatment.

The PCR exploits the mechanism of *in vivo* DNA replication to replicate a specific DNA fragment. The components required for PCR are primers, DNA polymerase and deoxyribonucleotides, dGTP, dATP, dCTP and dTTP. The primers consist of two synthetic oligonucleotides, whose sequences are complementary to those at the two ends of the target fragment. They anneal to their target sites on opposite strands of the template DNA, thereby defining the region to be amplified, and then function as the attachment points required for the functioning of DNA polymerase. The commonest form of the enzyme was initially isolated

from the thermophilic bacterium *Thermus aquaticus*, and is therefore termed *Taq* polymerase. Most currently available commercial *Taq* polymerases are recombinant products (derived from heterologous expression of the *T. aquaticus* gene in *Escherichia coli*). All DNA polymerases can perform single cycles of replication, but those used in the patented PCR process must be able to sustain repeated cycles of replication at high temperatures. The deoxyribonucleotides are the building blocks of the newly synthesised DNA molecules. The concentration of all reagents is designed to allow the assembly and replication of millions of copies of the fragment.

In the PCR (Figure 1), double-stranded (ds) template DNA is first heated in order to denature it to the single-stranded (ss) form. Then the primers (typically of length 20-30nt) are annealed to the template, by reducing the reaction temperature to allow this process to occur. The cycle is completed by a phase in which the DNA polymerase extends the primer sequence by sequential addition of further bases, to produce a complementary copy of the template strand. Because the products of each PCR cycle act as the template for the next PCR cycle, the number of newly synthesised copies in principle will double with each cycle. The exponential amplification achieved by the PCR can also be used to estimate the initial quantity of the template in the sample. For this purpose, the quantity of template, the number of reaction cycles, and the extent of inhibition of the theoretical doubling of product for each cycle must first be determined [1]. Quantification methods compare the signal intensity of a reference sequence to the signal from another sequence in the same reaction, either directly or by extrapolation from a standard curve [1].

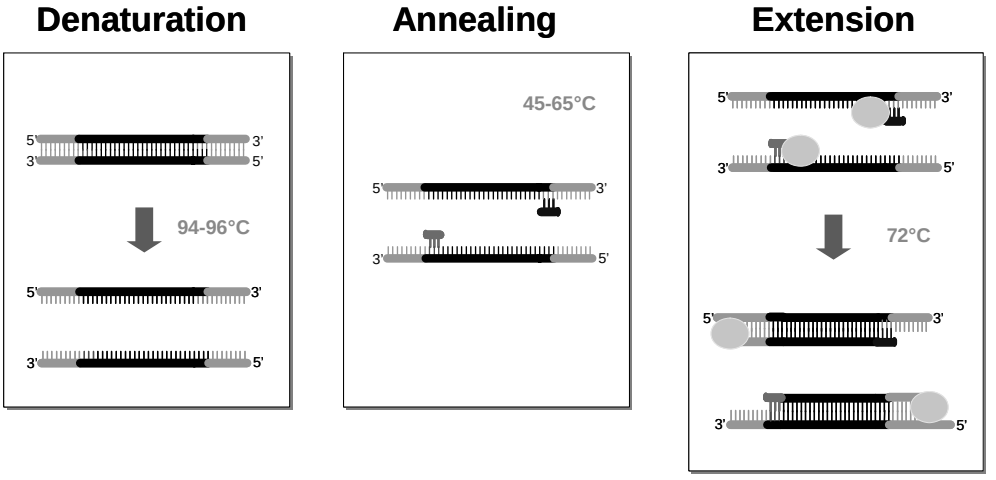


Figure 1- Scheme of the PCR reaction, illustrating the three separate phases.

PCR technology is unique in its ability to exponentially amplify a trace quantity of a specific nucleotide sequence present in a heterogeneous DNA background. This feature has led to the development of a large body of experimental and diagnostic molecular biology techniques, many of which would have been too time consuming or even impossible to perform before the early 1990s. In recent years, a number of modifications of the classic PCR technique has been developed, including multiplex, nested, reverse transcription (RT), quantitative, and real time PCR.

This chapter illustrates some successful examples of the application of PCR-based approaches for the analysis of food toxicants, which include allergens, microbial and fungal toxins, and genetically modified organisms. The use of PCR in assuring food traceability is currently an area of rapid expansion, because the ability to monitor toxicants via the detection of trace amounts of DNA fragments overcomes many of the practicality and sensitivity limitations suffered by other technologies [2].

2. Qualitative PCR

2.1. Multiplex PCR

Multiplex PCR represents a variant of PCR in which two or more DNA fragments are simultaneously amplified within a single reaction tube. This is achieved by including more than one primer pair to the reaction mixture [3]. The approach is particularly relevant to food analysis, where it is often necessary to test for the presence of a variety of toxicants in a single sample. Multiplex reactions can usefully discriminate between real and false negative results. For this purpose, one set of primers is targeted at a target known to be present in the sample, while the second set targets the sequence of interest. The former allows the experimenter to distinguish between a true negative and a reaction failure. Multiplex primers must be designed so that each separate amplification product is of distinct size, in order to ensure that all fragments can be identified following amplicon separation by either agarose gel or capillary electrophoresis [4]. An example is shown in Figure 2, illustrating the simultaneous amplification of seven targets in a mixture of DNAs extracted from independent transgenic events in maize and soybean and non-transgenic maize and soybean [5]. In this particular example, the primers were designed to amplify sequences within the transgenes, and targeted in addition fragments of endogenous genes (zein in maize and lectin in soybean) to provide a negative control [6] .Multiplex PCR – particularly when a significant number of primer pairs is involved – can require intricate optimization, and it may be in some instances be too difficult to achieve. Nevertheless, it represents an important technique for high-throughput analyses in a cost-effective manner.

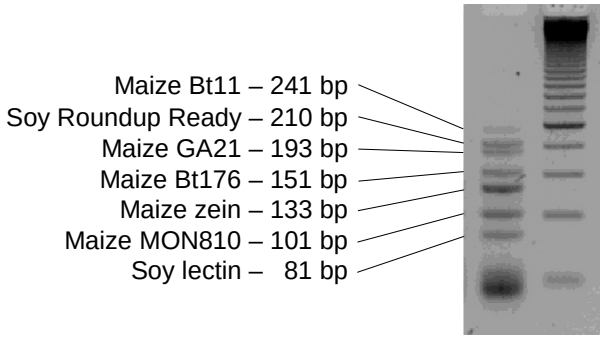


Figure 2: Multiplex PCR products obtained by amplification of seven targets in a mixture of GMO events. The lane on the right contains a 100-bp ladder.

In a representative application of multiplex PCR to detect the presence of food toxicants, Kumar et al. [7] spiked food samples (meat rinse and milk) with *Salmonella typhi*. The amplification targets were *invA* (invasion gene), *viaB* (synthesis of the capsule antigen Vi), *fliC-d* (synthesis of the flagellar H antigen) and *prt* (synthesis of the O antigen). The amplicons ranged in length between 370 and 890bp. The multiplex approach was justified by the fact that using a single gene assay could give positive results with other *Salmonella* serovars, whereas the presence of all four genes would unequivocally identify the *typhi* serovar. It was demonstrated that the presence of as few as 20-50 colony forming units (cfu) per ml could be detected. A limitation of this application is of course that the presence of a heterogeneous population of bacteria could deliver the same multiplex profile as that expected for the target serovar.

2.2. Nested PCR

In nested PCR, two (rather than just a single) pairs of primers target a single locus. The first pair amplifies the target fragment in a conventional PCR reaction. The second pair anneals to sites within the first amplicon, and amplifies an internal (shorter) sequence (Figure 3). Clearly, the sequence of the full amplicon must be known to design appropriate primers.

The purpose of nested PCR is to increase assay sensitivity by re-amplifying the target from a template previously enriched by the first PCR. Non-target sequences amplified non-specifically in the first PCR are not re-amplified in the second reaction as they would be unlikely to possess the internal priming sites targeted by the second PCR. However, an increased risk of contamination is a major disadvantage of this method, due to possible carry-

over contamination of PCR products, so great care must be exercised when performing it. Nested PCR has been used to detect the presence of verotoxinogenic *E. coli* in ground beef by targeting the genes *vt1* and *vt2* [8]. The sensitivity achieved was such that 110 cfu could be detected in a 10g sample. Similarly, when nested PCR was used to detect *Shigella flexneri* in lettuce samples spiked with the pathogen, the level of sensitivity was higher than that achievable with single PCR. In order to reach the same level of sensitivity, a prior phase of pathogen enrichment by culture was necessary [9].

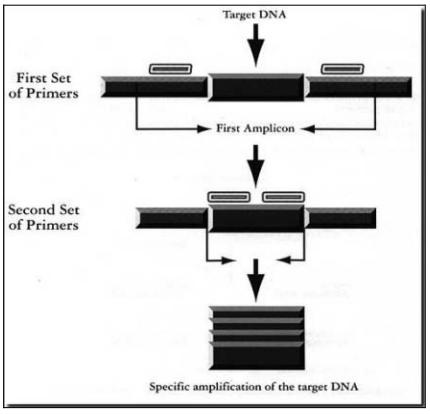


Figure 3: Schematic representation of the two primer sets used in nested PCR.

2.3. Reverse transcription PCR

Reverse Transcription PCR (RT-PCR) was developed to amplify, isolate or identify RNA sequences. The principle is to convert RNA into its complementary DNA sequence by reverse transcriptase, to synthesise a second strand with DNA polymerase, and finally to generate a ds cDNA molecule which can be amplified by PCR in the normal way [10]. RT-PCR is both specific and sensitive, and is particularly suitable as a means of detecting a small number of copies of the target RNA. Also in this case there is an increasing risk of contamination, because of the higher number of operations to be performed. Establishing amplicon identity is achieved by either sequencing, or by hybridisation with labelled probes. Recently, certain thermostable reverse transcriptases have become available; these can allow reverse transcription to be perfomed at an elevated temperature (typically 60°C), which improves reaction efficiency by minimising the secondary structure of the template RNA. r*Tth* polymerase (a recombinant enzyme derived from *T. thermophilus*) functions both as a reverse transcriptase and as a DNA polymerase. An application of RT-PCR in food toxicant detection

has been documented in the case of some pathogenic RNA viruses, particularly the NLVs (Norwalk-like viruses) [11].

3. Quantification with PCR

3.1. Quantitative conventional PCR techniques

Quantitative PCR techniques attempt to reliably measure the abundance of a specific sequence in a biological sample. Some approaches based on conventional PCR have been proposed, including a coupled PCR-ligase chain reaction assay [12], a homogenous Taq exonuclease activity-based assay [13], kinetic detection with intercalating dyes [14, 15], the use of labelled primers [16, 17] or amplicon capture by probes immobilized on membranes [18] or other solid phases [19, 20]. However, these methods have all been considered to be semi-quantitative in nature. Specific quantitative PCR applications must be accurately defined according to analytical requirements, including methods for amplicon quantification and the standards used for the assay. Quantification is absolute if the target can be compared with a standard, but only relative, if the output represents a ratio between a control sequence and the target.

3.1.1. Relative quantitative PCR

Relative quantification is informative when different samples are being compared with one another. In this technique, a constant amount of an internal control (calibrator) DNA fragment is added to each sample, and is amplified in a multiplex reaction containing primers directed at both the calibrator and the target sequence. The two primer pairs need to be compatible with one another (in terms of reaction parameters, particularly annealing temperature) but must not contain regions of complementarity, as this will encourage primer-dimer formation. The intensity of signal generated from the control is compared to that from the target in the various samples. This approach is particularly relevant when directed at the quantification of the temporal and/or functional variation of a specific mRNA species. The choice of calibrator sequence is important and requires some prior optimisation. A special case of calibrator is the engineered sequence used in quantitative competitive PCR (QC-PCR), where the standard is amplified with the same primers as the target, but yields a product of different (usually smaller) size, readily distinguishable by electrophoretic analysis. The range of competitor concentrations used must be established by a series of preliminary experiments.

The comparative PCR approach avoids a need for internal standards, and is frequently applied to assay RNA molecules. During reverse transcription, the product lengths of the two samples are deliberately engineered to be different by primer tailing. The products are then mixed and in the subsequent amplification, the two targets will generate PCR products of

different lengths. The relative quantification of the same target in the two samples being compared is inferred from signal intensity.

Since the advent of real time methods (see below), the quantification of PCR products is now rarely performed with comparative or relative approaches based on conventional PCR. A recent example of relevance to food toxicants featured the detection of viable shigatoxin-producing *E. coli* cells in ground beef and in broth. The amplification target was the A subunit of the *stx2* gene and the competitor fragment was constructed from the same gene to give a shorter amplification product. Quantification was used to monitor the time course of the signal generated from viable cells [21]. An application has also been described for the detection of gluten-containing cereals in various food products. The target chosen was a non-coding region of the chloroplast *trnL* gene, encoding a leucine transfer RNA, and the competitor fragment was prepared by adding 20bp with tailed primers. This approach is diagnostic for the presence of wheat, barley or rye flour in a processed food product [22].

3.1.2. Absolute quantitative PCR

The experimental determination of a precise number of copies of a nucleic acid target can only be achieved where an appropriate reference sequence is available. This needs to be as close as possible with respect to both size and sequence of the target. Its concentration must be accurately quantified and its sequence must allow an amplification efficiency comparable to that of the target. The latter can be best achieved where the reference sequence uses the same primer sites as the target, and differs from the target at only a small number of nucleotide positions. Where DNA (as opposed to RNA) levels are to be measured, the quantification can in some cases be achieved by comparison with a cell line or a tissue containing a known number of target gene copies. In this situation, the outcome can be expressed as the number of copies of the unknown gene per unit cell. QC-PCR can also deliver absolute quantification. As previously described, a single set of primers is used to amplify both the target gene and the standard, and since the amount of standard present at the start of the reaction is known, the initial amount of target template can be determined (Figure 4). This strategy assures the independence of the data from any experimental variables that may affect the rate of amplification.

3.1.3. PCR with clamping

PNAs (peptide nucleic acids) are synthetic DNA analogues, which have a peptide, rather than a sugar-phosphate backbone. PNAs interact with high specificity with both DNA and RNA via Watson and Crick hydrogen bonds and Hoogsteen interactions. PNAs can be introduced into a PCR to effect the specific suppression of amplification of a target fragment, a highly specific process referred to as “PCR clamping” [23]. Various hypotheses have been presented to explain the mechanism of PCR clamping. For overlapping (or adjacent) primer

and PNA binding positions, PNA may prevent the initiation of primer extension (primer exclusion) and/or may prevent access of the DNA polymerase to the primer (Figure 5, PCRa and PCRb). When the PNA binds to the middle of the target, clamping may operate by preventing read-through (elongation arrest; Figure 5, PCRc). The extent of suppression can be controlled by adjusting the relative concentration of target DNA and PNA. The lowest PNA concentration required to inhibit target amplification is directly correlated with the quantity of template present. By running experiments at various PNA concentrations, it is possible to derive a semi-quantitative estimation. This approach has been exemplified in an evaluation of the GMO content in food [24].

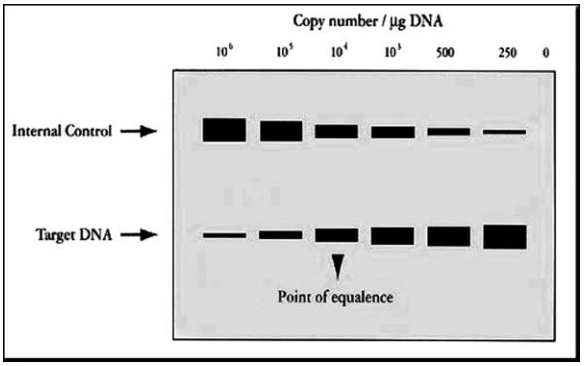


Figure 4: Quantitative PCR using standards. The relative intensities of the target and control PCR products are compared.

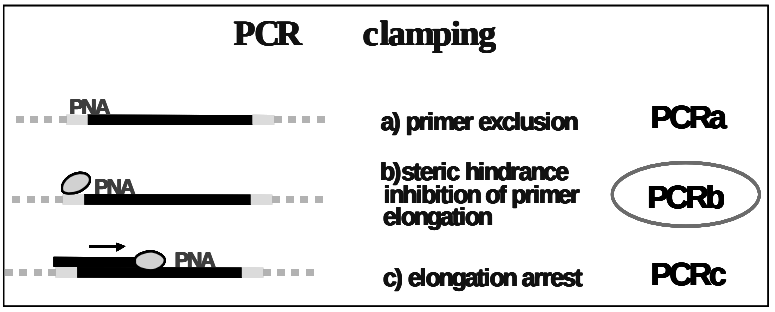


Figure 5: Types of PCR with clamping.

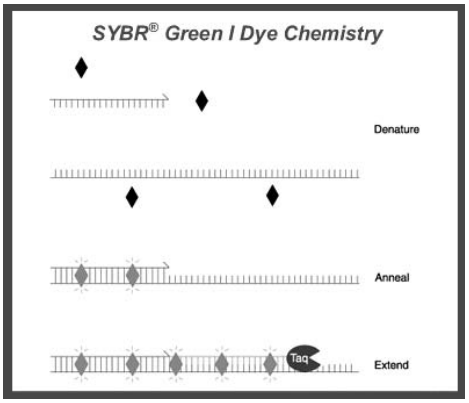
3.2. Quantitative PCR based on the "real-time PCR" system

Systems which use fluorescent dyes to monitor the progress of DNA amplification have been developed for the detection and quantification of specific PCR products. "Real-Time" PCR [25] relies on probes which hybridise to a site within the target sequence. During amplification, the number of cycles required to reach a threshold level of fluorescence is used to estimate the starting number of target sequences. The end-point amplicon is subjected to a series of increasing temperatures to determine when the ds product denatures; this melting point is determined by a combination of product length and nucleotide composition. The reason for the analysis is to identify the presence of contaminationm primer-dimer artifacts or other problems in the amplification. These tasks are accomplished by coupling conventional PCR with fluorescent chemistries and instrumentation. Real time PCR analysis can be applied to assess both DNA and RNA (via RT-PCR) amounts in a sample. It requires a dedicated instrument, because of the requirement to continuously detect and measure fluorescence throughout the PCR. Suitable machines and reagents are available from an increasing number of manufacturers. The platforms vary in the chemistry exploited, and user choice depends on a combination of throughput requirement and sensitivity. Excitation of the dye is achieved with lamps, light-emitting diodes (LED) or lasers. Since lamps produce a broad emission spectrum, instruments must be provided with filters to restrict emission to specified wavelengths. Few instruments at present utilise laser radiation for excitation. The emission detector may be a charge-coupled device (CCD) camera, or some other photodetector. An updated listing of machines and comparisons of their characteristics can be found in the internet, and a recent scan produced a list of more than 40 different instruments manufactured by ten companies.

Two reagents are in common use for amplicon quantification: (1) ds DNA-binding agents, such as SYBR® Green I, and (2) fluorescent dyes, such as those used in TaqMan probes. The following discussion describes mostly systems developed by Applied Biosystems. Several other companies have commercialised similar technologies.

3.2.1. SYBR® Green I chemistry

SYBR Green I is a dsDNA binding dye, which can be used to quantify amplicon amount during the course of the PCR by tracking overall fluorescence emission. The dye binds into the minor groove of dsDNA, and does not bind to ssDNA. When bound, it increases its fluorescence by up to 100 fold (Figure 6). During PCR, as the target sequence is amplified, SYBR Green I binds to each new copy of dsDNA during the extension step. The major disadvantage of this method is that its binding is non-specific. A relatively minor and more controllable problem is that the longer the amplicon, the stronger the signal, which can result in signal saturation. Clearly the system is inappropriate for multiplex reactions.



ADVANTAGES	DISADVANTAGES
Fluorescent intensity proportional to the amount of PCR products produced. Possibility of monitoring the amplification of any dsDNA. No requirement for specific probe design. Decreased assay set-up time and running costs.	No target specificity of the dye. Possibility of generation of false positive signals. Recognition of products require melting-curve analysis.

Figure 6: Schematic representation of Real-Time PCR using SYBR Green I dye.

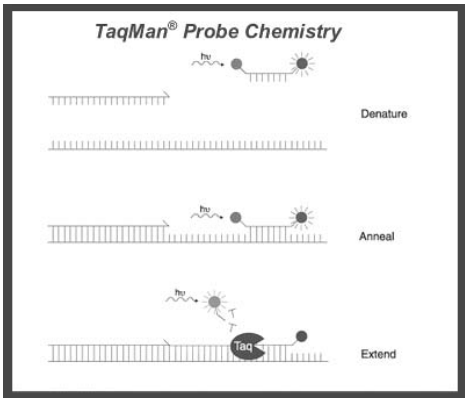
3.2.2. TAMRA quenched TaqMan probes

These probes, commonly referred to as the TaqMan assay [26], are fluorogenic oligomers designed to hybridise to an internal site within the target sequence as defined by the conventional PCR primers. The 5' end of the probe is labelled with a fluorescent reporter dye and the 3' end carries a fluorescent quencher dye. During PCR, the 5' exonuclease activity of *Taq* polymerase digests the hybridised probe, thereby separating the two dyes from one another (Figure 7). The resulting increase of fluorescence is proportional to the amount of specific PCR product present. The technology allows for multiplexed assays, since each amplicon can be targeted with a probe labelled with a distinct pair of reporter and quencher dyes [27].

Non-fluorescently quenched (NFQ) minor groove binder (MGB) probes

TaqMan MGB chemistry incorporates two further technologies: the addition to the probe of an inclusive MGB moiety greatly increases the stability and specificity of probe hybridisation, and the use of an NFQ in place of the TAMRA quencher dye enhances spectral performance (www.appliedbiosystems.com). The MGB is a small crescent-shaped molecule that fits into the minor groove of dsDNA. NFQ is essentially a chromophore acting as the energy transfer acceptor from the reporter molecule (which does not emit a detectable fluorescent signal of its own). NFQ is a proprietary molecule that is effective over a broad wavelength range (FAM™, VIC™ and TET™ dyes, www.appliedbiosystems.com). TaqMan MGB probes (Figure 8)

comprise a reporter dye (6-FAM) linked to the 5' end of the probe, and an NFQ at the 3' end. As with the standard TaqMan assay, when the probe is intact, the proximity of the reporter dye to the quencher suppresses reporter fluorescence, primarily via the Förster-type of energy transfer [28]. Because the quencher does not fluoresce, background is eliminated, and the signal-to-noise ratio is increased (www.genecore.embl.de).



ADVANTAGES	DISADVANTAGES
Specific hybridisation of probe-target sequence. Possibility of multiplexing by labelling multiple probes with different dyes. Cost-effective when the same sequence is analysed numerous times.	Cost for the synthesis of different specific probes.

Figure 7: **:** Schematic representation of real time PCR with TaqMan primers. In the intact TaqMan probe, energy is transferred (via fluorescence resonance energy transfer - FRET) from the short-wavelength fluorophore on one end (**green circle**) to the long-wavelength fluorophore on the other end (**red circle**), quenching the short-wavelength fluorescence.

During PCR, the MGB stabilises the annealing of the TaqMan probe by folding into the minor groove created between the probe and the target sequence. Stabilisation is most effective when the duplexes are perfectly matched. The addition of the MGB significantly stabilises probe-template complexes [29], enabling the use of probes in the 13 to 20mer size range [30] (www.appliedbiosystems.com). The reporter dye-fluorescence increases in each PCR cycle, reflecting the physical separation of the reporter dye from the NFQ.

The TaqMan real time assay procedure

In the TaqMan real time assay, the fluorescent signal arising from the degradation of the hybridisation probe and the consequent release of reporter dye is monitored in real time using the 7000 Sequence detector (Applied Biosystems, Foster City, CA). This instrument combines a conventional 96 well thermal cycler, a laser for the excitation of fluorescent dyes and a

detection and software system for the automatic acquisition and calculation of specific fluorescence released during the reaction. The fluorescence emission is detected by a CCD camera. For each sample, the camera collects the emission data between 520nm and 660nm once every few seconds. Every few seconds, the analytical algorithm calculates the reporter dye (R) and the quenching dye (Q) emission. The normalized fluorescence values (DRn values) reflect the quantity of fluorescent probe degraded and generally fit an exponential function for amplification over time (Figure 9).

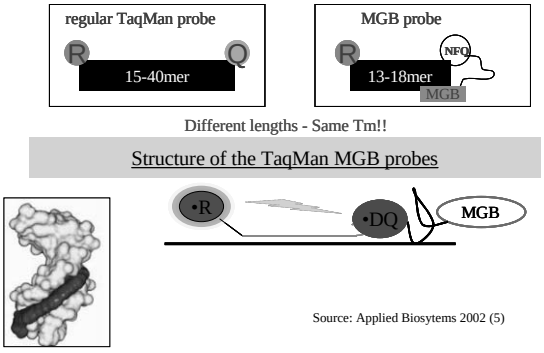


Figure 8: Molecular structure of the MGB probes.

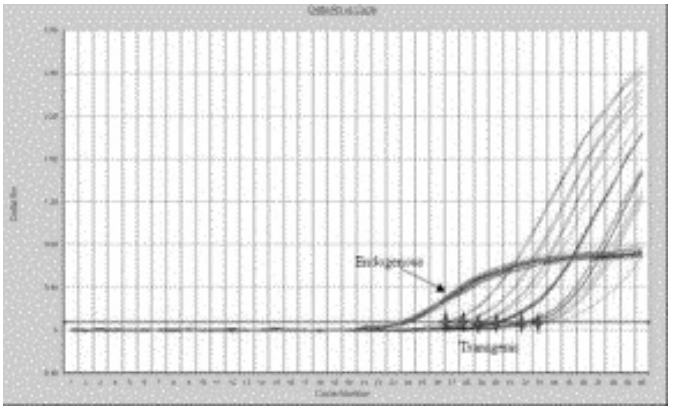


Figure 9: Amplification plot of duplex PCR using the Real-Time system

Simultaneously, the algorithm determines an experimental threshold on the basis of the mean baseline signal after the first 15 cycles plus 10 standard deviations. The cycle number at which the fluorescence of each sample reaches this threshold (crossing point – Cp - or threshold cycle - Ct) is correlated with the number of copies of the target present in the template. The Ct of each sample is used to quantify the samples, using a standard curve generated with a precisely quantified DNA or RNA standard that is amplified in the same PCR run (Figure 10). In the model PCR kinetic equation, $k = t_0 (E)^{Cp}$, where k represents the number of target sequences present at the crossing point Cp, E the efficiency and t_0 the initial concentration of the standard. This equation can be linearised as $\log k = \log t_0 + Cp \log E$, from which Cp can be simply derived from the expression $Cp = - (1/ \log E) \log t_0 + (\log K / \log E)$. Being a linear equation of the form $y = -mx + b$, the y intercept is given by the log of the number of copies present at the Cp, divided by the log of E. The system minimises carry-over contamination between samples, because it monitors *in situ* fluorescent signal in tubes with closed caps and avoids any need to open sample tubes during the run.

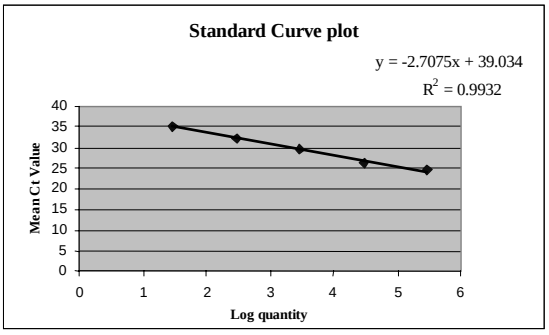


Figure 10: Amplification plot from a standard template.

The possibility of monitoring reaction efficiency in real time has revolutionised the approach to PCR-based DNA and RNA quantification. Reactions are characterized by the cycle at which the PCR product is first detected, rather than the amount of end-point product. The higher the initial copy number of the target sequence, the sooner the threshold level of fluorescence is reached. The quantification of target amount in unknown samples is achieved by the measurement of Ct, and relative or absolute quantification is used to determine the starting copy number. The sensitivity of fluorescence detection provided by the Applied Biosystems7000 sequence detector allows the threshold to be set at a cycle during the

exponential phase of amplification, thereby avoiding problems associated with quantification during the plateau stage. For this reason, Ct is a more reliable measure of starting copy number than an end-point measurement of accumulated product. Furthermore, well-to-well variation in fluorescence can be normalized using a reference fluorescent dye. High specificity and sensitivity can be achieved provided that care is taken in the choice of primer and probe sequence, with particular reference to the avoidance of inefficient probe cleavage which occurs in highly variable sequences. The real time PCR method enjoys several advantages over other related methods. In particular, it offers an efficient assay development, reproducible results and a wider dynamic range than end-point quantification methods, and can readily be extended into a high-throughput format.

3.3. Other types of probes

Several other types of sequence-specific probes have been developed for quantitative PCR [25], including hybridisation probes, “Molecular Beacons” and “Scorpion” probes. Hybridisation probes consist of two separate oligonucleotides, designed to anneal to adjacent positions on the same strand of the target sequence. The two oligonucleotides are differentially labelled, with one carrying a donor fluorochrome at the 3’ end, and the other an acceptor dye at the 5’ end. When both probes are correctly annealed, FRET delivers an increase in fluorescence proportional to the increase in the amplicon quantity. If a mismatch is present in the annealing site of one probe, hybridisation will not occur correctly and there will be no increase in fluorescence. Therefore, this technique also allows for positive mutation identification [31]. “Molecular Beacon” molecules self anneal into a stem and loop structure, and carry a fluorescent dye on one end and a quencher on the other. The loop incorporates a sequence complementary to the target, and when hybridisation occurs, separation between quencher and dye leads to the development of fluorescence, proportional to the amount of target DNA. In this case also, a single mismatch can prevent annealing, allowing for the identification of mutations [32, 33]. Molecular beacons have been applied only recently to food analysis. The presence of *Salmonella* species was detected at a level of 1-4cfu in real time PCR [34], using as target the *iagA* (invasion associated) gene specific for *Salmonella* spp. The probe increased assay sensitivity compared to the SYBR Green method. “Scorpion” probes represent a modification of molecular beacons, in which the same molecule acts as both probe and PCR primer. The molecule has a stem and loop structure, with a fluorophore at the 5’ end, and a quencher close to a sequence at the 3’ end, which is designed to prime Taq polymerase. When the primer is extended, the loop sequence is free to hybridize to the target, leading to the emission of fluorescence in proportion to the amount of target [35].

4. High-throughput genotyping with PCR

PCR based genotyping can contribute to the detection of toxic organisms in food. These methods are identical to those applied for genetic profiling or for measuring genetic variation within species, but can be readily adapted for the identification of specific gene variants in food matrices. The genotyping platforms appropriate for the various available molecular markers can be classified into three main types:

- Separation techniques, based on electrophoresis, either through a gel or a capillary, via chromatography (e.g. denaturing high performance liquid chromatography), or using mass spectrometry (e.g. Matrix-assisted laser desorption ionisation, MALDI): e.g. RFLP and microsatellite (simple sequence repeats or SSR) markers
- Homogeneous methods, in which reagents and products need not be separated and data are collected by fluorescence-based assays, such as the molecular beacons technology (see previous section): e.g. Single Nucleotide Polymorphism (SNP) markers
- Array methods using probes bound to a solid surface

4.1. Restriction fragment length polymorphism (RFLP) of PCR-amplified fragments

Electrophoretic separation distinguishes between DNA fragments of differing size. Where a sequence polymorphism introduces (or abolishes) a restriction enzyme recognition site, the length of the relevant restriction fragment will depend on whether or not the site is present. Thus the presence / absence of the fragment can be used as a genetic diagnostic. Representing the earliest DNA-based polymorphism assay, the analysis requires electrophoretic separation followed by transfer to a membrane and finally probe hybridisation - overall a highly time consuming technique. However, once such an RFLP has been identified, it can often be converted into a PCR assay, by obtaining the relevant local DNA sequence from which informative primers can be designed. After PCR, the product is digested with the appropriate restriction enzyme and a simple electrophoretic separation will then allow the presence or absence of the restriction site to be visualised (Figure 11).

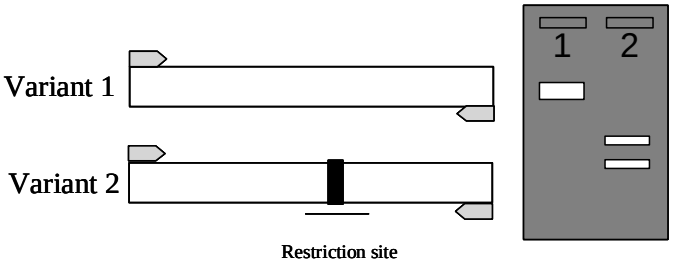


Figure 11: Schematic procedure for PCR-RFLP

The application of PCR-RFLP to food analysis has been useful for genotyping and characterising microbial isolates. For instance, when *Campylobacter coli* and *jejuni* contamination were detected in retail packs of chicken, PCR-RFLP was applied, using as target the flagellin gene *flaA*, followed by digestion with *Dde*I. This showed that specific subtypes were present over a one year period in the meat sourced from a particular producer, suggesting a common source rather than sporadic contamination [36].

4.2. Other genetic markers

Other genetic markers applicable to the identification of toxic organisms in food are SSRs and SNPs. SSRs are distributed throughout the genome, mostly in non-coding regions. However SNPs are ubiquitous, and represent the most common type of genetic variation in all organisms analysed. SSR variation takes the form of differences in the number of repeated elements present at a locus, and thus alleles can be recognised by the length of the amplicon. For this reason, a separation technique is necessary, and is generally based on electrophoresis (or less commonly MALDI) [37]. By contrast, SNP detection is not usually achieved via separation, because the lengths of the allelic amplicons are identical to one another. Instead, detection is usually based on homogeneous or array methods in which the mismatched base is recognised either by a failure of fragment hybridisation, or via the sequencing of localised parts of the amplicon [38]. In all SNP assays, sequence information is required for the design of allele-specific primers. This sequence information can also be used to generate molecular beacons or hybridisation probes, as previously described.

4.3. Array systems

Array systems, or chips, in which probes are immobilised on a solid surface, provide a platform for multiplexed genotyping. Three chip types are in common use, arraying either oligonucleotides, cDNAs and expressed sequence tags), or genomic probes. The specific hybridisation of a heterogeneous sample, as detected by fluorescence emission, to a feature(s) on the array allows the identification of a specific target(s). Differential labelling of two samples can then be used to simultaneously define their genetic differences at many loci in a single experiment. Where the sample is the product of a PCR amplification, however, quantification is not possible [39].

4.4. New trends in genotyping

4.4.1. Micro total analytical systems (μTAS)

Also called “lab-on-a-chip”, μTAS is a microfluidic device, in which the PCR is itself carried out on a chip. This down-scaling is particularly advantageous with respect to heat transfer, so that extremely rapid cycling becomes possible. The sample is moved continuously

through regions held at different temperatures, allowing for the completion of a full PCR amplification process in under 20 minutes. Capillary electrophoresis of the products is performed on the same device [40].

4.4.2. Single molecule PCR

PCR has the potential to amplify from a single target copy, although the risk of contamination becomes rather high, and the cycle number needs to be increased by about 100%. The approach generally involves a nested PCR (see above, and [41]).

4.4.3. Immobilized PCR and DNA colonies

The immobilisation of primers to a surface is advantageous as it eliminates the formation of primer dimers and other PCR artefacts. Oligomers can be covalently bonded to surfaces at very high densities. The presence of template promotes extension from one primer, and hence generates an immobilised strand; in a following step, after denaturation, the immobilised strand can bind to the second primer, leading to synthesis of the complete amplicon (www.promega.com). This greatly facilitates multiplexing procedures because it eliminates primer-dimer artifacts, it decreases cross contamination and reduces the problem of unequal efficiency in amplification between primer sets [42]. As each area of the array can be considered as a “DNA colony”, the cloning of selected amplicons is straightforward. Primers can also be immobilised in gels, allowing DNA colonies to be screened *in situ*.

4.4.4. GOOD assay

The GOOD assay was developed to allow MALDI detection of SNPs with the minimum amount of purification effort. The target sequence, including the SNP, is amplified and from this product, primers are extended with modified nucleotides to create allele-specific products. The products are alkylated to neutralise all negative charges except for those associated with the phosphate group which serves as the necessary charge tag, and the molecules are detected by MALDI [43].

4.4.5. Arrayed primer Extension (APEX)

The APEX method allows for the genotyping of several different SNPs on a chip. The probes bound to the chip represent the target sequences, with their 3’ ends placed at the base prior to the polymorphic one. Short amplified products from the test genomes are hybridised to the chip, and an extension reaction using fluorescently labelled terminator nucleotides is allowed to occur. In this way, each colour represents a base and all possible polymorphisms can be identified [44].

4.4.6. Fluorescent amplified fragment length polymorphism (FAFLP)

This technique is a modification of the standard AFLP technique [45] particularly suited for the analysis of bacterial genomes. The test DNA is digested with two restriction enzymes, and the fragments are ligated to ds adaptors. Primers (one labelled with a fluorochrome) annealing to the adaptors are added to amplify the fragments, which are then separated in an automatic DNA sequencer. Fragments are sized by comparison with an internal standard. As in the AFLP technique, various combinations of restriction enzyme and primers can be imposed [46].

5. Analysis of toxicants

Some food toxicants are detectable using PCR or other nucleic acid assays. Nucleic acids *per se* have not been shown to be toxic, and therefore these assays only detect a marker for contamination, rather than the contaminant itself. The marker may consist of a DNA fragment corresponding directly to the gene involved in synthesis of a specific toxicant, or it may be diagnostic for the species of the organism which produces a specific toxicant. Major targets for PCR analysis are proteinaceous allergens and toxins, but non-proteinaceous toxicants can in some cases be detected by targeting the gene(s) responsible for their biosynthesis. The favoured target for PCR analysis of food are DNA (or RNA) sequences associated with the synthesis of toxicant molecules. Since DNA is generally less susceptible than protein to degradation and modification, and since DNA (unlike protein) can be amplified, this approach can lead to false positive results (when the gene is present, but the toxicant is not). However, from a precautionary point of view, the presence of target DNA is taken as suggestive of previous contamination by the toxicant-producing organism, and therefore such false positives are still considered as informative.

The detection of genes involved in toxicant synthesis by PCR is not possible where either the sequence of the candidate gene(s) is not known, or where the genes responsible have not yet been identified. In these cases, the analysis must rely on sequences specific to the species or to the particular strain producing the toxicant. This approach is also indirect and can only demonstrate that a food product has contained or has come into contact with an organism capable of producing the toxicant. Table 1 summarises approaches reported in the literature for the three main classes of toxicant, while Table 2 reports several specific applications.

Table 1-Possible targets for the PCR detection of food toxicants

Toxicant	Organism	PCR target
Allergenic proteins	Various animal and plants	Genes encoding allergenic proteins Species-specific sequences to identify the organism
Mycotoxins	Various fungal strains	Genes encoding enzymes involved in the biosynthetic pathway Species-specific sequences to identify the organism Polymorphic markers identifying the strain or species
Bacterial proteinaceous toxins	Various bacterial strains	Genes encoding the toxic proteins Species-specific sequences to identify the organism

5.1. Food allergens

Food allergens and the methods for their detection have been described in previous chapters. Distinct from other food toxicants, allergens have no dose-dependent toxicity, but are dangerous to a portion of the population. Therefore, the detection of allergens in food is needed to serve as a warning to consumers suffering from known allergies. Since there is no therapy to treat the allergic reaction, the avoidance of allergenic food products is the only option. The bulk of allergens are proteinaceous, so detection methods based on PCR directed to the corresponding genes and/or mRNAs are appropriate (Table 2). As alluded to above, the particular advantage of PCR based diagnostic methods is that it allows for the amplification of the target from a very low initial concentration. However, the absence of the gene or its transcript does not absolutely guarantee absence of the protein, so a level of false negative results can be expected to occur.

Table 2. Summary of techniques applied for food analysis

Technique	Examples	References
PCR amplification of target genes		
Nuclear genome	Hazelnut *Cor a 1*	36, 47, 53, 56, 57, 60, 61, 62, 65, 66, 67, 73, 74, 82, 86, 87
	Wheat gliadin	
	Buckwheat rDNA genes from spiked wheat	
	Fusarium tri5	
	Penicillium pepg1	
	Aspergillus calmodulin from grapes	
	Aspergillus nor-1, *ver-1*, *aflR*, *omt-1*	
	Fusarium beta-tubulin	
	Fusarium calmodulin, rDNA	
	Escherichia coli eaeA, *hlyA*, *fliC*	
	Staphylococcus nuc from different foods and spiked beef samples	
	Staphylococcus se genes from various foods	
	Campylobacter jejuni flaA, *cad F*, *ceuE*, *cdt* cluster, *virB11* from turkeys	
	Bacillus cereus bceT, *nheABC*, *hblACD*, *cytK* in chicken	
Mitochondrion or chloroplast genome	Hazelnut *nad1* in chocolate	22, 48
	Cereals *trnL*	
Molecular markers	*Aspergillus carbonarius* (SCARs from AFLPs) in coffee beans	62, 63, 64, 88
	Aspergillus carbonarius (SCARs from RAPD) in coffee beans	
	Bacillus cereus (STS) from various foods	
Nested PCR	*Shigella flexneri ial* from spiked lettuce	9

Technique	Examples	References
Multiplex PCR	*Aspergillus avfA, omtA, ver-1* *Fusarium tri6, fum1*, rDNA from cornmeal *Vibrio* and *Salmonella hns, spvB, vvh, ctx, tI* from spiked oysters *Salmonella typhi invA, viaB, fliC-d, prt* in spiked meat rinse and milk	7, 68, 69, 89,
PCR-ELISA test	Hazelnut *Cor a 1* in various foods *E. coli* $SILO_{157}$	49, 83
Immobilised probes	*Vibrio* and *Salmonella hns, spvB, vvh, ctx, tI* from spiked oysters	89
Cloth-based hybridisation array system (CHAS)	*Salmonella stn*, *Escherichia coli vt1, vt2, lt1, stx1, Vibrio cholerae ctxA*	78
PNA	Hazelnut *Cor a 1* from various foods	50
Quantitative Competitive PCR	Cereals *trnL* in various foods *Escherichia coli slt2* from broth and milk	21, 22
Real-Time PCR for identification or quantitation	Peanut *Ara h 2* in different foods *Clostridium botulinum BoNT* from spiked foods *Vibrio cholerae hlyA* from oysters *Escherichia coli vt1* and *vt2* in meat samples *Staphylococcus aureus nuc* from various foods	51, 52, 73, 81, 85, 90,
Molecular beacons	*Salmonella iagA* in fresh vegetables	34
Multiplex Real-Time PCR	*Fusarium tri6, fum1*, rDNA from corn	58, 59, 79, 80

Technique	Examples	References
	Fusarium species-specific fragments from grains *Staphylococcus aureus se* genes *Escherichia coli rfb* and *stx2* in juice and beef	
Reverse Transcriptase PCR	*Aspergillus nor-1*	60
Microarrays	*Staphylococcus aureus se* genes *Clostridium perfringens cpb1, etxD, cpe, cpa, iota, cpb2* *Campylobacter fur, glyA* *Listeria iap* *Escherichia coli eaeA, stx1, stx2, hlyA* (low density) from chicken rinsate	72, 75, 76, 77

The first published applications of PCR for allergen detection targeted the hazelnut *Cor a 1* gene (a major allergen), and wheat prolamin (ω-gliadin), important for celiac disease sufferers [47]. An alternative approach for the detection of hazelnut targeted the mitochondrial *nad1* gene [48]. The choice of a mitochondrial gene can be justified by the high copy number of mitochondria in the plant cell, thereby conferring a higher probability of detection, since the initial copy number of the target will be higher than for a nuclear gene. In contrast to the highly specific *Cor a 1* gene, this mitochondrial sequence is not specific for hazelnut, being similar to those in other Betulaceae species. However, little or no cross-reactivity with other plant species (cocoa, sugar beet and soya bean) was present. This emphasises the importance for care in the choice of target gene and the design of primers.

Holzhauser et al. [49] developed a PCR-ELISA test, in which the amplified *Cor a 1* fragment was biotinylated to allow its binding to streptavidin-coated microtitre plate wells. As little as 2pg of hazelnut DNA could be identified using this method, equivalent to less than 10 copies per reaction of the target. PCR-ELISA provides the possibility of verifying the identity of the amplicon by hybridisation.

A recent development exploited a 15mer PNA, designed to hybridise to an internal sequence of the 156bp *Cor a 1* amplicon, to confirm the identity of the amplified product [50].

A real time PCR assay for the peanut allergen *Ara h 2* has been developed with a sensitivity of 10 copies per reaction of the target gene [51]; as DNA is often degraded in processed

foods, such a short (86bp) amplicon sequence is desirable. The choice of a real time approach was not made for quantification purposes, since this may be affected by variation in extraction efficiency and the presence of inhibitor. Rather, it was selected to allow confirmation of the identity of the amplicon, as demonstrated by hybridisation with a specific probe. An identical strategy was proposed by Hird et al. [52], who compared PCR and ELISA assays, and showed that both could detect the presence of peanut material in chocolate at 10ppm. The PCR method was marginally more sensitive than ELISA.

Equivalent methods have been developed for several other allergens. For example, traces of both common (*Fagopyrum esculentum*) and Tartary *(F. tataricum)* buckwheat can be detected by amplifying the internal transcribed spacer region of the ribsomal locus (rDNA ITS) [53]. Gluten from wheat and some other cereals is hazardous for sufferers of celiac disease. Thus it is important that foods labelled as gluten-free are tested with a highly sensitive assay. PCR analyses can detect the presence of wheat, barley and rye DNA by targeting various chloroplast genes [22]. As for the mitochondrial genome, chloroplast gene targets are advantageous, since green plant cells contain many chloroplasts.

DNA sequences not involved in the synthesis of allergens can be chosen as targets for two main reasons: (i) allergen heterogeneity, which would require a high number of allergen-specific PCR targets, and/or (ii) a lack of sequence information for the allergenic protein or/and its encoding gene(s).

5.2. Mycotoxins and bacterial toxins

Some fungal and bacterial contaminants only cause rotting and spoilage of food, whereas others produce toxins which can have short- and long-term effects on human health. Conventional techniques for the identification micro-organisms require microbiological and physiological tests, which are both time-consuming and require considerable operator expertise. Moreover, culturing techniques cannot detect the presence of dead micro-organisms, which represent an index of past contamination. PCR assays of specific nucleic acids clearly are an improvement over these techniques. Assays based on PCR have the specific advantage of allowing early detection of a toxin-producing strain. A further advantage of PCR is that it can generate a precise estimate of the amount of microbial contamination, and can also be used for species identification in the absence of any morphological indicators [54].

The most straightforward assays rely on a target gene directly involved in the biosynthetic pathway of a specific toxin (Tables 1 and 2). The presence of the gene is then taken as providing evidence for past or present contamination by a toxin-producing strain. Sequence information regarding these genes in the various species is therefore required. Ongoing genomic sequencing of a number of pathogens is contributing to the identification of many new gene targets. Sample preparation involves the isolation of microbial cells from the food

product and the extraction/purification of DNA [55]. These cells are typically present in low concentrations, the DNA present in the processed food product may be degraded, and inhibitors may be present. There is, unfortunately, no globally accepted protocol for the isolation of microbial DNA. Instead, when dealing with food matrices, optimisation is necessary on a case-by-case basis.

5.2.1. Fungal contaminants

Several assays for mycotoxin-producing strains have been based on the identification of specific genes or molecular markers via PCR [56]. *Fusarium* fungi produce a series of mycotoxins of relevance to human health. Deoxynivalenol (vomitoxin) contamination of wheat grain results from heavy infection by *F. graminearum* and *culmorum*. The toxins are produced both before and after harvest. The Tox5 PCR assay [57] detects the presence of *Fusarium* spp. which produce tricothecenes, by targeting the gene *tri5 (*syn. *Tox5;* encoding tricothecene synthase). A specific requirement of these tests is to discriminate between the presence *graminearum* and that of other species, which are also commonly found on the grain surface. A PCR assay directed to the three most important mycotoxin producing species (*graminearum*, *culmorum* and *sporotrichioides*) and which lacks any cross-amplification with six other *Fusarium* species has been described [58]. This represents a considerable improvement over classical microbiological methods, because it is both quicker and discriminates between morphologically similar species. These assays have been upgraded to allow both QC-PCR and real time PCR in the framework of an EU financed project (DETOX-FUNGI) (http://detox.ba.cnr.it/). Further targets, such as *tri6*, involved in tricothecene biosynthesis, and *fum1* in fumonisin biosynthesis, are now available in a real time platform, and are sensitive over a wide range of genomic DNA concentrations [59].

The highly carcinogenic aflatoxin B1, produced by *Aspergillus flavus*, *A. nomius* and *A. parasiticus*, is a particularly important mycotoxin. Its detection can be achieved by targeting genes for toxin biosynthesis, specifically *nor-1* (norsolorinic acid reductase), *ver-1* (versicolorin A dehydrogenase), *aflR* (regulator) and *omt-1* (sterigmatocystin-o-methyltransferase) (cited in [60]). The limits of detection are of the order 100cfu/g [61]. The measurement of mRNA abundance with RT-PCR has been proposed as a more accurate means of monitoring toxin levels [60]. RT-PCR results were found to correlate well with conventional plate counts, but the enhancement in gene expression occurs two days before any observable increase in mycotoxin concentration. The suggestion was not that this approach could be used for the detection mycotoxigenic strains in food products, but rather was suggested as a means to obtain information on aflatoxin biosynthesis at critical points of the food chain.

The sequence of *Pepg1*, encoding a polygalacturonase responsible for the decay of fruit tissues, has been used to assay for the presence of *Penicillium expansum*, the source of the

mycotoxin patulin [62]. The presence of contamination was followed by the amplification of a gene involved in the process of infection, rather than one involved in toxin synthesis.

Ochratoxin A, a nephrotoxic and carcinogenic mycotoxin, is produced by *Aspergillus* and *Penicillium* strains, which are common as food contaminants. *A. carbonarius* is considered to be the more important contaminant of coffee and grape-based products, although both *A. ochraceus* and *niger* can produce the toxin. The situation is further complicated by difficulties in *Aspergillus* taxonomy. Molecular approaches in this case have been based on the amplification of species- or strain-specific markers, even when the function of the sequence, or even the the sequence itself is not known. Appropriate targets have been identified from anonymous molecular multilocus profiles, which can be used to select DNA fragments which discriminate between mycotoxogenic and non-mycotoxogenic species and/or strains. These fragments are excised from the gel and cloned, and the resulting sequence used to generate primers which are specific in their amplification. Markers of this type are commonly referred to as "sequence characterized amplified regions" (SCARs) (Figure 12). An *A. carbonarius* SCAR was generated from RAPD profiling [63], but the amplicon is probably too long (809bp) to be a practical target for amplification from food matrices. However, it was possible to amplify it from DNA extracted from coffee beans. A similar approach has produced a 189bp *A. carbonarius* specific SCAR from an AFLP profile [64].

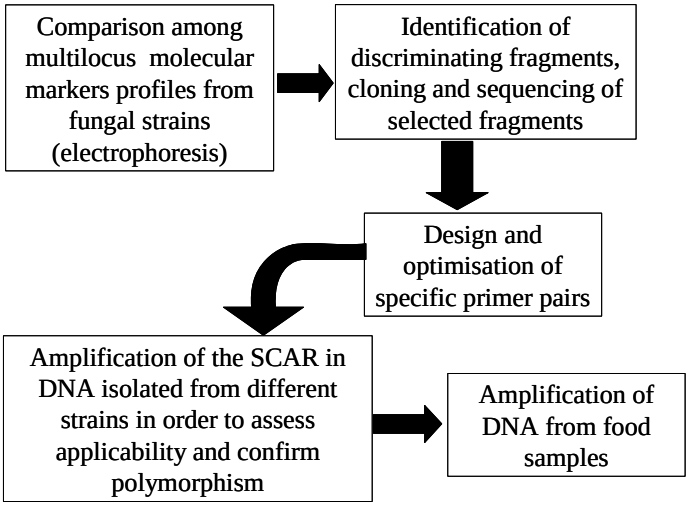


Figure 12: The process of SCAR identification for the PCR detection of fungal contaminants

A different approach uses as target other genes for which sequence information is available, such as tubulin [65], ribosomal internal transcribed spacer, calmodulin [66]. All instances in which sequence polymorphisms can be identified among fungal strains are amenable to transformation into diagnostic markers. The main consideration becomes the dimension of the amplified fragment, especially in those cases in which DNA in food products may be degraded. It is also necessary to acquire sequence data on a high number of strains and species before the assay can be considered informative.

Where DNA sequence is available, such as for tubulin [65], rDNA ITS and calmodulin [66], SNPs diagnostic for fungal strains can be exploited to generate functional markers. A major consideration is the length of the amplicon, especially where the template is derived from processed food products. Clearly sequence data must be generated from an adequate number of strains and species before the assay can be considered robust. SNP variation in the calmodulin gene correlated with ochratoxin production in *A. carbonarius* has been described [67]. Species-specific primers were generated using an sequence alignment from 30 strains of various geographical provenance. A high level of within-species sequence homology was the first requirement, and a primer pair was generated which amplified a 371bp fragment from *A. carbonarius*, while another amplified a 583bp fragment from *A. japonicus*. The former did not amplify from template of either other *Aspergillus* species or other fungal genera. Serial dilution experiments showed that detectable amplification was obtained from 12.5pg target DNA, corresponding to about 260 haploid genomes (calculated on the basis of the haploid genome size of *A. niger*).

Certain multiplex methodologies have been developed, in which two or more genes have been targeted, as recently demonstrated for both *Aspergillus* [68] and *Fusarium* [69]. Three genes involved in aflatoxin biosynthesis formed the targets for *Aspergillus*, while for *Fusarium* the choices were *tri6* (*graminearum*), *fum1* (*verticillioides*) and rDNA ITS as a means of identifying the *Fusarium* genus. Multiplex PCR saves both time and labour, and its adaptation to real time systems also allows for target quantification, and the estimation of the extent of contamination [59].

5.2.2. Bacterial contaminants

Bacterial contamination can also lead to toxin presence in food products. An enrichment step is typically required for its detection, involving culture in specific media. However, when multiple bacterial species are present in the same sample, differences in growth rate and culture requirements can introduce bias into the final results. Attempts have been made to avoid the enrichment step by using PCR amplification of DNA extracted from the sample, but this may be not sufficiently sensitive. Moreover, the DNA extracted from food can be hampered by the presence of inhibitors, which are absent from DNA extracted from bacterial

cultures [70, 71]. Tables 1 and 2 list the various approaches adopted and the genes targeted for bacterial toxin detection.

The major problem encountered in bacterial toxin contamination is related to heterogeneity. Closely related pathogen species may differ in their toxin production, a single species may produce several different, although related toxins, and even a single strain may produce multiple toxins [72]. A typical example is presented by *S. aureus*, which produces at least 17 different toxins. In cases of food poisoning caused by this bacterium, strain genotyping and identification of the enterotoxin(s) are important to define related clusters of infection, and to trace the source and spread of contaminated food. Thus assays are necessary to track the various toxin genes. Common targets for PCR amplification are the thermonuclease (*nuc*) and enterotoxin (*sea-see*) genes. The amplification product of the *nuc* gene is specific for *S. aureus*, allowing it to be distinguished from other *Staphyloccocus* species [73]. It has therefore been proposed as a rapid identifier in routine food analyses [74]. A search for a real time assay has compared TaqMan chemistry with SYBR Green I [73]. Testing was carried out on standards containing from 1.5 – 1,500,000 genome equivalents, and from 3 to 2,600,000cfu/ml. Using spiked food samples, SYBR Green was able to detect 490cfu/g, whereas the limit for TaqMan was an order of magnitude higher. For the effective detection of all serological types and of all the 17 toxins, a microarray approach, focusing on multiple enterotoxin genes, has been proposed [75]. The critical genomic region of the various strains is amplified with primers annealing to the conserved regions of the genes which flank the variable regions. These amplicons are then hybridised to an array containing sets of four 21-30nt oligonucleotides with average melting temperature of 50°C specific to each gene, in order to account for occasional cross-hybridisation (Figure 13).

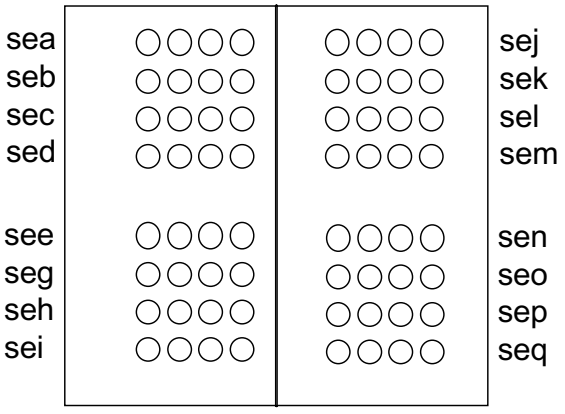


Figure 13: Lay-out of staphylococcal genes on a microarray [58]. Each gene is represented by four spots, corresponding to four specific oligonucleotides. The microarray contains also spots for quality control, not shown.

A microarray assay for *C. perfringens*, based on hybridisation with the genes encoding enterotoxin (*cpe*) and beta (*cpb*), epsilon (*etx*), alpha (*cpa*) and iota (*iA*) toxins, differentiated eight strains [76]. Similarly, *E. coli* O157:H7 can be identified via a microarray containing probes for intimin (*eaeA*), shiga-like toxins I and II (*stx1* and *stx2*), and hemolysin A (*hlyA*) [77]. A single microarray has been reported as able to simultaneously detect a number of different pathogens (Figure 14) [72]. In this platform, a single primer pair is used to amplify the *fur* (ferric uptake regulator) gene from four *Campylobacter* species. A second primer pair amplifies the *glyA* (hydroxymethyltransferase) gene from the same species, a third 16 *S. aureus* enterotoxin genes, and a fourth the *iap* (secreted invasion-associated protein) genes from six *Listeria* species. A further six primer pairs target the *cpt* toxin-producing genes of *C. perfringens*. An important consideration for the latter targets was to ensure that the probe sequences avoided regions rich in point mutations.

The advantage of the microarray approach, particularly when preceded by a multiplex PCR, is the simultaneous analysis of several targets in one experiment, in which the hybridisation to specific probes confirms the identity of the amplified product and therefore enables recognition of the contaminant.

A novel approach, with most of the advantages of the array technique coupled with ease of detection, has been developed for the detection of various bacterial genes. The arrays were low-density and were formed on a polyester cloth [78]. The amplified genes were *stn* (*Salmonella* enterotoxin), *vt1, vt2, lt1, stx1* (*E. coli*) and *ctxA* (*Vibrio cholerae*). The 120 to 180nt probes were generated by PCR, and cross-linked to the cloth by UV irradiation. Multiplex PCR products were labelled with digoxygenin, and detected, after hybridisation, with an anti-digoxygenin-peroxidase conjugate followed by incubation with the peroxidase substrate. The detection limit was bacterial species dependent and ranged from 0.015 to 0.12pg genomic DNA.

Triplex assays based on MGB-TaqMan real time PCR have been described for multiple *Staphyloccocus* genes [79]. The specificity of the probes effectively suppresses non-specific signals. After designing the sequences for the monoplex PCR detection of nine *se* genes, three triplex assays were assembled, in which the probes were labelled with different reporter dyes (VIC, FAM or TET). The detection limits were 16 – 2,000 copies of the target gene, but application to food matrices was not attempted. Shigatoxin and verotoxin producing strains of *E. coli* O157:H7 can be identified by PCR amplification of *stx (1, 2)* and *vt (1, 2)*. A sensitive assay based on duplex real time PCR can discriminate between strains producing the O antigen and the shiga-like toxin 2. It also allows for quantification in food samples, using as targets the *rfb* (necessary for O antigen biosynthesis) and *stx2* genes [80]. The sensitivity of the method was sufficient to eliminate the need for prior enrichment. For milk samples, DNA could be amplified from directly boiled samples, because no PCR inhibitors were present. From other food sources (e.g., apple juice), the presence of inhibitors required some DNA

purification prior to amplification. A real time amplification of *vt1* and *vt2* was able to detect 10 copies of purified plasmids, 100fg of genomic DNA, or about 3,000 bacterial cells in meat samples, a remarkable level of sensitivity. Real time RT-PCR was further used to monitor the expression of the *vt* genes in food samples [81]. Multilocus profiling of *E. coli* has also targeted other virulence genes, such as intimin (*eaeA*), hemolysin (*hlyA*), and the fimbrial gene *fliC* [82].

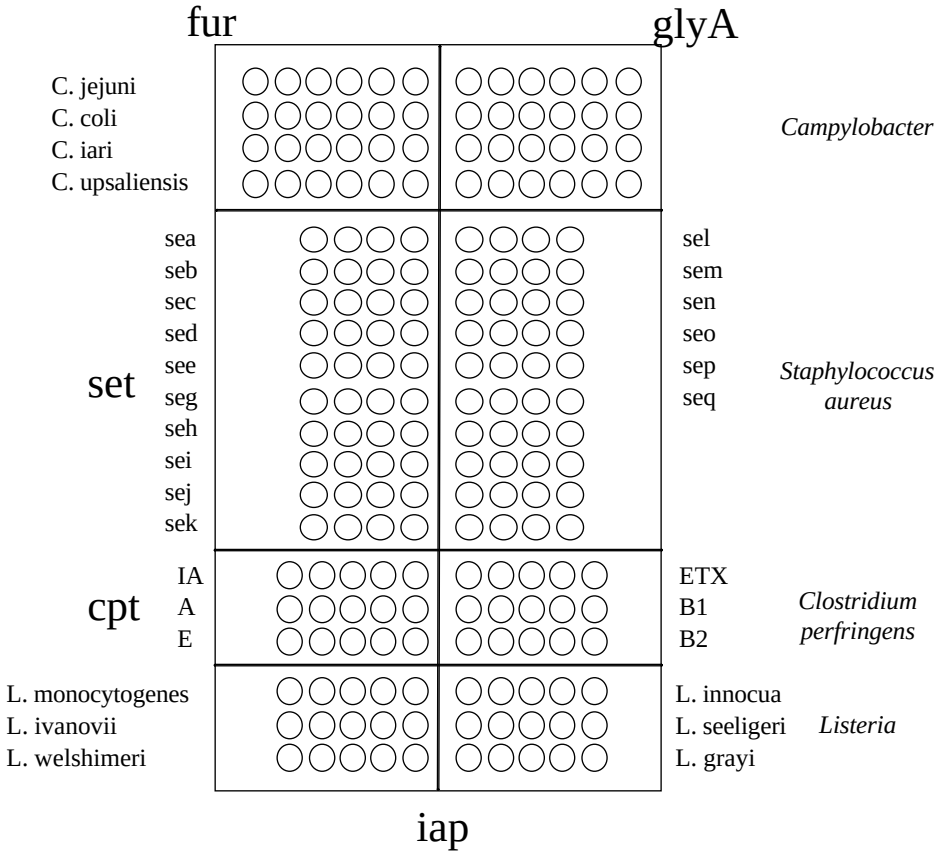


Figure 14: Layout of the FDA-1 chip [72], with probes specific for *Campylobacter* sp *fur* and *glyA*, *S. aureus* enterotoxin genes, *C. perfringens cpt* toxins, and *Listeria* sp. *iap* genes.

A QC-PCR assay was developed as a means of identifying specifically viable cells present in food samples [21]. A fragment of the *stx2* gene was used as marker, and its increase in concentration over time was taken as indicator of the increase in viable cell number. QC-PCR was strongly correlated with the number of cells as estimated by cfu counting. However, the correlation was less strong when the cells reached the stationary phase, because of the contribution of dead cells to the template DNA.

Fach et al [83] compared six PCR assays for *E. coli* O157, based on the genes *rfbE, rfbB, eaeA, fliC*$_{H7}$ (flagellin) and *SILO*$_{157}$ (small inserted locus). The *rfb* assays detected both toxigenic and non- toxigenic strains. The non-toxigenic strains could be identified with the other assays, but only the *SILO*$_{157}$ assay was able to identify O157 strains. An ELISA-PCR assay was then based on the *SILO*$_{157}$, in which the amplicon was hybridised in solution with two probes (one coupled to biotin, the other labelled with digoxigenin). The capture probe ensured that the material bound to streptavidin-coated microtitre wells, and the detection probe allowed for visualisation via peroxidase anti-digoxigenin conjugation. The sensitivity of the system was less than 10 genomes per sample. Finally, an integrated system for the purification of *E. coli* cells from rinsings of poultry carcasses has been proposed [84]. This uses an immunomagnetic separation, followed by PCR amplification (*eaeA* promoter) and a low-density microarray hybridisation [77], with the overall aim of developing a fully automated pathogen detection system from food and environmental matrices.

C. botulinum produces one of four neurotoxins (BoNT A, B, E, and F) responsible for human botulism, and several PCR assays have been developed for its detection. Recently, a TaqMan real time assay was developed to quantify levels present in type A strains [85], and its efficacy was tested on DNA extracted from spiked food samples. The primers designed from the *BoNT* sequence were specific for the A strain, giving no amplification from other *Clostridium* strains and species, or other food-borne pathogens. The advantage of the assay with respect to prior test was speed (3h) and the elimination of the enrichment step. The limit of detection was 0.1ng DNA per ml.

Campylobacter jejuni contamination can also be identified by targeting virulence and toxin genes; of these *flaA* (flagellin subunit A), *cadF* (adhesion to fibronectin), *ceuE* (siderophore transport), *cdt* cluster (A, B, C encoding the cytolethal distending toxin), and *virB11* (from virulence plasmid) have been tested to date. The targets were amplified with varying efficiency from 117 strains isolated from turkey meat. *virB11* could be amplified only from one third of the samples, but all strains which were PCR negative for the *cdt* genes also produced little or no CDT toxin [86].

The targets for *Bacillus cereus* toxins are *bceT* (enterotoxin T), *nheABC* (non-hemolytic enterotoxins), *hblACD* (hemolytic entorotoxin) and *cytK* (cytotoxin K) [87]. These have been used in monoplex PCR analyses of samples from chicken meat (cooked and breaded) obtained from retail stores. A positive PCR result correlated well with conventional

microbiological assays (growth, staining, and various biochemical tests). In particular, a novel approach has been sought to identify carriers of the cereulide toxin, responsible for emesis; the target in this case is a fragment of a gene of unknown function, shown to be specific for strains producing the toxin [88].

Vibrio cholerae can be detected by amplification of the *ctx* gene. A multiplex test simultaneously identifies *cholerae*, *vulnificus*, *parahaemolyticus* and *Salmonella* sp. [89]. The targets were *hns* (a histone-like *Salmonella* nucleoid protein), *spvB* (a plasmid virulence factor for pathogenic *Salmonella*), *vvh* (*V. vulnificus* hemolysin), *ctx* (*V. cholerae* cholera toxin) and *tl* (*V. parahaemolyticus* thermolabile hemolysin). All five amplicons were generated in a multiplex PCR from mixed samples of bacteria. The sensitivity was 100 cells per g of oyster tissue. Further validation of the amplified products was obtained from microtitre plate hybridisation to immobilised oligonucleotide probes, each of which was specific for one of the amplicons. Another target gene is *hlyA* encoding a non-classical hemolysin, expressed in both environmental and clinical strains of *V. cholerae*. This target has been chosen because heterogeneity between serotypes can lead to false negatives in assays based on specific targets, such as *ctx* genes. The *hlyA* gene sequence is specific for all strains of *V. cholerae*, but is distinct from that of other species. The application of a TaqMan PCR assay yielded high sensitivity and specificity without enrichment. In spiked oysters, the limit of detection was 6-8 cfu per g [90].

Listeria monocytogenes is another important food pathogen, causing listeriosis. A recent review [91] listed approaches used for its detection and identification in food, clinical and environmental samples. Several targets for PCR amplification have been chosen among the genes involved in the pathogenesis process. These include *iap* (invasion associated protein), *hly* (hemolysin) and *inlA-D* (internalin). Other targets are genes which can discriminate among species and subtypes, such as ribosomal genes and spacers, transcriptional activators, enzyme-coding genes, and flagellins. Assays available for *Listeria* include qualitative PCR for specific genes, multiplex PCR assays, molecular markers and SCARs, quantitative PCR in real time, microarrays, ELISA-PCR, and RT-PCR [91].

5.3. Identification of genetically modified organisms (GMOs)

An area of food analysis where PCR techniques have become of particular importance is the detection of the presence of GMOs. Transgenes are not generally toxicants, but because their presence in food can be accidental, the means for their detection are analogous to those used for microbial contaminants. Several excellent relevant reviews have been recently published [92, 93, 94], and another chapter in the present book addresses the topic in more depth. PCR detects DNA targets present at very low concentrations, and therefore is the technique which can best effect GMO detection in potentially contaminated food products. Since DNA can survive harsh treatment, its signature is still present, even if degraded, in most

food matrices. The targets for GMO analysis are the relevant transgene constructs. Most components of the construct can be exploited, be it promoters, terminators, coding regions, or junction regions between elements. A major hindrance in the development of specific tests is imperfect knowledge of commercial (and hence confidential) transgenic events. To discriminate between events derived in the same species using the same constructs, the only possible target is the junction between the construct and recipient plant DNA, and this can be obtained only through sequencing.

Qualitative analyses are important for the identification of transgenic events, but quantitative analyses are also relevant to enforce labelling thresholds imposed by legislation. Quantitative analyses require reference genes, which are typically species-specific single-copy genes, such as zein for maize or lectin for soybean. All the techniques listed in Table 2 have been applied to GMO detection: PCR assays, nested and multiplex assays [5], QC-PCR, PNA clamping [24], mono- and multiplex real time, and microarrays [95]. Method development is an activity which has been stimulated in Europe by the Framework Programmes 5 and 6 of the European Union, which supported several research projects. An innovative approach for GMO detection is the topic of the Framework 6 project Co-Extra (http://www.coextra.org), which seeks to evaluate a modular analytical procedure, based on the assumption that the procedure will only be valid if all the methods used in the different steps (modules) are themselves robust. Whereas PCR methodologies and assays are commonly validated, the prior steps of the procedure (sampling, processing and extraction) are seldom validated according to international guidelines. The modular approach offers the possibility of validating individual modules, which can then be combined in several ways, helping the implementation of the guidelines and the regulations concerning food safety.

6. Predictive toxicogenomics

Toxicogenomics describes the use of gene expression changes to follow reaction to toxicant exposure. It is likely to become an increasingly useful tool, as it has the potential to identify toxicity problems during the process of product development rather than after the product has already been already marketed. The technology could provide a platform for the identification and ranking of potential toxicity of “new chemical entities” (NCEs) thus promoting levels of food safety at the pre-marketing stage. Conventional toxicological studies of food and food ingredients are time-consuming and costly to conduct, and may not always accurately predict toxicity to humans. The number of endpoints (toxicity, mortality, developmental endpoints) evaluated in food toxicology is very low and may not adequately explore the potential toxicity to humans of a particular NCE. On the other hand, a transcriptome-wide gene expression study can be designed to explore the full impact of a food toxicant across highly diversified, multifunctional gene sets and numerous biochemical pathways. This property can be expected

to change dramatically the way in which food safety issues will be evaluated in the future (Figure 15).

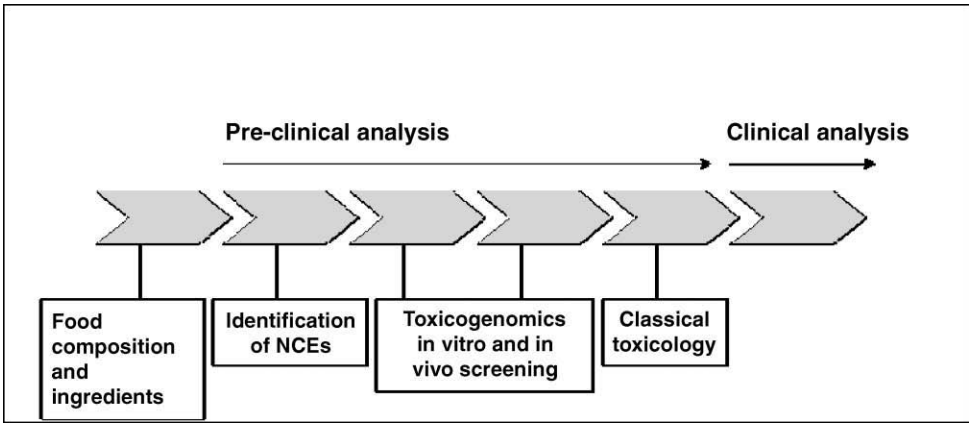


Figure 15. Toxicogenomics and food analysis

Predictive modelling involves statistical inference and does not necessarily answer mechanistic questions. However the behaviour of individual genes within a predictive model can be used to infer specific mechanisms in reference to knowledge derived from wider extant data. The construction of a reference database and the implementation of several analytical and visualisation methodologies are fundamental requirements for the establishment of a toxicogenomics programme. Several sorts of problems can be addressed using predictive modelling algorithms, including:

(i) early stage compound toxicity ranking with in vitro and in vivo testing,
(ii) mechanistic based analysis for toxicity
(iii) discovery of unique biomarkers for pre-clinical and clinical evaluations

Microarrays and multiplex PCR are both well suited for the classification of toxicity, since together they allow the measurement of gene expression levels across the entire transcriptome. A small number of multiplex PCRs or a single chip experiment can be sufficient to yield expression data for hundreds or even thousands of parameters. For both sorts of data, however, some statistical validation is essential. As a first step, sets of genes informative for the classification of expected toxicity types must be identified (Figure 16). A general toxicity response, for example, can be defined by tracking the expression of any genes whose expression is altered to approximately the same degree irrespective of the toxin involved. On the other hand, markers for a specific toxicity, such as the allergenic response,

can be defined by their tendency to be similarly regulated by the presence of a spectrum of allergenic agents. Not all genes change their expression in response to a specific toxicant, and what constitutes a “change” also needs definition related to the assay platform in use. Microarray platforms assay the expression of thousands of genes, but the quantification resolution is poor; in contrast, PCR can only deal with a modest number of targets, but their expression can be much more accurately quantified. The gene selection procedure has the good of ranking genes with respect to distinct differences that analysts or biologists deem important. Ranking should be based on the probability that the expression profiles are reproducible and different in control and treated samples, and not due to random noise. But all genes differ in their dynamic ranges of expression as well as in their overall biological variability and only a powerful statistical approach can sort out “real compound effects” from “background effects”. A reliable database with all these parameters can allow for reliable statistical inferences. Parametric and non parametric techniques can be applied to identify marker genes for food toxicological applications. The selection of target genes is based on a ranking which must reflect their importance to the biological process under study. However this ranking also needs to take into account the reproducibility of expression profiles, and not just be governed by a difference between control and treated samples. Biological noise interferes with expression level, so a robust statistical analysis is always necessary to identify real effects. Both parametric and non-parametric analyses are appropriate for the identification of suitable marker genes for food toxicants.

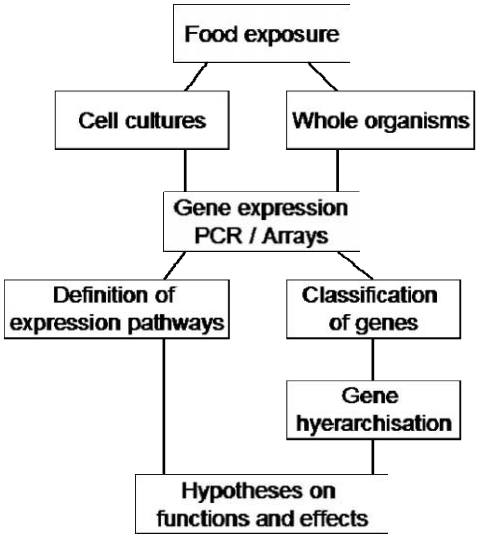


Figure 16. The concept of toxicogenomics

A further important role of predictive toxicogenomics relates to the potential toxicity of food compounds or components, for which there is no, or only limited experimental data available. A classification of “normal” or “pathological” should consider all possible normal states and pathologies which requires that a large number of treated samples must be analysed. Many methods are used to classify samples described by large numbers of parameters, such as in gene expression analysis: i.e. logistic regression, linear discriminant analysis, neural networks, support vector machines and co-clustering approaches.

Gene function is commonly predicted on the basis of sequence analysis at either the protein or nucleotide level, and genes can be classified by their overall observed ontology and by family membership. Co-regulation and co-expression in response to a number of treatments also provides relevant evidence. The prediction of co-regulation on the basis of sequence similarity (clustering) is a widely used and accepted approach, but has some drawbacks. For instance, few clustering algorithms allow for membership in more than one cluster. Hierarchical clustering has been extensively used to relate one gene to another by means of an association which groups genes together and then subdivides the groups. Proper marker gene selection, comparison of multiple clustering methods using a variety of matrices, and robust validation measures are all necessary, as is the construction of a substantial reference database. Ideally, a toxicogenomics programme should be applicable to several types of related analyses. Many procedures, including the treatment and the sacrifice of animals, and chip or PCR processing protocols are expected to develop over time, and a reference database has to be able to incorporate these changes in a flexible manner.

A final advantage of the toxicogenomics approach is its potential to provide insights into the molecular mechanisms of toxicity. PCR or microarray gene expression data can be linked to the Kyoto Encyclopaedia of Genes and Genomes (KEGG), which attempts to illustrate all known biochemical pathways and define their genetic control. This tool allows a toxicologist to determine which genes are being affected by the toxicant in a particular pathway of interest, and provide a framework for the evaluation of gene expression changes in multiple pathways.

7. Conclusions and future trends

The ever-increasing requirement for speed and reliability in analyses concerning food quality and safety has driven the development of new tests and assays based on molecular biological, and in particular, nucleic acid technologies. PCR-based approaches are becoming widespread in food analysis, substituting for and integrating with conventional approaches based on the immunological recognition of molecules, and chemical-physical and microbiological analyses [96] (Table 3). Among the various PCR approaches (Table 4), the tendency has been to move from monoplex to multiplex methods, and on to microarray

assays, which have a large capacity for multiple gene recognition and identification. An additional trend has been the shift from qualitative to quantitative assays, culminating in real time technologies.

The standardisation of PCR assays, with respect to methodology and target gene(s), will be easily accomplished. What still defies easy standardisation are the sampling and sample preparation steps, including DNA extraction and purification. Mistakes at these stages can often invalidate any analytical result. Thus there is still a requirement for research and development in these areas. In the meantime, case-by-case approaches will remain the rule.

Table 3. Comparison of the major groups of analytical approaches to toxicant identification

Technique	Advantages	Disadvantages
Culture and serological methods	Convenience Cost-effectiveness Reliability Reproducibility	No possibility of subtyping Labour-intensive Time-consuming No automation No detection of dead cells
ELISA-based tests	Speed Possibility for automation Cost-effectiveness High throughput	Limited possibility for subtyping
PCR-based tests	High discriminatory power Possibility for subtyping Robustness Reliability Reproducibility Standardization Fast assays Possibility for automation High accuracy Early detection Possibility for quantitative assays	Equipment requirements Reagent costs Highly trained personnel Indirect test Limited regulatory approval

Table 4. Advantages and disadvantages of PCR based approaches

Approach	Advantages	Disadvantages
Qualitative PCR assays	Inexpensive apparatus Possibility of multiplexing High specificity	Time/labour-consuming Requirement for post-PCR analysis, difficult to automate Requirement for enrichment procedures Possibility of false positive results
Real-Time PCR	Fast reactions Less opportunity for cross-contamination in post PCR processing High specificity and sensitivity Potential for automation Dynamic range of quantification	Expensive apparatus Difficult to multiplex
Microarray	Simultaneous detection of distinct PCR products Same sensitivity and specificity as PCR assays Avoidance of non specific reactions Identification based on multiple characteristics Possibility of multiple sample analysis	Expensive development Expensive apparatus Bias introduced by multiplex reactions for different targets No quantification

A requirement which will grow in importance is suitability for automation, which should bring analytical technologies to bear on-line, in-line or at-line along food chains at specific critical points [94]. The aim will be to deploy sensors (devices which respond to a specific physical or chemical change by producing a measurable output). Of particular interest are bio-sensors, which incorporate a biological recognition element, e.g. a nucleic acid or an

antibody, connected to a mechanism for data acquisition and processing. The miniaturisation of such a device increases the flexibility of the measurement. Microarrays represent a step towards the development of sensors for toxicant detection along food chains. The development of such devices is one of the main aims of the EU Framework 6 project TRACEBACK, in which micro-devices will be connected into an integrated traceability system.

Acknowledgements

The authors wish to acknowledge the technical assistance of Dr. Maria Cristina Samson Torres. Prof Marmiroli acknowledges the financial support of: EC projects CO-EXTRA, CHILLON and TRACEBACK; Ministry of University and Technological and Scientific Research project PRIN 2005 and FIL2005; NATO-CCMS project ECOTER. The authors thank www.smartenglish.co.uk for linguistic advice in the preparation of this manuscript.

References

[1] M. C. Edwards and R. A. Gibbs, in C. W. Dieffenbach and G. S. Dveksler (eds.), PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1995.
[2] N. Marmiroli, C. Peano and E. Maestri, In: M. Lees (ed.), Food Authenticity and Traceability, Woodhead Publishing, Cambridge (UK), 2003, pp. 3-33.
[3] J. S. Chamberlain, R.A. Gibbs, J.E. Ranier, P. N. Nguyen and C. T. Caskey, Nucleic Acids Res., 16 (1988) 11141.
[4] V. García-Cañas, R. González and A. Cifuentes, Tr. Anal. Chem., 23 (2004) 637.
[5] C. Peano, R. Bordoni, M. Gulli, A. Mezzelani, M. C. Samson, G. De Bellis and N. Marmiroli, Anal. Biochem., 346 (2005) 90.
[6] M. J. Loeffelholz, C. A. Lewinski, S. R. Silver, A. P. Purohit, S. A. Herman, D. A. Buonagurio, and E. A. Dragon, J. Clin. Microbiol., 30 (1992) 2847.
[7] S. Kumar, K. Balakrishna and H. V. Batra, Lett. Appl. Microbiol., 42 (2006) 149.
[8] M. Gilgen, P. Hubner, C. Hofelein, J. Luthy and U. Candrian, Res. Microbiol., 149 (1998) 145.
[9] R. Lindqvist, J. Appl. Microbiol., 86 (1999) 971.
[10] A. Rashtchian, PCR Methods Appl. 4 (1994) 83
[11] K. J. Schwab, F. H. Neill, F. Le Guyader, M. K. Estes and R. L. Atmar, Appl. Envir. Microbiol. 67 (2001) 742.
[12] F. Barany, PCR Methods Appl., 1 (1991) 5.
[13] P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, Proc Natl Acad Sci U.S.A., 88 (1991) 7276.
[14] K. J. Livak, S. J. Flood, J. Marmaro, W. Giusti and K. Deetz, PCR Methods Appl., 4 (1995) 357.
[15] R. Higuchi, G. Dollinger, P. S. Walsh and R. Griffith, Biotechnology (N.Y.), 10 (1992) 413.
[16] P. J. Sykes, S. H. Neoh, M. J. Brisco, E. Hughes, J. Condon, A. A. Morley, Biotechniques, 13 (1992) 444.
[17] P. Dahlen, A. Iitia, V. M. Mukkala, P. Hurskainen and M. Kwiatkowski, Mol. Cell. Probes, 5 (1991) 143.
[18] R. K. Saiki, P. S. Walsh, C. H. Levenson and H. A. Erlich, Proc. Natl. Acad. Sci. U.S.A., 86 (1989) 6230.

[19] N. DiDomenico, H. Link, R. Knobel, T. Caratsch, W. Weschler, Z. G. Loewy and M. Rosenstraus, Clin. Chem., 42 (1996) 1915.
[20] H. Rong, H. Ji, Y. Pernow, U. Sjostedt and E. Bucht, Clin. Chem., 43 (1997) 2268.
[21] W. Li and M. A. Drake, J. Food Protect., 66 (2003) 1277.
[22] I. Dahinden, M. von Büren and J. Lüthy, Eur. Food Res. Technol., 212 (2001) 228.
[23] H. Orum, P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt and C. Stanley, Nucl. Acids Res., 21 (1993) 5332.
[24] C. Peano, F. Lesignoli, M. Gulli, R. Corradini, M. C. Samson, R. Marchelli and N. Marmiroli, Anal. Biochem., 344 (2005) 174.
[25] M. Kubista, J. M. Andrade, M. Bengtsson, A. Forootan, J. Jona, K. Lind, R. Sindelka, R. Sjoback, B. Sjogreen, L. Strombom, A. Stahlberg and N. Zoric, Molec. Aspects Med., 27 (2006) 95.
[26] C. F. Terry, D. J. Shanahan, L. D. Ballam, N. Harris, D. G. McDonnell and H. C. Parkes, J. A.O.A.C. Int., 85 (2002) 938.
[27] M. Vaitilingom, H. Pijnenburg, F. Gendre and P. Brignon, J. Agric. Food Chem., 47 (1999) 5261.
[28] J. R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Publishing, New York, 1983.
[29] I. V. Kutyavin, I. A. Afonina, A. Mills, V. V. Gorn, E. A. Lukhtanov, E. S. Belousov, M. J. Singer, D.K. Walburger, S.G. Lokhov, A.A. Gall, R. Dempcy, M.W. Reed, R.B. Meyer and J. Hedgpeth, Nucl. Acids Res., 28 (2000) 655.
[30] F. M. De La Vega, D. Dailey, J. Ziegle, J. Williams, D. Madden and D. A. Gilbert, Biotechniques, Suppl S (2002) 48.
[31] V. V. Didenko, Biotechniques, 31 (2001) 1106.
[32] S. Tyagi and F. R. Kramer, Nat. Biotechnol., 14 (1996) 303.
[33] C. A. Heid, J. Stevens, K. J. Livak and P. M. Williams, Genome Res., 6 (1996) 986.
[34] S. H. Liming and A. A. Bhagwat, Int. J. Food Microbiol., 95 (2004) 177.
[35] N. Thelwell, S. Millington, A. Solinas, J. Booth and T. Brown, Nucl. Acids Res., 28 (2000) 3752.
[36] R. H. Madden, L. Moran and P. Scates, J. Appl. Microbiol., 84 (1998) 703.
[37] J. S. Ziegle, Y. Su, K. P. Corcoran, L. Nie, P. E. Mayrand, L. B. Hoff, L. J. McBride, M. N. Kronick and S. R. Diehl, Genomics, 14 (1992) 1026.
[38] A.-C. Syvänen, Nat. Genet., 37 (2005) S5.
[39] M. J. Heller, Annu. Rev. Biomed. Eng. 4 (2002) 129.
[40] S. J. Lee and S. Y. Lee, Appl. Microbiol. Biotechnol., 64 (2004) 289.
[41] P. H. Dear, in T. Weissensteiner, H. G. Griffin and A. Griffin (eds.), PCR Biotechnology. Current Innovations, CRC Press, Boca Raton, 2004.
[42] D. H. Bing, C. Boles, F. N. Rehman, M. Audeh, M. Belmarsh, B. Kelley and C. P. Adams, http://www.promega.com/geneticidproc/ussymp7proc/0726.html.
[43] S. Sauer, D. Lechner, K. Berlin, C. Plançon, A. Heuermann, H. Lehrach and I. G. Gut, Nucl. Acids Res. 28 (2000) e100
[44] A. Kurg, N. Tönisson, I. Georgiou, J. M. Shumaker, J. Tollett and A. Metsplau, Gen. Testing, 4 (2000) 1.
[45] P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kulper and M. Zabeau, Nucl. Acids Res., 23 (1995) 4407.
[46] C. Arnold, L. Metherell, G. Willshaw, A. Maggs and J. Stanley, J. Clin. Microbiol., 37 (1999) 1274.

[47] M. Besler, Tr. Anal. Chem., 20 (2001) 662.
[48] L. Herman, J. De Block and R. Viane, Int. J. Food Sci. Technol., 38 (2003) 633.
[49] T. Holzhauser, O. Stephan and S. Vieths, J. Agric. Food Chem., 50 (2002) 5808.
[50] A. Germini, E. Scaravelli, F. Lesignoli, S. Sforza, R. Corradini and R. Marchelli, Eur. Food Res. Technol., 220 (2005) 619.
[51] O. Stephan and S. Vieths, J. Agric. Food Chem., 52 (2004) 3754.
[52] H. Hird, J. Lloyd, R. Goodier, J. Brown and P. Reece, Eur. Food Res. Technol., 217 (2003) 265.
[53] T. Hirao, S. Imai, H. Sawada, N. Shiomi, S. Hachimura and H. Kato, Biosci. Biotechnol. Biochem., 69 (2005) 724.
[54] J. Varga, B. Toth and A. Mesterhazy, Cereal Res. Commun. 32 (2004) 193.
[55] K. Rudi, H. K. Nogva, B. Moen, H. Nissen, S. Bredholt, T. Møretrø, K. Naterstad and A. Holck, Int. J. Food Microbiol., 78 (2002) 171.
[56] G. Mule', M. T. Gonzalez-Jaen, L. Hornok, P. Nicholson and C. Waalwijk, Food. Addit. Contam. 22 (2005) 316.
[57] M. L. Niessen and R. F. Vogel, Syst. Appl. Microbiol., 21 (1998) 618.
[58] T. Demeke, R. M. Clear, S. K. Patrick and D. Gaba, Int. J. Food Microbiol., 103 (2005) 271.
[59] B. H. Bluhm, M. A. Cousin and C. P. Woloshuk, J. Food Prot., 67 (2004) 536.
[60] Z. Mayer, P. Faerber and R. Geisen, Appl. Environ. Microbiol., 69 (2003) 1154.
[61] H. K. Manonmani, S. Anand, A. Chandrashekar and E. R. Rati, Process Biochem. 40 (2005) 2859.
[62] P. Marek, T. Annamalai and K. Venkitanarayanan, Int. J. Food Microbiol., 89 (2003) 139.
[63] M. H. Pelegrinelli Fungaro, P. C. Vissotto, D. Sartori, L. A. Vilas-Boas, M. C. Furlaneto and M. H. Taniwaki, Curr. Microbiol., 49 (2004) 123.
[64] H. Schmidt, M. H. Taniwaki, R. F. Vogel and L. Niessen, J. Appl. Microbiol., 97 (2004) 899.
[65] R. L. Mach, C. M. Kullnig-Gradinger, A. H. Farnleitner, G. Reischer, A. Adler and C. P. Kubicek, Int. J. Food Microbiol., 95 (2004) 333.
[66] G. Mule', A. Susca, G. Stea and A. Moretti, Eur. J. Plant Pathol. 110 (2004) 495.
[67] G. Perrone, A. Susca, G. Stea and G. Mule', Eur. J. Plant Pathol., 110 (2004) 641.
[68] Z. Y. Yang, W. B. Shim, J. H. Kim, S. J. Park, S. J. Kang, B. S. Nam and D. H. Chung, J. Food Prot., 67 (2004) 2622.
[69] B. H. Bluhm, J. E. Flaherty, M. A. Cousin and C. P. Woloshuk, J. Food Prot., 65 (2002) 1955.
[70] P. A. Chapman, World J. Microbiol. Biotechnol., 16 (2000) 733.
[71] B. Glynn, S. Lahiff, M. Wernecke, T. Barry, T. J. Smith and M. Maher, Int. J. Dairy Technol., 59 (2006) 126.
[72] N. Sergeev, M. Distler, S. Courtney, S. F. Al-Khaldi, D. Volokhov, V. Chizhikov and A. Rasooly, Biosens. Bioelectron. 20 (2004) 684.
[73] B. Alarcón, B. Vicedo and R. Aznar, J. Appl. Microbiol., 100 (2006) 352.
[74] B. Pinto, E. Chenoll and R. Aznar, Syst. Appl. Microbiol. 28 (2005) 340.
[75] N. Sergeev, D. Volokhov, V. Chizhikov and A. Rasooly, J. Clin. Microbiol. 42 (2004) 2134.
[76] S. F. Al-Khaldi, D. Villanueva and V. Chizhikov, Int. J. Food Microbiol., 91 (2004) 289.
[77] D. R. Call, F. J. Brockman and D. P. Chandler, Int. J. Food Microbiol., 67 (2001) 71.
[78] M. Gauthier and B. W. Blais, J. Rapid Meth. Autom. Microbiol., 13 (2005) 243.
[79] C. Letertre, S. Perelle, F. Dilasser, P. Fach, Mol. Cell. Probes, 17 (2003) 227.
[80] C. F. Hsu, T. Y. Tsai and T. M. Pan, J. Clin. Microbiol. 43 (2005) 2668.
[81] J. Fitzmaurice, M. Glennon, G. Duffy, J. J. Sheridan, C. Carroll and M. Maher, Mol. Cell. Probes, 18 (2004) 123.

[82] S. L. Foley, S. Simjee, J. H. Meng, D. G. White, P. F. McDermott and S. H. Zhao, J. Food Prot. 67 (2004) 651.
[83] P. Fach, S. Perelle, J. Grout and F. Dilasser, J. Microbiol. Meth., 55 (2003) 383.
[84] D. P. Chandler, J. Brown, D. R. Call, S. Wunschel, J. W. Grate, D. A. Holman, L. Olson, M. S. Stottlemyre and C. J. Bruckner-Lea, Int. J. Food Microbiol., 70 (2001) 143.
[85] S.-Y. Yoon, G. T. Chung, D.-H. Kang, C. Ryu, C.-K. Yoo, W.-K. Seong, Microbiol. Immunol., 49 (2005) 505.
[86] D. D. Bang, B. Borck, E. M. Nielsen, F. Scheutz, K. Pedersen and M. Madsen, J. Food Prot., 67 (2004) 2171.
[87] D. P. Smith, M. E. Berrang, P. W. Feldner, R. W. Phillips and R. J. Meinersmann, J. Food. Prot., 67 (2004) 1770.
[88] M. Ehling-Schulz, M. Fricker and S.Scherer, FEMS Microbiol. Lett., 232 (2004) 189.
[89] C.-Y. Lee, G. Panicker and A. K. Bej, J. Microbiol. Meth., 53 (2003) 199.
[90] W. J. Lyon, Appl. Environ. Microbiol., 67 (2001) 4685.
[91] U. Gasanov, D. Hughes and P. M. Hansbro, FEMS Microbiol. Rev., 29 (2005) 851.
[92] A. Holst-Jensen, S. B. Rønning, A. Løvseth and K. G. Berdal, Anal. Bioanal. Chem. 375 (2003) 985.
[93] A. K. Deisingh and N. Badrie, Food Res. Int. 38 (2005) 639.
[94] M. Lees (ed.), Food Authenticity and Traceability, Woodhead Publishing, Cambridge (UK), 2003.
[95] R. Bordoni, A. Mezzelani, C. Consolandi, A. Frosini, E. Rizzi, B. Castiglioni, C. Salati, N. Marmiroli, R. Marchelli, L. Rossi Bernardi, C. Battaglia and G. De Bellis, J. Agric. Food Chem., 53 (2004) 1049.
[96] T. A. McMeekin (ed.), Detecting pathogens in food, Woodhead Publishing Limited, Cambridge, 2003.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 7

Analysis of food allergens. Practical applications

Arjon J. Van Hengel[a], Elke Anklam[a], Steve L. Taylor[b], and Sue L. Hefle[b]

[a]European Commission, DG Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, B-2440 Geel, Belgium

[b]Food Allergy Research & Resource Program, University of Nebraska, Lincoln, Nebraska USA 68583-0919

1. Introduction

Food allergy has emerged as a serious health problem and is estimated to affect up to 8 % of children and up to 2 % of the adult population [1, 2]. An increase in awareness of allergic diseases in industrialised countries has resulted in an increase in their perceived prevalence with 25 % of adults believing that their children are afflicted with a food allergy [3]. Food allergies develop most commonly in infancy but can often be outgrown. However, food allergies can also develop later in life.

Food allergens should not be categorized in the same manner as food toxicants. Food toxicants have the capacity to elicit adverse reactions in all consumers if sufficient doses are ingested. In contrast, food allergies are individualistic adverse reactions to foods [4] because such allergies affect only a small percentage of the population. Food allergies are characterised by adverse immunological reactions provoked by the uptake of a particular food. In contrast, other individualistic adverse reactions to foods also occur in the form of food intolerance that in principle lacks an immunological response, but is often caused by disorders of digestion, absorption or metabolism of food components.

Food allergies involve abnormal (heightened) immunological responses to specific components of certain foods [4]. Naturally occurring proteins occurring in certain foods are the typical instigators of allergic sensitization [5]. For the majority of consumers, food allergens serve as safe and nutritious proteins in the diet, while ingestion of the same food allergens by sensitized individuals provokes allergic reactions. Table 1 gives an overview of common clinical features triggered by the consumption of allergenic foods by sensitized individuals.

Two different types of abnormal immune responses can occur – immediate hypersensitivity and delayed hypersensitivity reactions [4]. Immediate hypersensitivity reactions involve abnormal responses of the humoral immune system with the formation of allergen-specific immunoglobulin E (IgE) antibodies [6]. These reactions are referred to as

immediate hypersensitivity reactions because symptoms ensue within minutes to a few hours after ingestion of the offending food. In contrast, symptoms develop more slowly after 24 or more hours following ingestion of the offending food in delayed hypersensitivity reactions. Delayed hypersensitivity reactions involve cell-mediated, typically T cell-mediated, immune reactions [4]. With the exception of celiac disease, which involves an abnormal response to wheat, rye, barley and related grains, the role of delayed hypersensitivity in adverse reactions to foods remains poorly understood.

Table 1. Overview of common clinical features caused by adverse reactions to food.

Organ	**Clinical features**
Skin	Atopic dermatitis Urticaria Angio-oedema
Gastrointestinal tract	Oral allergy syndrome Abdominal pain Colic, nausea Vomiting Diarrhoea Constipation Bloating Gastro-oesophageal reflux Enteropaties Failure to thrive
Respiratory tract	Asthma Rhinitis
Eyes	Conjuctivitis Watering eyes
Central nervous system	Headache Abnormal behaviour (ADHD)
Generalised	Anaphylaxis (with all it's complications)

Despite the existence of a multitude of allergenic foods, more than 90% of the severe food hypersensitivity responses in the U.S. were found to be caused by 8 foods or food groups: namely milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybean [7]. While these 8 foods or food groups are recognized as the most commonly allergenic foods in

the U.S., the European Commission has recognised three additional common food allergenic foods: celery, mustard and sesame seed. In Europe, a high prevalence of allergy against celery has been observed [8, 9] which is often associated with a primary sensitization against birch or mugwort pollen [10]. Sesame seeds are also recognized as commonly allergenic foods in Canada and Australia/New Zealand [4, 11]. Buckwheat is recognized as a commonly allergenic food in Japan and Korea [12]. Figure 1 gives an overview of the major food allergens as recognised in different parts of the world.

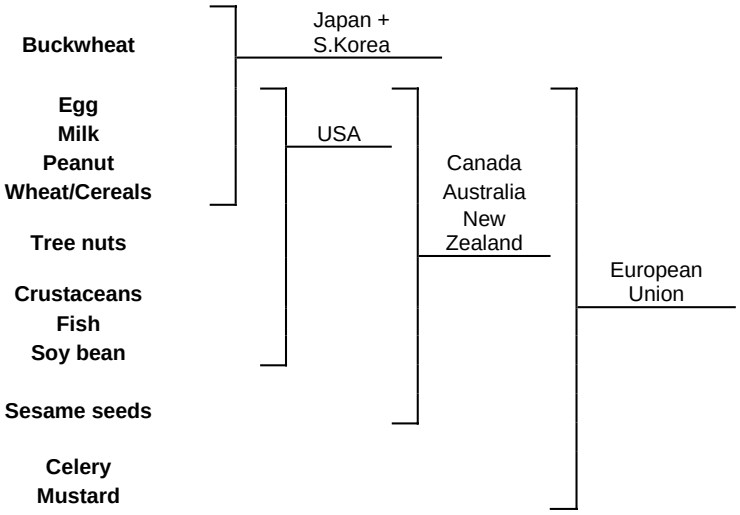


Figure 1. Major food allergens whose presence needs to be declared on the label of food products.

Several factors may be involved in the identification of additional allergenic foods in certain countries including the frequency of consumption of the particular food. However, many commonly consumed foods are not frequently allergenic apparently because they lack sufficient quantities of proteins able to provoke allergic sensitization. Currently more than 160 allergenic proteins have been identified in foods [13], with a number of allergenic foods containing several different allergenic proteins. Food allergens must have the capacity to provoke the immune system in a manner leading to hypersensitivity (complete allergen) or must be able to cross-react with antibodies elicited to other allergens (incomplete allergens). Many food allergens have the capacity to survive proteolytic digestion and arrive in the intestinal tract in immunologically intact form [14]. However, other food allergens interact with IgE antibodies in the oral mucosa provoking a generally mild form of food allergy

termed the oral allergy syndrome [15]. Although the vast majority of food allergens are proteins, only a few of the numerous proteins present in foods are known to be allergens [16].

Clinical reactions resulting from the ingestion of an allergenic food vary widely between individuals and may include hives, pruritus, atopic dermatitis, swelling of the throat or facial tissues, vomiting, diarrhoea, asthmatic wheeze, difficulty in breathing, and hypotension [17]. For some allergic individuals the ingestion of a certain food allergen can even provoke life-threatening reactions (severe anaphylaxis). It is estimated that in the U.S. about 30,000 anaphylactic reactions to food are treated in emergency departments and that food allergy results in 150-200 deaths each year [18].

Most severe anaphylactic reactions are caused by the ingestion of peanuts or tree nuts. The prevalence of peanut and tree nut allergies in the U.S. are estimated to be 0.6 % and 0.5%, respectively [19]. Inadvertent ingestion of peanut and tree nuts is responsible for most of the deaths attributed to food allergies [20, 21]. The intake of minute amounts of peanut (e.g. 200 μg) can be sufficient to trigger a mild, subjective allergic reaction, confirming its high allergenic potential [22, 23]. Given the incurable nature of food allergy and its potential life-threatening consequences, the management of food allergy concentrates on a strict avoidance diet that has to be implemented by food allergic individuals or their care givers [24]. The accurate and unambiguous labelling of food products is therefore of paramount importance. World-wide regulatory initiatives are aimed at the mandatory declaration of certain allergenic foods and ingredients derived from those foods. An example of this is Directive 2003/89/EC, issued by the European Commission [25]. This piece of legislation has resulted in a mandatory labelling of the eleven most commonly allergenic foods (plus sulphites) and ingredients derived from those foods. A similar labelling approach is now mandated in the U.S. with enactment of the Food Allergen Labelling & Consumer Protection Act of 2004. This demand for a declaration of allergenic ingredients has led to a variety of different types of labels appearing on food products. Such labels can declare the presence of allergenic ingredients or alternatively they can indicate that although the allergenic food has not been used as ingredient there is a chance that the food product contains the allergenic food unintentionally. Some examples of this are given in Figure 2.

The availability of accurate and sensitive detection methods for food allergens is crucial for the food industry to inspect and control their production processes, and to ensure the correct labelling of their products which can prevent costly product recalls. Those methods are also needed for government regulatory agencies in order to enforce legislation and for allergic consumers whose health is at risk.

The development of any detection method relies on the identification of a target analyte. In the case of food allergens this is not so straightforward. A particular allergenic food can often contain a number of proteins that can cause allergic reactions. However, patients that are allergic against this food often react to different protein constituents of the food. Furthermore, the detection of the allergen is not always feasible when its chemical properties

are not well characterised or the detection limit of the methodology used is insufficient. Currently multiple technical approaches are designed to detect the presence of allergenic foods used as ingredients in food products. Several methods do not target a specific allergenic protein, but rather a marker indicative for the presence of the offending food. Any component that is specific for the offending food can serve as a marker to detect its presence. In practice, proteins or DNA are targeted for this purpose. Protein-based methods usually involve immunological techniques that use either human antibodies like serum IgE of allergic patients, or antibodies raised in animals against purified allergens or allergenic food extracts (mixtures of proteins). DNA-based methods are based on the amplification of specific DNA fragments by means of the polymerase chain reaction (PCR) in which specificity is achieved by the use of primers that only facilitate amplification of DNA originating from the offending food.

Example biscuit No. 1
INGREDIENTS: wheat flour, butter (26 %), sugar, chocolate chips (10 %), salt
NOT SUITABLE FOR NUT ALLERGY SUFFERERS

Example biscuit No. 2
INGREDIENTS: wheat flour, sugar, vegetable oils and fats, roasted almonds (10 %), baking powder (E500, E336), salt, lecithin, spices
MANUFACTURED IN PRODUCTION HALL WHERE ALSO OTHER NUTS AND SESAME SEEDS WERE USED

Example dark chocolate
INGREDIENTS: cocoa mass, sugar, cocoa butter, butter fat, flavours, soy lecithin.
MAY CONTAIN TRACES OF NUTS, ALMONDS AND EGG

Figure 2. Examples of information found on the label of cookies.

The detection of allergens in food products can often be very difficult, since they are often present in trace amounts or are masked by the food matrix. Processing may also affect the detection of the allergenic food residues. Another question that remains to be answered is how sensitive the detection methods need to be, as there is a lack of data required to determine threshold levels [26]. In human oral challenge studies double-blind placebo controlled food challenges (DBPCFC) have shown that threshold levels range from 1 mg to 1 g of allergenic protein, depending on the food concerned and the sensitivity of selected allergic individuals [26]. There is a general agreement that the detection limits for food allergens need to be somewhere between 1 and 100 mg allergenic protein per kg food product [27, 28]. A further complication exists in the detection of allergens in ingredients derived from allergenic sources. In some cases, only a subset of the total proteins from the allergenic source is present; soy lecithin with its over-representation of lipid-soluble proteins would serve as an example. Obviously, methods can differ in their ability to detect allergens

remaining in such ingredients. In other cases, the allergenic proteins are hydrolyzed to varying degrees or subjected to fermentation. While residual allergenic activity may remain in partially hydrolyzed ingredients, the ability to detect the proteins is often dependent upon the existence of intact, unhydrolyzed protein. The reliability of PCR methods can be questioned in situations where DNA may be less stable than protein.

A review on a variety of methods for the determination of food allergens in food products has recently been published [28]. In the next section, we describe advantages and drawbacks of currently available methods for allergen detection.

2. Methods for the detection of allergens

2.1. RAST/EAST inhibition

RAST (radio-allergosorbent) or EAST (enzyme-allergosorbent) assays are designed for the detection of allergen-specific IgE. These *in vitro* assays are mainly used in the diagnosis of food allergy [29]. In addition to their clinical application, RAST and EAST inhibition tests have been used for allergen detection and for the assessment of the potential allergenicity of a wide range of foods [30–34]. RAST and EAST inhibition analyses are based on a competitive binding of human IgE. The principle of the methods is based on an allergen which is bound to a solid phase. This allergen functions as an antigen for specific human IgE which binds to it. This binding is inhibited by free antigen/allergen present in the sample solution. Subsequent detection of bound IgE by anti-IgE antibodies labelled with an isotope (RAST) e.g. ^{125}I , or an enzyme (EAST) e.g. horseradish peroxidase allows quantitative measurements of the allergen concentration in the sample. A limited number of publications that report the use of RAST inhibition for quantitative allergen determinations mention detection limits of around 1 mg per kg [35, 36].

RAST and EAST both rely on human sera from allergic patients. The limited availability of human serum, the variability of sera from individual patients, and difficulties in the standardization of such assays does not allow extensive use of these methods and has prevented commercialization of the applications [37].

2.2. Immunoblotting

One-dimensional sodium dodecyl sulphate (SDS) polyacryl gel electrophoresis (PAGE) followed by immunoblotting represents the standard procedure to separate proteins and identify allergens. Proteins in a test sample are denatured and given a strong negative charge by the SDS, subsequently they are separated based on their molecular weight, irrespective of the original electrochemical charge. After separation proteins are transferred from the gel onto a membrane. Antibodies labelled with an isotope or an enzyme are then used to detect individual allergenic proteins, either directly, or more likely indirectly when for instance

human IgE is used to bind allergens and labelled anti-IgE antibodies are employed to detect bound IgE. This method has allowed the detection, identification and characterization of a large number of individual allergens [38]. In addition to this SDS PAGE followed by immunoblotting can be used for a qualitative determination of potentially allergenic food contaminants down to a detection limit (LOD) of 5 mg/kg [39]. Like with RAST and EAST, this method often relies on the sera of patients which can be quite variable since allergic individuals do not always recognize the same allergen. However, after an allergen has been characterized, human IgE can be replaced by using antibodies raised in animals for immunoblotting procedures.

Dot immunoblotting allows a simpler and less expensive screening of food samples for the presence of food allergens. This method omits the separation of proteins in the sample, and the sample is directly spotted onto a membrane. The detection procedure is identical to the one described above. The intensity of the dot is proportional to the amount of antigen/allergen, which allows a semi quantitative detection of the target protein or mixture of proteins (e.g. peanut) in food with a LOD of 2.5 mg/kg [40]. Since this method does not include a separation of proteins, cross-reactivity of antibodies with food matrix components goes unobserved, which could lead to false positive results.

2.3. Rocket immuno-electrophoresis

Rocket immuno-electrophoresis, a technique using antibodies, can also be utilized to detect allergenic proteins. The antibodies are contained in a gel, while samples are migrated through this gel by means of electrophoresis. Antigen-antibody complexes will form in the gel resulting in rocket-shaped precipitates. The formation of such complexes will only take place at a constant antigen/antibody ratio. Therefore, the height of the rocket is proportional to the amount of antigen in the sample.

This method has been used for the detection of several allergens in various food products [41, 42] with LODs of 2.5 to 30 mg/kg. Rocket immuno-electrophoresis is not used widely because of the laborious gel preparation and immuno-staining procedures.

2.4. ELISA

Enzyme-linked immunosorbent assay (ELISA) is probably the method that is used most commonly by the food industry and official food control agencies. ELISA tests allow a determination of the presence of (hidden) allergenic proteins in food products which can be detected by a colorimetric reaction. A standard curve generated with the use of samples containing purified reference allergen of known concentrations allows (semi)quantification of allergens in food products.

Two types of ELISA systems are employed for the detection of food allergens, competitive ELISA and sandwich ELISA. The latter is the most common type of

immunoassay and utilizes a capture antibody immobilized on a solid phase, which is usually a microtiter plate or a multiple well strip. Specific (allergenic) proteins are captured by this antibody and detected by a second protein-specific antibody that is labelled with an enzyme capable of facilitating a colorimetric reaction. The concentration of the antigen/allergen is proportional to the colour intensity, which can be measured in a spectrophotometer (Figure 3). Sandwich ELISA methods have been developed for the detection of several different food allergens [36, 43-48] and numerous commercial test kits have become available during the last decade.

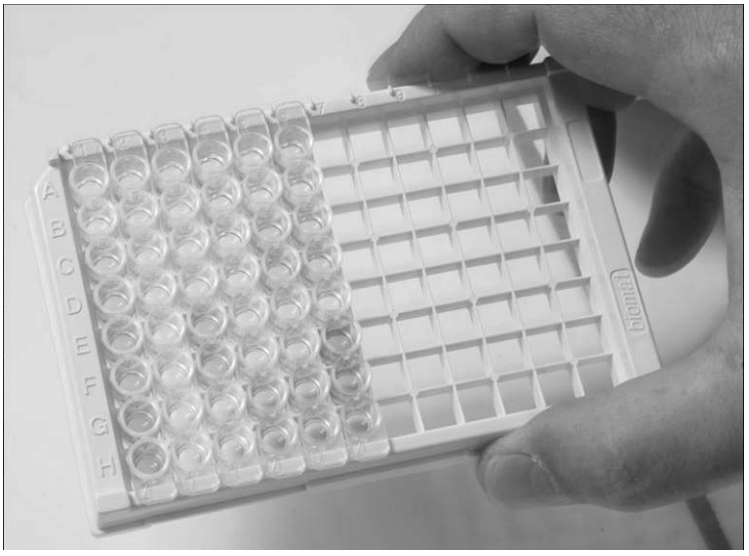


Figure 3. ELISA assay for the detection of peanut protein. The amount of peanut protein in the sample can be assessed by the colour reaction.

Competitive ELISA is often the preferred format for the detection of relatively small proteins. This type of ELISA utilizes immobilized antigen in contrast to immobilized antibody that is used in the sandwich ELISA. Antibodies are added to the sample extracts resulting in the formation of antibody-antigen complexes. The pre-incubated samples are then added to the solid phase with immobilized antigen. When the sample contains no antigen, an optimal binding will be observed, visualized by a high absorption of the coloured reaction product. Antigens present in the sample will inhibit this binding resulting in a decreased absorbance. Therefore the absorbance is inversely proportional to the concentration of antigen in the sample. Competitive ELISA systems have been described for the detection of

several food allergens [36, 48-53]. Competitive ELISAs for the detection of food allergens are also available commercially.

Recently the performance of commercially available ELISA test kit for the detection of peanut in food products has been analyzed in three different studies. Four ELISA kits were evaluated in a single laboratory [54], while two validation studies have been reported in which 3 [55] or 34 [56] laboratories participated to assess the performance of respectively 3 and 5 ELISA test kits. The precise measurement of peanut concentrations was found to be affected by variables like the type of test kit, the food matrix, the spiking method and the peanut material used to spike. Despite this, the validation studies have shown that all ELISA test kits used were capable of correctly identifying test samples containing 5 mg peanut per kg food matrix.

However, food processing methods might affect the detection of allergenic ingredients in food products. Koch et al. [57] have shown that the response of ELISA test kits for the detection of peanut decreases with increased roasting times, while the analysis of raw and roasted peanuts by human sera-based immunological methods was not found to show remarkable differences.

2.5. Dipsticks

Lateral flow immunochromatographic assays, commonly known as dipsticks can serve as extremely fast methods for the detection of food allergens. This method is also based on an immunological detection of proteins from an allergenic food. When those proteins are present in a test sample they are captured by specific antibodies that are conjugated to coloured particles. This mobile antibody-antigen complex flows along the test strip and is captured by a zone of antibodies specific for the antigen. The capture of the complex results in the development of a visible line on the test strip. Dipsticks are used as qualitative methods, although the intensity of the band is correlated to the concentration of antigen in the sample, suggesting a limited potential for (semi) quantification.

Despite the fact that their use is limited because of their inappropriateness for good quantification purposes, dipsticks have many advantages. The tests are inexpensive, rapid, portable, do not require instrumentation and are extremely simple to perform. A validation of 2 different types of commercially available dipsticks in which 18 laboratories participated has shown that the LOD of those products lies between 5-20 mg/kg [58]. Therefore the sensitivity of dipsticks is higher, but approaches, that achieved with ELISA test kits.

2.6. Biosensors

Biosensors provide a novel approach for the analysis of food products and the detection of food allergens [59]. Biosensor instruments allow the real time detection of compounds interacting with an immobilized target molecule. This molecule can be an antibody raised

against an allergen or a single stranded DNA molecule capable of hybridizing with an allergen-specific DNA fragment. Such molecules are attached to a sensor chip surface, and binding of sample compounds (allergens / DNA from allergenic foods) to the immobilized molecules can be detected and quantified by measuring changes in the refractive index. The advantages of this technology are the short analysis time and the high degree of automation [60]. The sensitivity of this method largely depends on the characteristics of the sensor chip. LODs in the range of 0.45 to 2.0 have been described for the detection of milk proteins in food products [61-62]. In addition to this, biosensors can be used to discriminate between intact and degraded protein/allergen [61, 63].

2.7. PCR

Polymerase Chain Reaction (PCR) -based methods are characterized by the amplification of stretches of DNA. The target DNA is generally species-specific and functions as a marker for the presence of a particular food ingredient. This technique is currently used for the detection of microbial pathogens [64], genetically modified crops in food products [65-66] and food allergens in food products [67].

At the basis of the method is a set of oligonucleotides or primers. Those primers can anneal to complementary single stranded DNA, which can be obtained by heat denaturation of normal double-stranded DNA. The enzyme polymerase can add extra nucleotides to the primer by using the genomic DNA as a template. Subsequent heat denaturation and annealing of the second primer to the newly synthesised single-strand DNA allows synthesis of a complementary strand of DNA. Several cycles of denaturation – annealing – extension result in the amplification of the target DNA fragment that is bordered by the primers used in the PCR reaction. The amplified product can be visualised by staining after gel electrophoresis. This can also provide information on the size of the amplified product, however it does not prove the identity of the PCR product. Southern blotting in which the amplified product is detected on the basis of hybridization to a labelled version of the target DNA provides a means for identification of the amplified product, while DNA sequencing allows a complete identification of such a PCR product.

In general PCR results are qualitative, but the inclusion of internal standards can provide semi-quantitative measurements [68-69]. Superior quantification can be achieved by PCR-ELISA or real-time PCR.

PCR-ELISA combines the high specificity of DNA amplification with the sensitive detection that is characteristic for ELISA. Target DNA is amplified by means of PCR. The newly synthesized DNA is labelled either by the use of a modified primer that will allow binding of the DNA to coated microtiter plates, or by the incorporation of labelled nucleotides (e.g. DIG labelled UTP). In the first case, binding of the DNA is followed by denaturation and subsequent hybridization with a labelled DNA probe. Enzyme-linked antibodies are then used to detect the target DNA (which is present as a complex of amplified target DNA –

labelled probe – enzyme-linked antibody). When labelled nucleotides are incorporated during PCR, the DNA can be bound to a solid phase and enzyme-linked antibodies capable of binding the labelled nucleotides are then used for detection. The colour development that is driven by the enzymatic reaction can be measured and provides a way for semi-quantification of the target DNA. This method combines the advantages of PCR and ELISA, but the combination of the two techniques makes it also more laborious and time-consuming.

Another PCR-based method is provided by real-time PCR. This technique requires expensive laboratory equipment, but it provides an accurate method for quantification of the target DNA [70]. In contrast to conventional PCR, real-time PCR does not require post-PCR detection of the amplified product by means of gel electrophoresis. Instead it utilizes detection 'in real time'. For this purpose, the tube in which the PCR reaction takes place also contains a target-specific oligonucleotide probe with a fluorescent reporter dye and a quencher attached to it. The proximity of the quencher to the dye prevents the detection of fluorescence, but, when the probe hybridizes to the amplified target DNA, the 5' exonuclease activity of the polymerase cleaves the probe and thereby separates the quencher from the dye which is displaced by the newly synthesised DNA strand. The fluorescence of the free reporter dye can then be measured and the increase in fluorescence is proportional to the amount of target DNA (Figure 4). The sigmoid curve obtained when the fluorescence level is plotted against the number of amplification cycles is used to quantify the target DNA present in the sample [67].

DNA-based methods offer both advantages and disadvantages by comparison to protein-based methods. Where the concentration of an allergenic protein within the allergenic food depends on the species/variety and the growth conditions, the DNA level is stable. Food processing also affects the stability of proteins and DNA in different ways. In addition to this, the extraction efficiency of DNA or proteins from a food matrix is likely to differ. Therefore DNA-based methods supplement protein-based methods. The applicability of a DNA-based method does depend on the food allergen that is to be detected. Some allergenic foods have a very low DNA content versus a high protein content (e.g. eggs) and are therefore not suited for detection by means of PCR. Of course, the allergens in foods are proteins so the protein-based methods more directly measure the allergens by comparison to PCR.

Currently there are several DNA-based methods for the detection of food allergens on the market. However, the robustness of those methods remains to be proven in proper method validation trials. The sensitivity and specificity of some commercially available DNA-based methods for the detection of food allergens were found to be too low, which prevented their validation in an interlaboratory study so far (van Hengel and Anklam unpublished results).

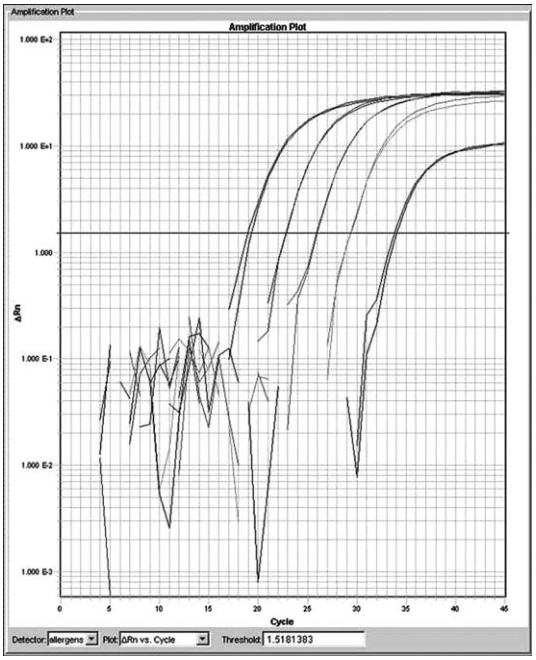


Figure 4. Real-time PCR assay for the detection of peanut protein. The amount of peanut protein in the sample can be assessed by the number of amplification cycles.

2.8. Cell response factor release assay

In vitro basophil activation assays can be used as a method for confirmation of the identification of allergens and for allergy screening. This method attempts to mimic the mechanism of anaphylaxis and to replace animal models. Degranulation of basophils is measured by the release of 'allergic mediators' (histamine, leukotriene etc). The development of basophil degranulation is assessed by flow cytometry methods that can quantify the presence of cell surface-bound allergen-specific IgE antibodies as well as the degranulation induced after incubation with the allergenic product via a marker of degranulation (e.g. CD63). This allows the confirmation of the allergenicity of the test sample. The concentration of a specific allergen in a sample can also be measured using this assay.

Mucosal mast cells are also used to measure the allergenic potential of food. In this type of assay the release of β-N-acetylhexosaminidase has been employed for the quantification of allergenic proteins from soybean in various food products [71-72].

A third cell-based method for the analysis of food allergens is provided by dendritic cell maturation assays. Immature dendritic cells obtained from human or animal donors are cultured *in vitro* and exposed to allergens. This can induce maturation of dendritic cells which can be analysed by chemokine secretion or flowcytometric detection of cell surface markers. Purified wheat gluten has been shown to cause dendritic cell maturation in a dose-dependent manner over the range of 1-100 μg/ml [73].

Cell-based methods suffer from reproducibility problems and the natural variability of the immunological responses of individual donors often prevents standardization of these types of methods. Unfortunately, the limited availability of blood from (allergic) donors and its restricted life time prevent cell-based methods from playing an important role in the detection of food allergens.

2.9. Proteomics

The proteome is the collection of all protein components present in a complex system. Whereas the genome of living organisms is stable, variable levels of gene transcription result in a higher complexity of the transcriptome. A much higher degree of complexity is observed for the proteome. This is caused by variation in the relative amounts of individual proteins as well as post transcriptional processes like protein modification and association with other proteins or molecules of different types.

Proteomic techniques that allow the study of the proteome are now applied for the detection of nutritionally relevant proteins and proteomics is expected to have a major impact on nutritional science [74]. Most food allergens are components of a proteome and since they are usually glycoproteins, post translational processing like glycosylation does affect the allergen proteome.

Proteomic research is usually based on two components, the first being a separation of proteins and the second an identification of individual proteins. Many improvements in separation and identification of proteins, such as two-dimensional electrophoresis, nano-liquid chromatography and mass spectrometry, have rapidly been achieved [75]. These techniques have enabled a high throughput analysis of complex protein mixtures.

Despite the need for costly specialized equipment, this method has the potential to provide an unambiguous identification of allergenic proteins present in a food product. This is a major advantage compared to methods based on immunological techniques (indirect identification which might be hampered by cross reactivity of antibodies) or DNA based methods (identification of DNA markers instead of allergens).

At present proteomic research is mainly focused on the identification of allergens [76], but it is expected to be used to study allergen modifications and allergen detection in food products. No clear data on the limit of detection of allergens are currently available.

3. A selection of food allergens and their detection

In this section we will give a more detailed description of a selection of several major food allergens, and the variety of methods that have been employed for their detection in food products. The food allergens described below are all recognised as major food allergens and their presence in food products needs to be declared on food labels in the European Union, as well as North America, Australia and New Zealand.

3.1. Peanuts

3.1.1. Characteristics of peanut allergy

Peanuts are the most allergenic food known, and in sensitive individuals, can cause adverse reactions ranging from mild urticaria to life-threatening anaphylaxis [77-79]. The number of people allergic to peanuts and tree nuts in the U.S. has been estimated at 0.6% [19] and 0.48% in the U.K [80]. While the threshold level necessary to cause a reaction is not known with confidence, minute amounts may trigger anaphylaxis. Most studies to date have shown that low mg amounts (1-3 mg) of peanut protein are sufficient to cause objective reactions in peanut-allergic subjects [26, 81].

3.1.2. Peanut allergenic proteins

Peanuts contain an average of 29% protein. Approximately 20% of the total protein can be attributed to the major allergen Ara h 1 (vicilin-like protein, molecular weight 63-64 kD), and about 10% to another major allergen Ara h 2 (conglutin-like protein, molecular weight of about 17 kD) [82-83]. Other allergens in peanut include Ara h 3 and 4 (glycinin proteins) [84-86], Ara h 5 (profilin), 6 and 7 (conglutin-like proteins) [86], and Ara h 8 [87] There are several excellent overviews on peanut allergens that the reader can consult on peanut allergens [82, 88].

3.1.3. Peanut detection methods

IgE-based detection methods

Rocket immunoelectrophoresis (RIE) was first used by Barnett and Howden in 1984 [89] to detect heat-treated peanut protein, and this marked the first time an immunological method was used to detect peanut protein. Rocket immunoelectrophoresis was also used by Malmheden-Yman et al. [90] to detect the undeclared presence of peanuts in a small number of food products. The detection limit of the method was approximately 30 ppm.. Holzhauser et al. [91] a few years later used a commercially available anti-raw peanut antibody in

developing a RIE method and were able to obtain a 10 ppm detection limit. One disadvantage of this technique was that the amount of antiserum used in the gel has to be optimized; this is dependent upon whether or not the sample contains a large or small amount of peanut, details of which are seldom known in advance.

The first report of Radioallergosorbent assay (RAST) inhibition being used to detect peanut contamination of a non-peanut-containing food was described by Yunginger et al. in 1983 [92]. The technique was used to document the presence of peanut butter in sunflower butter (likely made on shared production lines) in investigating a reaction in a peanut-allergic consumer. This method was expanded upon by Keating et al. in 1990 [93]. In contrast to the work discussed above, a roasted defatted peanut meal was used for the solid phase and standards in a RAST inhibition method. The detection limit was reported to be 87.5 ppm peanut; most experts agree at this time that allergen detection methods should have an ability to detect down to at least 10 ppm.

An IgE-dot-immunoblotting method using peanut-allergic sera was described by Schappi et al. [94] to detect the presence of peanut proteins in foods. The standards were made from roasted peanuts. The detection limit was approximately 50 ppm, too high for practical allergen management.

While IgE-based techniques such as RAST and EAST inhibition and IgE-immunoblotting are useful technique that can be used in many clinical and research settings, it must be stressed that they require well-characterized peanut-allergic human sera, a substance which needs special handling, and the use of this reagent is precluded in food processing environments.

Immunoassays

The first ELISA method for detection of peanut residues was described by Hefle et al. in 1994 [44]. The method used monoclonal antibodies against selected peanut proteins with molecular weights (in SDS-PAGE) of 14-44 kD as capture antibody and a polyclonal anti-peanut rabbit antibody as detector. The rabbit antibody was raised against a crude roasted peanut extract. The method could detect <40 ppm peanut in most food matrices. This new method opened the door for other ELISA formats with increased sensitivity [95].

In 1996, both Yeung and Collins [50] and Koppelman et al. [27] published ELISA methods for detection of peanut proteins in foods. Yeung and Collins developed a competitive format ELISA using a rabbit polyclonal antibody raised against a mixture of defatted roasted, raw, denatured, and unfolded raw peanut. This ELISA method reportedly had a detection limit of 400 ppb. Koppelman et al. [27] described a sandwich-type ELISA using rabbit polyclonal antibodies against partially purified Ara h 1 derived from raw peanuts. This sandwich ELISA utilized the same antibody for detector and capture; part of the antibody was labelled with

horseradish peroxidase to be the detector antibody. The assay was capable of measuring raw or roasted peanut, and the reported detection limit was 100 ppb.

Much later, Pomes et al. [96] published another sandwich ELISA based on monoclonal antibody against Ara h 1. In this study, Ara h 1 could not always be recovered in spiked chocolate samples nor always detected in the presence of chocolate. However, it was found that in general, Ara h 1 content correlated with a commercial ELISA method, but the results were not compared to any other commercial ELISA kits. Newsome and Abbott [97] coupled an immunoaffinity column to capture peanut proteins from foods, with a subsequent ELISA. The antiserum used was raised against roasted peanuts. The antiserum was coupled to a commercial gel preparation and then the ELISA was performed according to the protocol of Yeung and Collins [50]. The detection limit was 0.1 ppm.

Holzhauser and Vieths [52] also developed an indirect ELISA method using commercial anti-raw peanut rabbit antisera (absorbed to remove possible soy reactivity) and ground roasted peanuts as a standard. This method was reported to have a detection limit of 2 ppm detection limit. Later the same research group [98] reported both a sandwich ELISA and PCR method for detection of peanut residues. Both sheep and rabbit anti-peanut antibodies were used in the development of the ELISA.

A cloth-based immunoassay was described by Blais and Phillippe [99]. The method utilized polyester cloth as the solid phase and the capture and detector (peroxidase-labelled) antibody was chicken anti-peanut IgY. Peanut immunogen prepared as according to Yeung and Collins [50] was used to immunize chickens. The authors felt that the cloth solid phase provided advantages of conferring a high surface area for rapid immunoreactions and ease of washing between steps. Food samples connected with consumer illnesses were analyzed using the cloth-ELISA; of 11 samples not declaring peanut, 9 contained peanut residues and were positive in the cloth-ELISA. Levels were reported to be 1.2 – 116 ppm (determined using the Yeung and Collins [50] quantitative ELISA method). The authors reported that the detection limit of the cloth-ELISA was less than 1 ppm peanut protein..

The first peanut dipstick method described in 1997 by Mills et al. [100]. Dipstick or lateral flow methods dipsticks are only semi quantitative or qualitative in nature. In this study, polyclonal rabbit antisera were raised to conarachin, the 7S globulin of peanut, which had been isolated from defatted raw peanuts. The detection limit was reported as being 100-1000 ppm depending on matrix; this is much too high for allergen concerns.

Stephan et al. [101] developed a dipstick/lateral flow method for the detection of trace amounts of peanut and hazelnut in foods. The reported detection limit was 1 ppm protein. Dipsticks are a useful tool for rapid and convenient testing of food and environmental samples. There are two commercial lateral flow/dipstick devices on the market for peanut, made by Neogen (Reveal) and Tepnel (Biokits Rapid Peanut test), and other diagnostic kit companies are planning to produce kits in this type of format also.

MS-based technologies

A publication by Shefcheck and Musser [102]describes the detection/confirmation of Ara h 1 in a food matrix using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method. This technique provides confirmation of allergenic proteins in food systems as a confirmatory test for immunoassays, which has been identified by some regulatory agencies as a significant need. It is likely best suited to regulatory agencies or academic labs rather than food manufacturers. It is felt that these types of methods could possibly have detection limits approaching 1 ppm protein.

PCR Methods

In addition to protein/immunochemical methods, some PCR methods for peanut have been described. Two types of formats are currently commercially available; real-time PCR and DNA-ELISA. Reported detection limits are below 10 ppm. Hird et al. [103] published a real-time PCR method for detection of peanut residues. The amplification consisted of a part of the DNA encoding Ara h 2, a major peanut allergen. The investigators reported that the method could detect 2 ppm lightly roasted peanut flour spiked into cookies. Stephan and Vieths [98], as noted above, also developed a PCR method for the detection of peanut. A commercial DNA extraction method was used and the primer was also specific for a coding region on Ara h 2. This real-time PCR method was able to detect down to 10 ppm peanut.

3.1.4. Comparisons/validation of commercial peanut detection kits

Hurst et al. [104] compared commercial test kits for peanut on samples of dark and milk chocolate. Dark and milk chocolate samples were made in the levels of 0, 10, 40, and 200 ppm peanut, although details of how this was done and how homogeneity was determined were not disclosed. Four commercial test kits for the detection of peanut (Neogen Veratox®, Pro-Lab Prolisa, Tepnel Biokit®, and r-Biopharm RIDASCREEN®) were evaluated using the chocolate samples. In general, the kits varied in their ability to quantitate the levels of spiked peanut. However, with no details given on the nature of the peanut material used to make the spiked chocolate, etc., it is difficult to truly compare one kit to another. In addition, refinements have been made to most of these kits since this work was published.

In another study, Koch et al. [57] compared spiked samples using some commercial peanut ELISA kits and IgE-immunoblotting using peanut-allergic sera. Samples of peanut-free cookie matrix were spiked with various levels of ground raw or roasted peanuts. The protein extractions for blotting were done with urea, a powerful disrupting agent. However, when samples were analyzed in the commercial ELISAs, manufacturer instructions were followed, and none of these include urea in their extraction buffer. Dot blotting was used to estimate the amount of peanut in the samples, whereas IgE-immunoblotting was used to analyze the

extracts further and identify specific peanut allergens. The levels of peanut spiked ranged from 500 ppm – 2500 ppm, well above allergen concern levels. In addition, extracts of spiked samples were diluted in extraction buffers to 10 ppm in the comparison of the three kits; it must be pointed out that this is not how the kits are designed to be used. The authors made some assumptions that test kit companies used raw peanuts as immunogen to explain their results showing that raw peanuts were detected at higher levels, but in fact some kit manufacturers use roasted peanuts as immunogen for producing their antibodies. The authors also concluded that roasted peanuts were less detectable than raw peanuts in commercial ELISA kits, but only used one of the three commercial kits to test this hypothesis. A direct comparison of IgE-blotting using urea extraction and use of commercial test kits (with their non-urea extraction methods) casts some doubt on the validity of some of the conclusions made in this study.

Recently the performance of commercially available ELISA test kits for the detection of peanut protein in food matrices has been analysed by several laboratories. Whitaker et al. [54] reported the results of a single laboratory study in which 4 ELISA kits were evaluated for the detection of peanut proteins at 4 levels of peanut contamination (including a negative matrix control), in 4 food matrices (breakfast cereal, milk chocolate, ice cream and cookies). A multiple laboratory validation study in which 3 ELISA test kits were validated with 3 laboratories that analysed 4 different matrices (cereal, milk chocolate, ice cream and cookies) has been reported by Park et al. [55]. In the latter study the focus was placed on the qualitative analysis of samples containing 0 and 5 mg peanut per kg food matrix. Poms et al. [56] have reported an inter-laboratory validation study of 5 ELISA test kits, in which 34 laboratories analysed 2 food matrices (biscuit and dark chocolate) for the detection of peanut proteins at 4 levels of peanut contamination (including a negative matrix control). A direct comparison of the above mentioned studies is hampered by the differences in the experimental design. In the three studies the peanut-containing food matrices were prepared in different ways, one study used defatted peanut flour to spike food matrices [54], while another one used peanut butter to spike [55] and in the third one peanut flour was added to biscuit dough prior to baking [56]. The precise measurement of peanut concentrations in food is known to be affected by variables like the type of test kit, the food matrix, the spiking method and the peanut material used to spike or contaminate the food matrix. Despite the differences in experimental design, two international validation studies [55-56] have shown that all ELISA test kits used in these studies correctly identified test samples containing 5 mg peanut per kg food matrix.

An interlaboratory validation study of two commercially available dipsticks was recently reported by van Hengel et al. [58]. In this study 18 laboratories analysed cookies containing peanut at 7 different concentrations. The results showed that the both dipstick test kits are capable of correctly identifying test samples containing peanut at levels of 21 mg/kg or more.

3.2. Milk and dairy products

3.2.1. Characteristics of milk allergy

Allergy to cow's milk (CMA) can be considered one of the most common food allergies especially in early childhood with an incidence of 2-3% in the first year. Different food products could be responsible for this type of allergy since cow's milk (CM) proteins are used as processing aids and therefore a large number of food products may contain residual amounts of CM protein. Breast milk from mothers who have eaten products containing CM might be another threat for the development of CMA due to the absorption of cow proteins, their passage through the gut mucosa and their release in human milk [105]. The exposure to food proteins, especially CM proteins during the neonatal period can also trigger some clinical and immunological effects correlated to immaturities of the immune and "digestive" systems of the newborn [106]. Factors such as low stomach pepsin activity at birth, an immature stomach acid generating mechanism (stomach pH is ~3.5) and malfunction of pancreatic and intestinal enzymes contribute to the stability of CM proteins (α-lactoglobulin and β-lactalbumin) by limiting their gastric proteolysis and therefore expose neonates to "allergic" responses [107].

Symptoms of CMA and adverse reactions to CM proteins are usually attributed to CMA or CM intolerance (CMI) because there is no single reliable laboratory test available for the diagnosis of one of these just on the basis of symptoms [108]. From an immunological point of view, CMA is a IgE-mediated reaction to CM and may induce cutaneous (atopic dermatitis, urticaria, angioedema), respiratory (rhinitis, asthma, cough) and gastrointestinal (vomiting, diarrhoea, colic, gastroesophageal reflux) reactions, and in some extreme cases even systemic anaphylaxis. CMI should instead refer to a non-immunological reaction to CM such as disorders of digestion, absorption or metabolism of certain CM components, like lactose [109-111].

3.2.2. Milk allergenic proteins

CM contains 3-3.5% protein which can be divided into two main classes: caseins (80%) and whey proteins (20%). The latter group of milk proteins remains soluble in milk serum after acidic precipitation of caseins at pH 4.6 that forms the coagulum [112]. CM contains many proteins that are considered antigenic and capable of inducing immune responses and sensitivity to different CM proteins has proven to be widely distributed. Studies carried out on large populations of allergic patients have indicated that the most abundant proteins in CM especially lactoglobulins (β-LG), caseins (CN) and lactalbumin (ALA) are the major allergens; however also proteins that are present in low quantities such as bovine serum albumin (BSA), lactoferrin (LF) and immunoglobulins (Ig) have shown to be of importance in inducing milk allergies.

In conclusion, there is not just a single protein responsible for CMA. All milk proteins, encompassing whey proteins as well as CNs, can be considered potentially allergenic. A review on milk allergens, their hydrolysates and methods for their detection has recently been published [113].

3.2.3. Milk detection methods

In comparison to other allergens, CM allergens have been studied well employing a variety of different analytical approaches that are mostly based on separation and characterisation techniques. The two most popular methods for protein separation are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid phase separation which is becoming a more common method in proteomic studies [114]. A relatively new approach in proteomic analysis is the multidimensional liquid chromatography combined to mass spectrometric detection (LC-MS) which has been successfully used in many applications. Fast methods based on immunochemical detection protocols such as radio-allergosorbent tests (RAST), enzyme allergosorbent tests (EAST), rocket immuno-electrophoresis (RIE), immunoblotting and enzyme linked immunosorbent assays (ELISA) are available to check the presence of potential allergenic proteins [28].

The need to monitor and verify the presence of trace amounts of protein allergens in milk and dairy products has generated a demand for analytical methods capable of detecting, identifying and quantifying proteins at the lowest mg/kg levels. Here we will list a number of analytical methods that are currently available to monitor the presence of milk allergens in food products. Unfortunately all those methods remain to be validated in order to provide relevant information and confidence in measurements concerning the presence and the quantification of an offending allergenic food component in a product.

Immunoassays

The majority of immunoassay methods use the ELISA format for the detection of milk allergens. Both sandwich and competitive ELISA are available for the semi-quantification of milk proteins. Purified anti-β-LG was used for the development of a very sensitive sandwich type ELISA for the determination of very low levels of CM β-LG in infant formulae and human milk with a limit of detection around 0.002 ng/ml [115]. Monoclonal and polyclonal antibodies were also raised against casein components of CM with the aim to devise an ELISA method for the evaluation of residual antigenic activity in hypoallergenic infant formulae [116-117]. More recently a sandwich-type ELISA has been developed for the detection of undeclared casein in different non-milk-containing products such as juices, sorbets and chocolate. The present assay offers an appropriate detection limit (less than 5 mg/kg) suitable for the protection of milk-allergic consumers [118].

Fast biosensor immunoassays with a good sensitivity have also been developed for the detection of residual immunogenicity of food products [119]. These techniques are reported to

be fast, repeatable, fully automated and able to discriminate between intact and degraded protein while little or no pretreatment of the sample is required [120-121].

Recently the development of an immunoprobe made of polyclonal antisera raised against the whey proteins ALA and β-LG A and B has been described. This immunoprobe can be used for the analysis of thermally-treated milks. The limit of detection was 13 ng/ml for ALA, 27 ng/ml and 20 ng/ml for β-LG A and B respectively [122]. Monaci et al. [113] have reported an inventarisation of immunoassays, and various commercial ELISA test kits that have entered the market.

Capillary electrophoresis

In addition to immunoassays, the potential of capillary electrophoresis for the rapid separation of milk proteins has been widely demonstrated in recent years [123]. Different CE methods have been developed for the analysis of CNs and whey proteins in milk [124-127] milk powders [128] and cheese, mostly aimed at the characterisation of bovine milk in non-bovine and mixed cheeses [129-131]. A CE method for the determination of bovine whey proteins in soybean dairy-like products has been also described with detection limits for ALA and β-LG of 0.6 and 1.0 μg/g, respectively [132]. New improvements in capillary electrophoresis techniques have been introduced by the use of fluorescence detection and capillary derivatisation. This allows a quantitative analysis of whey proteins at nanomolar levels as well as the detection of β-LG traces in hypoallergenic formulae [133-134].

MS-based technologies

The recent advances in soft ionisation techniques such as electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) has made it possible to use this kind of mass spectrometers for the analysis of proteins with a high sensitivity. The last two approaches have quickly become important tools for the detection and characterisation of large biomolecules. For instance, ESI mass spectrometry (MS) has been demonstrated to be a powerful tool for the characterisation of CM proteins providing spectra of multiply charged ions from which information about the molecular weight can be obtained by using a deconvolution algorithm [135-136].

Liquid chromatography combined with MS detection (LC-MS) is also a valid and accurate analytical technique for the identification of peptides, and it has been mostly exploited for the rapid identification of interesting compounds present in a complex food matrix such as biologically active peptides derived from a hydrolysate or a fermented product [137]. However, the interpretation of mass spectra can be difficult when a complex mixture or a complicated food matrix is analysed, due to limitations in the detection of the ions formed as well as formation of adducts affecting the m/z parameter which can occur. Despite this, LC-MS generally offers a good signal-to-noise ratio and a rapid mass determination is feasible

without the need of purification steps. Liquid chromatography techniques can be combined with tandem mass spectrometry (MS-MS) for enhancing the capacity of ESI to elucidate the structure of proteins by sequence assignments from peptide mapping.

In order to improve the resolution and mass accuracy, recent instrumental improvements of MALDI-TOF have been introduced such as time-lag focusing (delayed extraction mode) and reflectron, resulting in a gain in both resolution and accuracy. A mass resolving power better than 10000 and mass accuracy below 5 mg/kg could be achieved using this technology [138]. Nonetheless MALDI-TOF suffers from restrictions in detecting low molecular mass proteins which deliver few peptides [139]. For this purpose the application of MS/MS technologies like TOF-TOF, Qq-TOF could provide further advantages.

The above discussed studies prove the potential of MALDI-TOF-MS, ESI-MS and ESI-MS/MS techniques as valid tools for mass determination purposes, which can be employed for the detection of allergenic milk proteins and peptides in food products.

3.3. Eggs and egg products

3.3.1. Characteristics of egg allergy

Egg allergy is one of the most frequent food allergies together with milk and peanut [140-145]. Eggesbo et al. [141] reported that in a population of over 3000 children at 2.5 years of age 2.6 % ± 1 % of were diagnosed with egg allergy. This prevalence was found to be similar to what has been reported for adults. Egg sensitisation in one year old children has been found to be highly predictive for the development of asthma in later life [146].

The clinical symptoms caused by henns' eggs in sensitised individuals are similar to those reported for milk and other allergenic foods.

Just like cow's milk (CM) proteins, egg proteins are used as processing aids and therefore a large number of food products may contain residual amounts of egg protein. Egg lysozyme is often used in the preparation of medications as well as in the preparation of a large variety of food products since it can prevent the growth of anaerobic bacteria. Another egg product, egg lecithin is used by the food industry as an emulsifier.

3.3.2. Egg allergens

Several egg proteins have been characterised as being capable of triggering an allergic reaction in sensitised individuals. These include Gal d 1 (ovomucoid), Gal d 2 (ovalbumin), Gal d 3 (conalbumin), Gal d 4 (lysozyme) and Gal d 5 (serum albumin). All of those are components of the egg white. Recently Walsh et al. [147] have reported an investigation into IgE binding to purified egg white and egg yolk proteins using RAST. This study demonstrated that egg allergic patient IgE also reacts to the yolk proteins apovitellenins I and VI and phosvitin.

3.3.3. Egg detection methods

A variety of methods have been employed for the detection of henn's egg in food products. Since the protein content of eggs is rather high and the DNA content is low all methods that are currently employed to detect (traces of) egg in food products concentrate on the detection of egg specific proteins. Here we will list a number of analytical methods that are currently available to monitor the presence of egg in food products. A proper validation by means of international collaborative trials is still lacking, but recently a working group consisting of representatives of the Food and Drug Administration (FDA), Health Canada, Food Allergy Research and Resource Program University of Nebraska (FARRP), Food Products Association (FPA), and the European Commission's Institute for Reference Materials and Measurements (EC - JRC - IRMM) has initiated the process to undertake such a validation study of test kits for the detection of egg.

Immunoassays

Egg has been detected by means of RIE in a variety of food products including processed meat products, noodles, chocolate and cake with a detection limit of 30 mg/kg [41].

An ELISA utilizing rabbit anti-henn's egg white antibodies or human IgE sera was compared to immunoblotting by Leduc et al. [148]. The ELISA assays showed higher sensitivities than immunoblotting and the authors reported sensitivities ranging between 0.03 % for raw and pasteurized egg and 0.125 % for sterilized egg products.

An ELISA test with a higher sensitivity was reported by Yeung et al. [149]. This method utilizes polyclonal antibodies specific to whole egg proteins. This method was used to analyse ice cream, noodles, pasta and bread and was shown to have a detection limit of 0.2 mg/kg. Hefle et al. [150] developed a sandwich ELISA that utilizes anti-egg white antibodies (to capture) as well as anti-ovalbumin antibodies (to detect). When tested on various pasta products a detection limit of 1 mg/kg was determined for this ELISA method. Recently Watanabe et al. [151] addressed the problem of the low recovery of egg proteins in extracts of food products containing heat denatured egg, and reported an ELISA that employs an extraction buffer containing a surfactant and a reducing agent. This extraction buffer was shown to improve recovery of heat denatured egg white albumin 10- to 100-fold.

The development of a lateral flow device or dipstick was reported by Baumgartner et al. [152]. This rapid immuno assay that employs polyclonal antibodies specific to egg proteins was used to analyse cookies and garlic dressing spiked with egg standards, whereby below concentrations of 2 μg/kg no egg protein could be detected.

An alternative immunological method for the detection of egg was reported by Sato et al. [153] who described the development of a fully automated system for the detection of egg lysozyme. This system utilised coupled to bacterially produced magnetite particles (BMPs). After incubation of lysozyme solutions with the BMP conjugated antibodies,

immunocomplexes were separated magnetically and different anti-lysozyme antibodies coupled to alkaline phosphatase were added to bind to the immunocomplexes. This was followed by another magnetical separation, and subsequently the alkaline phosphatase generated chemiluminescence intensity was measured. The limit of detection was reported to be 10 ng/ml. The suitability of this method to detect lysozyme in extracts from food products remains to be investigated.

Recently biosensors have also been employed for the detection of egg proteins in food products. An array biosensor for the detection of ovalbumin was used to analyse non-egg pasta extracts spiked with ovalbumin. The detection limit was found to be 12 ng/g pasta extract [154]. A study investigating the determination of egg proteins in snack food and noodles has shown that results obtained with a commercial egg ELISA test kit were similar to those obtained with this biosensor [155].

3.4. Crustaceans

3.4.1. Characteristics of crustacean allergy

Shrimp is the most commonly consumed crustacean shellfish and causes severe allergic reactions, but other related members of the Crustacean family such as lobster, and crab can also elicit severe reactions [78, 156-157]. In the U.S, shrimp allergy is believed to range from 0.6 to 2.8% of the population [157-158], which makes it more prevalent that peanut allergy. Data on the prevalence of crustacean allergy in Europe is very limited, but resulting from a questionnaire-based survey of ~40.000 individuals in France, an estimated prevalence of 3.2% was found [159]. This relatively high figure is most likely to be an overestimation since it is based on self-perception data and not on clinical studies.

In addition to the development of food allergy, crustacean sensitisation can also lead to respiratory allergy. In some crustacean-processing workplaces respiratory allergy to crustaceans developed into a considerable problem because of inhaled allergen [160-161].

3.4.2. Crustacean allergens

The major allergen of shrimp is tropomyosin, a muscle protein [162]. Tropomyosin is easily isolated from the boiling water and meat of cooked shrimp. The allergen nomenclature for this protein is Pen a 1. The molecular weight of various crustacean tropomyosins has been reported to be 34-39 kD; this protein is the target of some of the detection methods that will be discussed next. At least 80% of shrimp-allergic subjects have IgE binding to tropomyosin [163]. Another shrimp allergen (Pen m 2) was identified as having extensive similarity to crustacean arginine kinase and to possess arginine kinase activity [164]. Yu et al. [164] made specific polyclonal antibodies to Pen m 2 and used them to develop a competitive ELISA using shrimp-allergic sera to show that Pen m 2 was a cross-reactive crustacean allergen. Other minor IgE-binding proteins have been described in several other reports, but their

significance is unknown. For the protection of allergic consumers there is a requirement for methods capable of detecting the undeclared presence of shrimp residues in foods, this detection is focussed on seafood processing, soup, and frozen snack and meal industries. Of concern is also the transfer of shrimp allergens through frying oil in restaurants and in battering operations in the food industry, and also in the manufacture of shrimp-containing flavours and other ingredients on shared lines with non-shrimp ingredients.

3.4.3. Crustacean detection methods

There are few methods that have been described for the detection of crustacean residues. Only two methods have been reported to date to quantitate shrimp residues. Jeoung et al. [165] made monoclonal antibodies to shrimp tropomyosin and developed a sandwich ELISA for the purpose of quantitating the protein in commercial skin test extracts. In these clinical extracts, the ELISA could detect 4-125 ng tropomyosin/ml. In a method designed for detecting shrimp in foods, Ben Rejeb et al. [166] described an ELISA utilizing antibodies directed against shrimp tropomyosin which had a detection limit of 2.5 ppm. This method is only described in an abstract, but was apparently used to determine the presence of tropomyosin in some food matrices and products. There is one crustacean ELISA method on the market currently, made by ELISA Systems Pty. Ltd, Windsor, Queensland, Australia (http://www.elisas.com.au). The limit of detection of this sandwich ELISA tropomyosin assay is 0.05 mg/kg (0.05 ppm). Although the assay is positioned as a screening assay for tropomyosin, the reactivity of tropomyosin from crustaceans other than shrimp is not shown. Due to the being in the same phyla, anti-crustacean tropomyosins should be checked for cross-reactivity with insect tropomyosins. There is also one report in which direct ELISA and dot-immunoblotting were used to detect crab residues in surimi-based products [167]. Polyclonal anti-lobster arginine kinase antibody was utilized in an ELISA with a detection limit of 10-25 g of crab per kg of surimi-based product. However, this detection limit is much too high for allergen detection requirements.

3.5. Fish

3.5.1. Characteristics of fish allergy

Fish allergy is relatively common, especially in geographic regions where fish is an important part of the diet. Fatal reactions have occurred as a result of allergic reactions to fish [20, 78]. Many studies have attempted to estimate the prevalence of fish allergy, and found it to range from 0.3-0.5% of the population [157, 168-169].

3.5.2. Fish allergens

The best-characterized fish allergen is the major allergen of codfish, named Gad c 1, which belongs to a group of muscle tissue proteins known as parvalbumins [170]. Parvalbumins are found in high amounts in lower vertebrates and in smaller amounts in higher vertebrates [171]. Gad c 1 is an acidic protein of 12 kD which is stable to heating, extremes of pH, and mild proteolysis [172]. While many fish-allergic patients are allergic to multiple fish species, monospecificity has been reported to tuna and cod [173], and swordfish [174]. The existence of structurally related parvalbumins in divergent fish species may offer an explanation of cross reactivity to fish species in certain allergic individuals. Hansen et al. [175] noted that in adults with clinical sensitivity to cod, reactions were reported to mackerel, herring, and plaice. An IgE binding protein from salmon, named Sal s 1, was identified by in vitro analyses, and was shown to be salmon parvalbumin [176] In one study, almost 50% of cod-allergic patients had clinical sensitivity to salmon [177]. Sal s 1 is described as having two bands at 12 and 14 kD, and therefore has at least two isotypic variants. The allergens from other fish species are also parvalbumins including horse mackerel (Tra j 1), and bigeye tuna (Thu o 1) [178]. In one study, canned tuna did not present problems for five fish-allergic children [179], suggesting that canning may decrease the allergenicity of cod. However, amino acid sequencing has shown that the 12-13 kD band from tuna does not have the same sequence as does cod; this may explain why tuna does not cross react extensively with other species. Bernhisel-Broadbent et al. [180] found that in eleven subjects demonstrating in vitro allergy to fish, positive oral challenge only occurred to one species in seven subjects, to two species in one subject, and to three in two others. This observation has been also seen in other studies [173, 181-182]. Other studies have found that subjects clinically react to multiple fish [173, 183]. A recent survey found that the rate of reactions to multiple fish among those with fish allergy was 67% [157].

3.5.3. Fish detection methods

Because of the complexity of fish allergy, attempts to develop detection systems for fish residues have not been pursued by diagnostic kit companies. Several commercial monoclonal antibodies against different invertebrate parvalbumins are available, and a few methods have been described for detection of fish parvalbumins, and most use monoclonal antibodies. In one recent study [184], a rapid method using a surface plasmon resonance biosensor was developed that could detect parvalbumins from carp, sardine, and skipjack tuna (katsuonut) in 5 minutes. No detection limit in food for the biosensor technique was reported, but the authors felt the method would be useful in detecting undeclared fish residues in foods and that the biosensor chip has the advantage over ELISA methods of regeneration and continuous use. However, it is unknown whether this method will detect many different types of fish.

3.6. Conclusion

A multitude of methods have been employed for the detection of peanut, milk and egg residues in food products. Amongst this there are many commercial methods for the detection of those food allergens available, including ELISA, lateral flow devices, and PCR. The reader should consult the manufacturers for more information on those products.

Although crustaceans and fish are also recognised as major food allergens, methods for their detection in food products are very limited. The main reason for this lies in the large variety of crustaceans and fish species that are capable of triggering allergic reactions. Specificity is an important issue here since methods for the detection of crustacean and fish residues in food products should be specific so that only the allergenic ingredients are detected, but this would imply that every crustacean or fish species requires its own specific method of analysis. Thus it would be preferable to have methods at hand that are able to detect several different crustacean or fish species in a single analysis, but such methods might not be specific enough and there is a danger for cross-reactivities with non-crustacean or non-fish ingredients in the food products.

4. Case studies

This section will provide some practical examples of food allergen analysis. The primary uses of allergen detection methods in the industry environment are to test incoming raw materials (although quite rare), investigate consumer complaints, and the most-often used application is assessment and design of sanitation practices for allergens. Despite this existence of a multitude of control measures in which food producers, consumer organizations, and regulatory agencies all contribute to ensure a correct labelling of food products and to withhold food products with undeclared allergenic ingredients from the market, there is a collection of clinical cases whereby the food allergic reactions could be traced back to the consumption of particular food products. Subsequent analysis of the offending food products has led to the detection of allergenic ingredients in those consumed food products. Since cases like this are all unique and based on the health problems of individuals sensitized to particular food allergens the information about those cases is often confidential and therefore we will list only a few examples below (Hefle et al. unpublished).

Gingersnap cookies were being made on a shared line with peanut-containing cookies; for some reason, an adequate cleanout was not accomplished between the two products. A peanut-allergic consumer had a severe reaction after consuming on the gingersnaps; ELISA showed the cookie had 2.2 percent (22,000 ppm) peanut in it, a very high amount.

A peanut-allergic consumer called a company to complain that their sunflower seeds had given them a reaction; upon testing with ELISA, the company found out that the sunflower seeds contained 32 ppm peanut and 3 ppm almond; it was not sure where the contamination

occurred, but this company checks incoming sunflower seeds now before they come into the plant. Another nut-related case was that a consumer had a reaction to a product and the company traced it back to the ground almonds. By ELISA, the ground almost had 284 ppm peanut in them.

In the course of just testing some raw materials, one company found that their ground almond had 40 ppm peanut and their whole almonds had 50 ppm peanut.

In another case, organic hot chocolate was found to have 13 ppm by ELISA in it, but no reactions had occurred; the company simply wanted to see if their cleanout procedures between products was sufficient – they had to do a bit more cleaning to do during their sanitation schedule for this line before they could bring it down to the detection limit.

One company underwent a cleaning assessment project of pasteurizer lines that process both milk and juices. At the beginning of project, the juice had 10 ppm milk by ELISA, after more cleaning; they got it down do the detection limit of the test. ELISAs are very easy to use with juices and rinse waters in clean-in-place systems like this one.

A company was using whey to flush processing line after a soy containing product went through. At first, they thought a certain amount was enough – when the flush-through was tested by ELISA, it registered >5,000 ppm soy. When the company doubled the amount of whey flush used, ELISA gave 527 ppm soy; finally when tripled, ELISA gave <1 ppm soy.

5. Summary and Outlook

Food allergy constitutes an important health problem which is recognized by food producers, consumers and regulatory bodies. The current efforts to protect individuals sensitized to food allergens largely relies on a strategy of avoidance of the offending food. In order to support this, sensitive analytical methods with a high selectivity are required for the detection of traces of all major food allergens in food products. In this chapter we have listed the methods that are currently in use for this purpose, and we have described 5 major food allergens and the analytical methods that are employed for their detection.

Detection limits need to be at least in the range of established threshold levels that are known to trigger allergic reactions in oral food challenge studies [27]. Despite the fact that information on such threshold levels is very scarce, there is a general agreement that the detection limits for food allergens need to be somewhere between 1 and 100 mg allergenic protein per kg food product [27, 28]. For the detection of some allergens the sensitivity of the current assays falls within this range, but for others sensitivity and specificity of the currently available analytical methods is not satisfactory. Therefore a continuation of efforts to develop methods for food allergen detection is absolutely required.

Next to sensitivity and selectivity, reliability is another issue that is crucial for methods that are used for food allergen detection. The recent validation studies of ELISA and dipstick test kits that are employed for the detection of peanut residues in food products [54-56], together

with the establishment of a working group consisting of representatives of the Food and Drug Administration (FDA), Health Canada, Food Allergy Research and Resource Program University of Nebraska (FARRP), Food Products Association (FPA), and the European Commission's Institute for Reference Materials and Measurements (EC - JRC - IRMM) which has recently initiated a proper validation study by means of international collaborative trial of test kits for the detection of egg, clearly address the urgent need for validation of allergen detection methods and underline the importance of reliability of results obtained with those methods.

Another area of concern is the effects exerted by food processing procedures on the detection of allergenic ingredients in food products. The various heat processing techniques used in the manufacturing of food products have been shown to alter the allergenic potential of milk proteins and in a recent review [113] it was pointed out that the allergenicity of milk components may either decrease, increase or remain stable after exposure to processing techniques such as heating, pasteurization and homogenization. Karamanova et al. [122] have shown that the immunoreactivity of β-lactoglobulin increases considerably after heat treatments of milk, whereas such treatments are causing denaturation of this protein which usually has a negative effect on the LOD. Similar observations have been made for peanut. Analysis of extracts from peanut-containing cookies with increased baking times resulted in an increase in IgE binding as analysed by dotblotting for which human sera was used. In contrast to this the detectability/recovery for peanut as assessed by using an ELISA test kit decreased with increasing baking time (Anklam et al. unpublished results) (Figure 5). A deeper investigation into the effects of food processing is absolutely required in order to gain a better understanding of the allergenic potential of food allergens and the appropriateness of allergen detection methods to detect, or quantify allergen traces in processed food products.

As shown in figure 1 there are currently up to twelve allergenic foods that require a mandatory labelling, although more than 160 allergenic proteins have been identified in foods [13]. In order to keep up with scientific knowledge and technological means a revision of such lists is required and is indeed foreseen in the legislation [25]. Within the European Union the European Food Safety Authority (EFSA) is asked to advise the European Commission on the scientific basis supporting the identification of foods, food components and food ingredients which induce food allergies and food intolerances for labelling purposes. Within this context EFSA has recently issued an opinion on the possible inclusion of lupin in the list of allergenic foods whose presence has to be declared on the label of food products [185]. A justification for recognition of lupin as a major food allergen lies in a recent paper by Radcliffe et al. [186] that reports an increase in lupin allergy in a number of European countries and points to a link between peanut and lupin allergy. Lupin is used as an ingredient of food products for its high nutritional value and high protein content. An important trigger for the increased use of lupin lies in the controversy surrounding the introduction of

genetically modified soybeans. Since soybeans were brought into discredit as a result of this, European food producers are increasingly using lupin in order to exclude soybeans from their products.

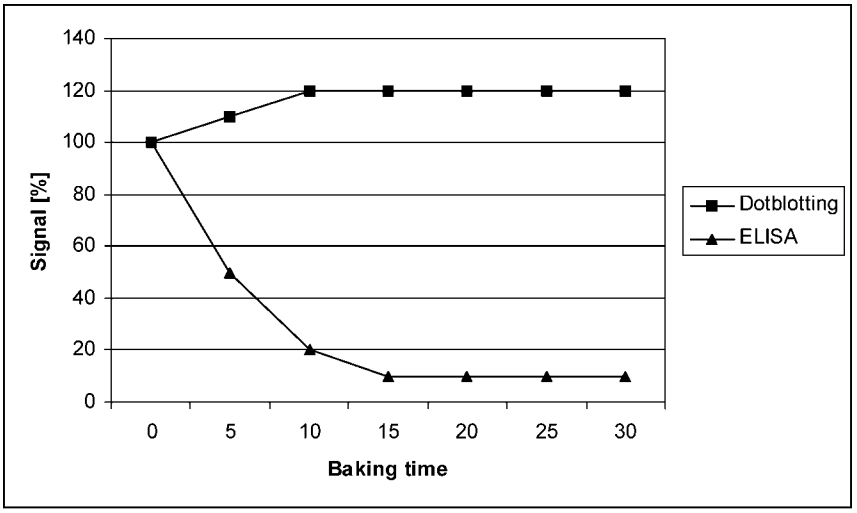


Figure 5. Analysis of peanut-containing cookies with increasing baking time showing a decrease in detection by means of ELISA, paralleled by an increase (and subsequent stability) of the allergenic potential as assessed by dotblotting (Anklam et al. unpublished results).

In parallel to lupin other foods known to contain allergens can and will be assessed with regard to their allergenic potential, the prevalence of allergic reactions triggered by them, and a possible inclusion into the legislation regulating food labelling. Possible extensions of the lists of food allergens that require a mandatory labelling will result in a necessity to have analytical methods at hand to detect their presence in food products. The availability of rapid, sensitive and specific detection methods for all (major) food allergens is of paramount importance in order to protect allergenic individuals and to prevent unexpected food allergic reactions from occurring.

References

[1] C. Ortolani, M. Ipsano, J. Scibilia and E. A. Pastorello, Introducing chemists to food allergy, Allergy. 56 (2001) 5.

[2] S. H. Sicherer, A. Muñoz-Furlong, R. Murphy, R. A. Wood and H. A. Sampson, Symposium: Pediatric Food Allergy. Pediatrics 111 (2003) 1591.

[3] H. A. Sampson, Food allergy--accurately identifying clinical reactivity, Allergy. 60 (2005) 19.
[4] S. L. Taylor and S. L. Hefle, Food allergies and other food sensitivities, Food Technol. 55 (2001) 68.
[5] S. L. Taylor and S. L. Hefle, Foods as allergens. In: Food Allergy and Intolerance, ed. J. Brostoff and S. J. Challacombe, Saunders, London, (2002a) 403.
[6] S. L. Taylor and S. L. Hefle, Allergic reactions and food intolerances, In: Nutritional Toxicology, 2nd ed., ed. F. N. Kotsonis and M. Mackey, Taylor & Francis, London, (2002b) 93.
[7] Food and Agricultural Organization of the United Nations, Report of the FAO Technical Consultation on Food Allergies, Rome, Italy, November 13-14, 1995.
[8] F. Andre, C. Andre, L. Colin, F. Cacaraci and S. Cavagna, Role of new allergens and of allergens consumption in the increased incidence of food sensitizations in France, Toxicology 93 (1994) 77.
[9] B. K. Ballmer-Weber, S. Vieths, D. Luttkopf, P. Heuschmann, and B. Wuthrich, Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root, J Allergy Clin Immunol. 106 (2000) 373.
[10] B. Wüthrich, J. Stäger and S. G. Johansson, Celery allergy associated with birch and mugwort pollinosis, Allergy 45 (1990) 566.
[11] S. L. Taylor, S. L. Hefle, G. Soylemez, and J. Kabourek, Sesame seed allergy, Food Allergy Intolerance 3 (2002a) 115.
[12] M. Ebisawa, K. Ikematsu, T. Imai and H. Tachimoto, Food allergy in Japan, Allergy Clin. Immunol. Int. 15 (2003) 214.
[13] S. L. Hefle, J. A. Nordlee and S. L. Taylor, Allergenic foods, Crit. Rev. Food Sci. Nutr. 36 (1996) Suppl:S69.
[14] J. D. Astwood, J. N. Leach, and R. L. Fuchs, Stability of food allergens to digestion in vitro, Nature Biotech. 14 (1996) 1269.
[15] C. Ortolani, M. Ipsano, E. Pastorello, A. Bigi and R. Ansaloni, The oral allergy syndrome, Ann. Allergy 61 (1988) 47.
[16] S. L. Taylor, Protein allergenicity assessment of foods produced through agricultural biotechnology, Ann. Rev. Pharmacol. Toxicol. 42 (2002) 99.
[17] S. L. Taylor and S. L. Hefle. Food allergies and intolerances. In: Modern Nutrition in Health and Disease, 10th ed., ed. M. E. Shils, M. Shike, A. C. Ross, B. Caballero, and R. J. Cousins, Lippincott, Williams & Wilkins, Philadelphia PA, (2006) 1512.
[18] H. A. Sampson, Anaphylaxis and emergency treatment, Pediatrics 111 (2003) 1601.
[19] S. H. Sicherer, A. Muńoz-Furlong, A. W. Burks and H. A. Sampson, Prevalence of peanut and nut allergy in the United States of America determined by a random digit dial telephone survey, J Allergy Clin. Immunol. 103 (1999) 559.
[20] S. A. Bock, A. Munoz-Furlong and H. A. Sampson, Fatalities due to anaphylactic reactions to foods, J. Allergy Clin. Immunol. 107 (2001) 191.
[21] R. Pumphrey, Fatal allergic reactions to food, Food Allergy Intolerance 2 (2001) 91.
[22] S. M. Tariq, M. Stevens, S. Matthews, S. Ridout, R. Twiselton and D.W. Hide, Cohort study of peanut and nut sensitisation by age of 4 years, B.M.J. 313 (1996) 514.
[23] M. Wensing, A. H. Penninks, S. L. Hefle, S.J. Koppelman, A. Bruijnzeel-Koomen and A. C. Knulst, The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy, J. Allergy Clin. Immunol. 110 (2002) 915.

[24] S. L. Taylor, S. L. Hefle and A. Munoz-Furlong, Food allergies and avoidance diets, Nutr. Today 34 (1999) 15.
[25] European Union, Directive 2003/89/EC of the European parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official J. Europ. Commun. L308 (2003)15.
[26] S. L. Taylor, S. L. Hefle, C. Bindslev-Jensen, S. A. Bock, A. W. Burks, L. Christie, D. J. Hill, A. Host, J. O'B. Hourihane, G. Lack, D. D. Metcalfe, D. A. Moneret-Vautrin, P. A. Vadas, F. Rance, D. J. Skrypec, T. A. Trautman, I. Mahlmeden-Yman, and R. S. Zeiger, Factors affecting the determination of threshold doses for allergenic foods: how much is too much? J. Allergy Clin. Immunol. 109 (2002b) 24.
[27] S. J. Koppelman, H. Bleeker-Marcelis, G. Duijn and M. Hessing, Detecting peanut allergens. The development of an immunochemical assay for peanut proteins, World Ingred 12 (1996) 35.
[28] R. E. Poms, C. L. Klein and E. Anklam, Methods for allergen analysis in food: a review, Food Addit. Contam.21 (2004) 1.
[29] S. T. Holgate, M. K. Church and L. M. Lichtenstein, Allergy 2nd edn St Louis Mosby. (2001)
[30] J. A. Nordlee, S. L. Taylor, R. T. Jones and J. W. Yunginger, Allergenicity of various peanut products as determined by RAST inhibition, J. Allergy Clin. Immunol. 68 (1981) 376.
[31] G. Oldaeus, B. Bjorksten, R. Einarsson and N. I. M. Kjellman, Antigenicity and allergenicity of cow milk hydrolysates intended for infant feeding, Pediatric Allergy and Immunology. 2 (1991) 156.
[32] A. M. Herian, S. L. Taylor and R. K. Bush, Allergenic reactivity of various soybean products as determined by RAST inhibition, J Food Science 58 (1993) 385.
[33] M. Wigotzki, H. Steinhart and A. Paschke, Influence of varieties, storage and heat treatment on IgE-binding proteins in hazelnuts (*Corylus avellana*), Food Agricultural Immunology 12 (2000) 217.
[34] M. Wigotzki, H. Steinhart and A. Paschke, Determination of the allergenicity of various hazelnut products by immunoblotting and enzyme allergosorbent test inhibition, J. Chromatogr. B 756 (2001) 239.
[35] S. Fremont, G. Kanny, S. Bieber, J. P. Nicolas and D. A. Moneret-Vautrin, Identification of a masked allergen, alpha-lactalbumin, in baby-food cereal flour guaranteed free of cow's milk protein. Allergy 51 (1996) 749.
[36] S. J. Koppelman, A. C. Knulst, W. J. Koers, A. H. Penninks, H., Peppelman, R. Vlooswijk, I Pigmans, G. van Duijn and M. Hessing, Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products, J. Immunol. Methods 229 (1999) 107.
[37] J. A. Nordlee and S. L. Taylor, Immunological analysis of food allergens and other food proteins, Food Technology 2 (1995) 129.
[38] E. A. Pastorello and C. Trambaioli, Isolation of food allergens, J. Chromatogr. B 756 (2001) 71.
[39] B. Scheibe, W. Weiss, F. Rueff, B. Przybilla and A. Gorg, Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate, J. Chromatogr. B 756 (2001) 229.
[40] B. W. Blais and L. Phillippe, Detection of hazelnut proteins in foods by enzyme immunoassay using egg yolk antibodies, J Food Prot. 64 (2001) 895.

[41] I. Malmheden Yman, A. Eriksson, G. Everitt, L. Yman and T. Karlsson, Analysis of food proteins for verification of contamination or mislabelling, Food and Agricultural Immunology 6 (1994) 167.

[42] T. Holzhauser, L. I. Dehne, A. Hofmann, D. Haustein and S. Vieths, Rocket Immunoelectrophoresis (RIE) for determination of potentially allergenic peanut proteins in processed foods as a simple means for quality assurance and food safety, Zeitschrift fuer Lebensmittel-Untersuchung und –Forschung 206 (1998) 1.

[43] S. Mäkinen-Kiljunen and T. Palosuo, A sensitive enzyme-linked immunosorbent assay for determination of bovine beta-lactoglobulin in infant feeding formulas and in human milk, Allergy 47 (1992) 347.

[44] S. L. Hefle, R. K. Bush, J. W. Yunginger and S. F. Chu, A sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification of selected peanut proteins in foods, J Food Protection. 57 (1994) 419.

[45] H. Tsuji, N. Okada, R. Yamanishi, N. Bando, M. Kimoto and T. Ogawa, Measurement of Gly m Bd 30K, a major soybean allergen, in soybean products by a sandwich enzyme-linked immunosorbent assay, Biosci. Biotechnol. Biochem. 59 (1995) 150.

[46] N. Bando, H. Tsuji, M. Hiemori, K. Yoshizumi, R.Yamanishi, M. Kimoto. and T. Ogawa, Quantitative analysis of Gly m Bd 28K in soybean products by a sandwich enzyme-linked immunosorbent assay, J Nutr Sci Vitaminol. 44 (1998) 655.

[47] T. Holzhauser and S. Vieths, Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes, J. Agric. Food Chem. 47 (1999a) 4209.

[48] J. J. Hlywka, S. L. Hefle and S. L. Taylor, A sandwich enzyme-linked immunosorbent assay for the detection of almonds in foods, J Food Prot. 63 (2000) 252.

[49] B. Mariager, M. Solve, H. Eriksen and C. –H. Brogen, Bovine β-lactoglobulin in hypoallergenic and ordinary infant formulas measured by an indirect competititive ELISA using monoclonal and polyclonal antibodies, Food and Agric. Immunol. 6 (1994) 73.

[50] J. M. Yeung and P. G. Collins, Enzyme immunoassay for determination of peanut proteins in food products, J. AOAC Int. 79 (1996) 1411.

[51] A. Plebani, P. Restani, A. Naselli, C. L. Galli, A. Meini, G. Cavagni, A. G. Ugazio and C. Poiesi, Monoclonal and polyclonal antibodies against casein components of cow milk for evaluation of residual antigenic activity in 'hypoallergenic' infant formulas, Clin. Exp. Allergy 27 (1997) 949.

[52] T. Holzhauser and S. Vieths, Indirect competitative ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrixes, J. Agric. Food Chem. 47 (1999b) 603.

[53] K. H. Roux, S. S. Teuber, J. M. Robotham and S. K. Sathe, Detection and stability of the major almond allergen in foods, J. Agric. Food Chem. 49 (2001) 2131.

[54] T. B. Whitaker, K. M. Williams, M. W. Trucksess and A. B. Slate, Immunochemical analytical methods for the determination of peanut proteins in foods, J. AOAC Int. 88 (2005) 161.

[55] D. L. Park, S. Coates, V. A. Brewer, E. A. Garber, M. Abouzied, K. Johnson, B. Ritter and D. McKenzie, Performance Tested Method multiple laboratory validation study of ELISA-based assays for the detection of peanuts in food, J. AOAC Int. 88 (2005) 156.

[56] R. E. Poms, M. E. Agazzi, A. Bau, M. Brohée, C. Capelletti, J. V. Nørgaard and E. Anklam, Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate, Food Addit. Contam. 22 (2005) 104.

[57] P. Koch, G. F. Schäppi, R. E. Poms, B. Wüthrich, E. Anklam and R. Battaglia, Comparison of commercially available ELISA kits with human sera-based detection methods for peanut allergens in foods, Food Additives and Contaminants 20 (2003) 797.

[58] A. J. van Hengel, C. Capelletti, M. Brohée and E. Anklam, Validation of two commercial lateral flow devices for the detection of peanut proteins in cookies: Interlaboratory study, J AOAC Int. 89 (2006) 462.

[59] W. Haasnoot, N. G. Smits, A. E. Kemmers-Voncken and M. G. Bremer, Fast biosensor immunoassays for the detection of cows' milk in the milk of ewes and goats, J Dairy Res. 71 (2004) 322.

[60] C. Elliot, Optical biosensors: from a research idea to an analytical necessity. Proceedings of the 3rd Joint CSL/JIFSAN Symposium on Food Safety and Nurition, York, UK. 2002.

[61] S. Muller-Renaud, D. Dupont and P. Dulieu, Quantification of beta casein in milk and cheese using an optical immunosensor, J. Agric. Food Chem. 52 (2004) 659.

[62] H. E. Indyk and E. L. Filonzi, Direct optical biosensor analysis of folate-binding protein in milk, J. Agric. Food Chem. 52 (2004) 3253.

[63] D. Dupont, O. Rolet-Repecaud and S. Muller-Renaud, Determination of the heat treatment undergone by milk by following the denaturation of alpha-lactalbumin with a biosensor, J Agric Food Chem. 52 (2004) 677.

[64] B. Malorny, P. T. Tassios, P. Radstrom, N. Cook, M. Wagner and J. Hoorfar, Standardization of diagnostic PCR for the detection of foodborne pathogens, Int. J. Food Microbiol. 83 (2003) 39.

[65] E. Anklam, E., Gadani, F., Heinze, P., Pijnenburg, H. and G. Van Den Eede, Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products, Eur Food Res Technol 214 (2002) 3.

[66] A. Holst-Jensen, S. B. Ronning, A. Lovseth and K. G. Berdal, PCR technology for screening and quantification of genetically modified organisms (GMOs), Anal. Bioanal. Chem. 375 (2003) 985.

[67] R. E. Poms and E. Anklam, Polymerase chain reaction techniques for food allergen detection, J. AOAC Int. 87 (2004) 1391.

[68] E. Studer, C. Rhyner and L. Lüthy, Quantitative competititive PCR for the detection of genetically modified soybean and maize, Zeitschrift fuer Lebensmittel-Untersuchung und – Forschung A. 207 (1998) 207.

[69] A. Jankiewicz, H. Broll and J. Zagon, The official method for the detection of genetically modified soybeans (German Food Act LMBG par. 35): a semi-quantitative study of sensitivity limits with glyphosphate-tolerant soybeans (Roundup Ready) and insect-resistant Bt maize (Maximizer), European Food Research and Technology 209 (1999) 77.

[70] L. E. Desjardin, Y. Chen, M. D. Perkins, L. Teixeira, M. D. Cave and K. D. Eisenach, Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis, J Clin Microbiol. 36 (1998) 1964.

[71] R. Yamanishi, K. Kondo, H. Tsuj and T. Ogawa, Micro-assay to measure the allergenicity of a Kunitz-type soybean trypsin inhibitor toward Balb/c mice by using RBL-2H3 cells, Biosci. Biotechnol. Biochem. 59 (1995) 1272.

[72] R. Yamanishi, H. Tsuji, N. Bando, I. Yoshimoto and T. Ogawa, Micro-assay method for evaluating the allergenicity of the major soybean allergen, Gly m Bd 30K, with mouse antiserum and RBL-2H3 cells, Biosci. Biotechnol. Biochem. 61 (1997) 19.
[73] M. Nikulina, C. Habich, S. B. Flohe, F. W. Scott and H. Kolb, Wheat gluten causes dendritic cell maturation and chemokine secretion, J. Immunol. 173 (2004) 1925.
[74] F. Kvasnicka, Proteomics: general strategies and application to nutritionally relevant proteins, J. Chromatogr. B 787 (2003) 77.
[75] H. Hirano, N. Islam and H. Kawasaki, Technical aspects of functional proteomics in plants, Phytochemistry. 65 (2004) 1487.
[76] M. Natale, C. Bisson, G. Monti, A. Peltran, L. P. Garoffo, S. Valentini, C. Fabris, E. Bertino, A. Coscia and A. Conti, Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry, Mol. Nutr. Food Res. 48 (2004) 363.
[77] S. A. Bock and F. M. Atkins, Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges, J. Pediatrics 117 (1990) 561.
[78] J. W. Yunginger, K. G. Sweeney, W. Q. Sturner, L. A. Giannandrea, J. D. Teigland, M. Bray, P. A. Benson, J. A. York, L. Biedrzycki, D. L. Squillace and R. M. Helm, Fatal food-induced anaphylaxis. J.A.M.A. 260 (1988) 1450.
[79] H. A. Sampson, L. Mendelson and J. P. Rosen, Fatal and near-fatal anaphylactic reactions to food in children and adolescents, New England Journal of Medicine 327 (1992) 380.
[80] S. E. Emmett, F. J. Angus, J. S. Fry and P. N. Lee, Perceived prevalence of peanut allergy in Great Britain and its association with other atopic conditions and with peanut allergy in other household members, Allergy 54 (1999) 380.
[81] M. Morisset, D. A. Moneret-Vautrin, G. Kanny, L. Guenard, E. Beaudouin, J. Flabbee and R. Hatahet, Thresholds of clinical reactivity to milk, peanut, and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges, Clin. Exp. Allergy 33 (2003) 1046.
[82] A. W. Burks, H. A. Sampson and G. A. Bannon, Peanut allergens, Allergy 53 (1998) 725.
[83] S. J. Koppelman, R. A. A. Vlooswijk, L. M. J. Knippels, M. Hessing, E. F. Knol, F. C. van Reijsen and C. A. F. M. Bruijnzeel-Koomen, Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different part of the world, Allergy 56 (2001) 132.
[84] G. A. Bannon, M. Besler, S. L. Hefle, J. O'. B. Hourihane and S. H. Sicherer, Allergen data collection: peanut (Arachis hypogea), Internet Symposium on Food Allergens 2 (2000) 87.
[85] S. J. Koppelman, E. F. Knol, R. A. A. Vlooswijk, M. Wensing, A. C. Knulst, AC, Hefle, S, Gruppen, H., Piersma S, Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization, Allergy 58 (2003) 1144.
[86] A. M. Scurlock and A. W. Burks, Peanut allergenicity, Ann. Allergy Asthma Immunol. 93 (2004) S12.
[87] D. Mittag, J. Akkerdaas, B. K. Ballmer-Weber, L. Vogel, M. Wensing, W. -M. Becker, S. J. Koppelman, A. Helbling, S. L. Hefle, R. van Ree and S. Vieths, Ara h 8; a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy, J. Allergy Clin. Immunol. 114 (2004) 1410.
[88] H. A. Sampson, Update on food allergy, J. Allergy Clin. Immunol. 113 (2004) 805.
[89] D. Barnett and M. E. H. Howden, A rocket immunoelectrophoretic method for the detection of heat-treated peanut protein, Food Technology in Australia 36 (1984) 510.

[90] I. Malmheden Yman, A. Eriksson, G. Everitt, L. Yman and T. Karlsson, Analysis of food proteins for verification of identification of contamination or mislabeling, Food Agric. Immunol. 6 (1994)167.
[91] T. Holzhauser, L. I. Dehne, A. Hoffmann, D. Haustein and S. Vieths, Rocket immunoelectrophoresis (RIE) for determination of potentially allergenic peanut proteins in processed foods as a simple means for quality assurance and food safety, Zeit. Lebensmittel. Unters. Forsch A 206 (1998) 1.
[92] J. W. Yunginger, M. B. Gauerke, R. T. Jones, M. E. Dahlberg and S. J. Ackerman, Use of radioimmunoassay to determine the nature, quantity and source of allergenic contamination of sunflower butter, J. Food Prot. 46 (1983) 625.
[93] M. U. Keating, R. T. Jones, N. J. Worley, C. A. Shively and J. W. Yunginger, Immunoassay of peanut allergens in food-processing materials and finished foods, J. Allergy Clin. Immunol. 86 (1990) 41.
[94] G. F. Schappi, V. Konrad, D. Imhof, R. Etter and B. Wuthrich, Hidden peanut allergens detected in various foods: findings and legal measures, Allergy 56 (2001) 1216.
[95] R. E. Poms, E. Anklam, C. L. Klein and F. Ulberth, Peanut allergens in food products, G.I.T. Laboratory Journal 3 (2003) 132.
[96] A. Pomes, R. M. Helm, G. A. Bannon, A. W. Burks, A. Tsay and M. D. Chapman, Monitoring peanut allergen in food products by measuring Ara h 1, J. Allergy Clin. Immunol. 111 (2003) 640.
[97] W. H. Newsome and M. Abbott, An immunoaffinity column for the determination of peanut protein in chocolate, J. AOAC Int. 82 (1999) 666.
[98] O. Stephan and S. Vieths, Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanuts (Arachis hypogaea) in processed foods, J. Agric. Food Chem. 52 (2004) 3754.
[99] B. W. Blais and L. M. Phillippe, A cloth-based enzyme immunoassay for detection of peanut proteins in foods, Food Agric. Immunol. 12 (2000) 243.
[100] E. N. C. Mills, A Potts, G. W. Plumb, N. Lambert and M. R. A. Morgan, Development of a rapid dipstick immunoassay for the detection of peanut contamination of food. Food Agricult. Immunol. 9 (1997) 37.
[101] O. Stephan, N. Moller, S. Lehmann, T. Holzhauser and S. Vieths, Development and validation of two dipstick type immunoassays for determination of trace amounts of peanut and hazelnut in processed foods, Eur. Food Res. Technol. 215 (2002) 431.
[102] K. J. Shefcheck and S. M. Musser, Confirmation of the allergenic peanut protein, Ara h 1, in a model food matrix using liquid chromatography/tandem mass spectrometry (LC/MS/MS), J. Agric. Food Chem. 52 (2004) 2785.
[103] H. Hird, J. Lloyd, R. Goodier, J. Brown and P. Reece, Detection of peanut using real-time polymerase chain reaction, Eur. Food Res. Technol. 217 (2003) 265.
[104] W. J. Hurst, E. R. Krout, and W. R. Burks, A comparison of commercially available peanut ELISA test kits on the analysis of samples of dark and milk chocolate, J. Immunoassay Immunochem. 23 (2002) 451.
[105] J. M. Wal, Bovine milk allergenicity, Ann. Allergy Asthma Immunol. 93 (2004) 2.
[106] P. Juvonen, M. Mansson, N. I. Kjellman, B. Bjorksten and I. Jakobsson, Development of immunoglobulin G and immunoglobulin E antibodies to cow's milk proteins and ovalbumin after a temporary neonatal exposure to hydrolyzed and whole cow's milk proteins, Pediatr. Allergy Immunol. 10 (1999) 191.

[107] K. Sakai, K. Yoshino, M. A. Satter, F. Ota, Y. Nii, K. Fukuta, N. Ueda, Y. Shimizu and S. Yamamoto, Effects of pH variation and NaCl on in vitro digestibility of cow's milk proteins in commercially available infant formulas, J. Nutr. Sci. Vitaminol. 46 (2000) 325.
[108] M. Eggesbo, G. Botten, R. Halvorsen and P. Magnus, The prevalence of CMA/CMPI in young children: the validity of parentally perceived reactions in a population-based study, Allergy 56 (2001) 393.
[109] R. Siciliano, B. Rega, A. Amoresano and P. Pucci, Modern mass spectrometric methodologies in monitoring milk quality, Anal. Chem. 72 (2000) 408.
[110] S. L. Bahna, Cow's milk allergy versus cow milk intolerance, Ann. Allergy Asthma Immunol. 89 (2002) 56.
[111] S. H. Sicherer, Determinants of systemic manifestations of food allergy, J. Allergy Clin. Immunol. 106 (2000) 251.
[112] H. M. J. Farrell, R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang and H. E. Swaisgood, Nomenclature of the Proteins of Cows' Milk--Sixth Revision, J. Dairy Sci. 87 (2004) 1641.
[113] L. Monaci, V. Tregoat, A. van Hengel and E. Anklam, Milk allergens, their characteristics and their detection in food: A review, European Food Research and Technology 223 (2006) 149.
[114] H. J. Issaq, T. D. Veenstra, T. P. Conrads and D. Felschow, The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification, Biochem. Biophys. Res. Commun. 292 (2002) 587.
[115] S. Makinen-Kiljunen and T. Palosuo, A sensitive enzyme-linked immunosorbent assay for determination of bovine beta-lactoglobulin in infant feeding formulas and in human milk, Allergy 47 (1992) 347.
[116] A. Plebani, P. Restani, A. Naselli, C. L. Galli, A. Meini, G. Cavagni, A. G. Ugazio and C. Poiesi, Monoclonal and polyclonal antibodies against casein components of cow milk for evaluation of residual antigenic activity in 'hypoallergenic' infant formulas, Clin. Exp. Allergy 27 (1997) 949.
[117] G. Anguita, R. Martin, T. Garcia, P. Morales, A. I. Haza, I. Gonzalez, B. Sanz and P. E. Hernandez, A competitive enzyme-linked immunosorbent assay for detection of bovine milk in ovine and caprine milk and cheese using a monoclonal antibody against bovine beta-casein, J. Food Prot. 60 (1997) 64.
[118] S. L. Hefle and S. L. Taylor, Food allergy and the food industry, Curr. Allergy Asthma Rep. 4 (2004) 55.
[119] W. Haasnoot, N. G. Smits, A. E. Kemmers-Voncken and M. G. Bremer, Fast biosensor immunoassays for the detection of cows' milk in the milk of ewes and goats, J. Dairy Res. 71 (2004) 322.
[120] S. Muller-Renaud, D. Dupont and P. Dulieu, Quantification of beta casein in milk and cheese using an optical immunosensor, J. Agric. Food Chem. 52 (2004) 659.
[121] D. Dupont, O. Rolet-Repecaud and S. Muller-Renaud, Determination of the heat treatment undergone by milk by following the denaturation of alpha-lactalbumin with a biosensor, J. Agric. Food Chem. 52 (2004) 677.
[122] L. Karamanova, L. Fukal, M. Kodicek, P. Rauch, E. Mills and M. Morgan, Immunoprobes for thermally-induced alterations in whey protein structure and their application to the analysis of thermally-treated milks, Food Agric. Immunol. 15 (2003) 77.
[123] I. Recio, M. Ramos and R. Lopez-Fandino, Capillary electrophoresis for the analysis of food proteins of animal origin, Electrophoresis 22 (2001) 1489.

[124] B. Vallejo-Cordoba, Rapid separation and quantification of major caseins and whey proteins of bovine milk by capillary electrophoresis., J. Capillary Electrophor. 4 (1997) 219.
[125] H. Olguin-Arredondo and B. Vallejo-Cordoba, Separation and determination of beta-lactoglobulin variants A and B in cow's milk by capillary free zone electrophoresis, J. Capillary Electrophor. 6 (1999) 145.
[126] B. Miralles, V. Rothbauer, M. A. Manso, L. Amigo, I. Krause and M. Ramos, Improved method for the simultaneous determination of whey proteins, caseins and para-kappa-casein in milk and dairy products by capillary electrophoresis, J. Chromatogr. A 915 (2001) 225.
[127] P. Riechel, T. Weiss, R. Ulber, H. Buchholz and T. Scheper, Analysis of bovine lactoferrin in whey using capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC), Adv. Exp. Med. Biol. 443 (1998) 33.
[128] J. E. Gutierrez and L. Jakobovits, Capillary electrophoresis of alpha-lactalbumin in milk powders, J. Agric. Food Chem. 51 (2003) 3280.
[129] J. Herrero-Martinez, E. Simo-Alfonso, G. Ramis-Ramos, C. Gelfi and P. Righetti, Determination of cow's milk and ripening time in nonbovine cheese by capillary electrophoresis of the ethanol-water protein fraction, Electrophoresis 21 (2000) 633.
[130] J. M. Herrero-Martinez, E. F. Simo-Alfonso, G. Ramis-Ramos, C. Gelfi and P. G. Righetti, Determination of cow's milk in non-bovine and mixed cheeses by capillary electrophoresis of whey proteins in acidic isoelectric buffers, J. Chromatogr. A 878 (2000) 261.
[131] E. Molina, M. De Frutos and M. Ramos, Capillary electrophoresis characterization of the casein fraction of cheeses made from cows', ewes' and goats' milks., J. Dairy Res. 67 (2000) 209.
[132] C. Garcia-Ruiz, M. Torre and M. L. Marina, Analysis of bovine whey proteins in soybean dairy-like products by capillary electrophoresis, J. Chromatogr. A 859 (1999) 77.
[133] M. T. Veledo, M. de Frutos and J. C. Diez-Masa, Development of a method for quantitative analysis of the major whey proteins by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection, J. Sep. Sci. 28 (2005) 935.
[134] M. T. Veledo, M. de Frutos and J. C. Diez-Masa, Analysis of trace amounts of bovine beta-lactoglobulin in infant formulas by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection, J. Sep. Sci. 28 (2005) 941.
[135] M. Mann, C. Meng and J. Fenn, Interpreting mass spectra of multiply charged ions, Anal. Chem. 61 (1989) 1702.
[136] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, Science 246 (1989) 64.
[137] B. Hernandez-Ledesma, L. Amigo, M. Ramos and I. Recio, Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion, J. Chromatogr. A 1049 (2004) 107.
[138] J. Leonil, V. Gagnaire, D. Molle, S. Pezennec and S. Bouhallab, Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides, J. Chromatogr. A 881 (2000) 1.
[139] S. Garbis, G. Lubec and M. Fountoulakis, Limitations of current proteomics technologies, J. Chromatogr. A 1077 (2005) 1.
[140] J. Bernhisel-Broadbent, H. M. Dintzis, R. Z. Dintzis and H. A. Sampson, Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice, J. Allergy Clin. Immunol. 93 (1994) 1047.

[141] M. Eggesbo, G. Botten, R. Halvorsen and P. Magnus, The prevalence of allergy to egg: a population-based study in young children, Allergy 56 (2001) 403.
[142] P. A. Eigenmann and A. M. Calza, Diagnosis of IgE-mediated food allergy among Swiss children with atopic dermatitis, Pediatr. Allergy Immunol. 11 (2000) 95.
[143] D. J. Hill, C. S. Hosking and R. G. Heine, Clinical spectrum of food allergy in children in Australia and South-East Asia: identification and targets for treatment, Ann. Med. 31 (1999) 272.
[144] G. Kanny, D. A. Moneret-Vautrin, J. Flabbee, E. Beaudouin, M. Morisset and F. Thevenin, Population study of food allergy in France, J. Allergy Clin. Immunol. 108 (2001) 133.
[145] T. Schäfer, U. Kramer, D. Dockery, D. Vieluf, H. Behrendt and J. Ring, What makes a child allergic? Analysis of risk factors for allergic sensitization in preschool children from East and West Germany, Allergy Asthma Proc. 20 (1999) 23.
[146] M. Kulig, R. Bergmann, B. Niggemann, G. Burow and U. Wahn, Prediction of sensitization to inhalant allergens in childhood: evaluating family history, atopic dermatitis and sensitization to food allergens. The MAS Study Group. Multicentre Allergy Study, Clin. Exp. Allergy 28 (1998) 1397.
[147] B. J. Walsh, D. J. Hill, P. Macoun, D. Cairns and M. E. Howden, Detection of four distinct groups of hen egg allergens binding IgE in the sera of children with egg allergy, Allergol. Immunopathol. (Madr) 33 (2005) 183.
[148] V. Leduc, C. Demeulemester, B. Polack, C. Guizard, L. Le Guern and G. Peltre, Immunochemical detection of egg-white antigens and allergens in meat products, Allergy 54 (1999) 464.
[149] J. M. Yeung, W. H. Newsome and M. A. Abbott, Determination of egg proteins in food products by enzyme immunoassay, J. AOAC Int. 83 (2000) 139.
[150] S. L. Hefle, E. Jeanniton and S. L. Taylor, Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foods, J. Food Prot. 64 (2001) 1812.
[151] Y. Watanabe, K. Aburatani, T. Mizumura, M. Sakai, S. Muraoka, S. Mamegosi and T. Honjoh, Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent, J. Immunol. Methods 300 (2005) 115.
[152] S. Baumgartner, I. Steiner, S. Kloiber, D. Hirmann, R. Krska and J. M. Yeung, Towards the development of a dipstick immunoassay for the detection of trace amounts of egg proteins in food, European Food Research and Technology 214 (2002) 168.
[153] R. Sato, H. Takeyama, T. Tanaka and T. Matsunaga, Development of high-performance and rapid immunoassay for model food allergen lysozyme using antibody-conjugated bacterial magnetic particles and fully automated system, Appl. Biochem. Biotechnol. 91-93 (2001) 109.
[154] L. C. Shriver-Lake, C. R. Taitt and F. S. Ligler, Applications of array biosensor for detection of food allergens, J. AOAC Int. 87 (2004) 1498.
[155] K. M. Williams, C. D. Westphal and L. C. Shriver-Lake, Determination of egg proteins in snack food and noodles, J. AOAC Int. 87 (2004) 1485.
[156] D.P. Steensma, The kiss of death: a severe allergic reaction to a shellfish induced by a good-night kiss, Mayo Clin. Proc. 78 (2003) 221.
[157] S. H. Sicherer, A. Munoz-Furlong and H. A. Sampson, Prevalence of seafood allergy in the United States determined by a random telephone survey, J. Allergy Clin. Immunol. 114 (2004) 159.

[158] M. Besler, C. B. Daul and P. S. B. Leung,. Shrimp allergy. (www.food-allergens.de), Internet Symposium on Food Allergens 3 (2001) 37.
[159] G. Kanny, D. A. Moneret-Vautrin, J. Flabbee, E. Beaudouin, M. Morisset anf F. Thevenin, Population study of food allergy in France, J. Allergy Clin. Immunol. 108 (2001) 133.
[160] D. W. Goetz and B. A. Whisman, Occupational asthma in a seafood restaurant worker: cross-reactivity of shrimp and scallops, Ann. Allergy Asthma Immunol. 85 (2000) 461.
[161] A. Desjardins, J. –L. Malo, J. L'Archeveque, A. Cartier, M. McCants and S. B. Lehrer, Occupational IgE-mediated sensitization and asthma caused by clam and shrimp, J. Allergy and Clin. Immunol. 96 (1995) 608.
[162] C. B. Daul, M. Slattery, G. Reese and S. B. Lehrer, Identification of the major brown shrimp (Penaeus aztecus) allergen as the muscle protein tropomyosin, Int. Arch. Allergy Immunol. 105 (1994) 49.
[163] R. Ayuso, S. B. Lehrer and G. Reese, Identification of continuous, allergenic regions of the major shrimp allergens Pen a 1 (tropomyosin), Int. Arch. Allergy Immunol. 127 (2002) 27.
[164] C. J. Yu, Y. F. Lin, B. L. Chiang and L. P. Chow, Proteomics and immunological analyses of a novel shrimp allergen Pen m 2, J. Immunol. 170 (2003) 445.
[165] B. J. Jeoung, G. Reese, P. Hauck, J. B. Oliver, C. B. Daul and S. B. Lehrer, Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibodies-based sandwich ELISA, J. Allergy Clin. Immunol. 100 (1997) 229.
[166] S. Ben Rejeb, D. Davies, J. Querry C. Cleroux, D. Langlois and P. Delahaut, Enzyme immunoassay for the detection of crustacean proteins in food. AOAC Annual Meeting Abstracts, Los Angeles, CA, Abstract C-124 (2002) 102.
[167] V. Verrez-Bagnis and I. Escriche-Roberto, The performance of ELISA and dot-blot methods for the detection of crab flesh in heated and sterilized surimi-based products, J. Sci. Agric. 63 (1993) 445.
[168] E. Bjornsson, C. Janson, P. Plaschke, E. Norrman and O. Sjoberg, Prevalence of sensitization to food allergens in adult Swedes, Ann. Allergy Asthma Immunol. 89 (1996) 327.
[169] M. de Martino, E. Novembre, L. Galli, A. de Marco, P. Botarelli, E. Marano, A. Vierucci, Allergy to different fish species in cod-allergic children: In vivo and in vitro studies, J. Allergy Clin. Immunol. 86 (1990) 909.
[170] S. Elsayed and H. Bennich, The primary structure of allergen M from cod, Scand. J. Immunol. 4 (1975) 203.
[171] P. Lehky, H. Blum, E. Stein and E. Fisher, Isolation and characterization of parvalbumins from skeletal muscle of higher vertebrates, J. Biol. Chem. 249 (1974) 4332.
[172] K. Aas and S. Elsayed, Physio-chemical properties and specific activity of a purified allergen (codfish), Dev. Biol. Standard 29 (1975) 90.
[173] J. M. James, R. M. Helm, A. W. Burks and S. B. Lehrer, Comparison of pediatric and adult IgE antibody binding to fish proteins, Ann. Allergy Asthma Immunol. 79 (1997) 131.
[174] J. M. Kelso, R. T. Jones and J. W. Yunginger, Monospecific allergy to swordfish, Ann. Allergy 77 (1996) 227.
[175] T. K. Hansen, C. Bindslev-Jensen, P. S. Skov and L. K. Poulsen, Codfish allergy in adults: IgE cross-reactivity among fish species, Ann. Allergy Asthma Immunol. 78 (1997) 187.
[176] C. D. Lindstrom, T. van Do, I. Hordvik, C. Endresen and S. Elsayed, Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (Salmo salar), Scand. J. Immunol. 44 (1996) 335.

[177] K. Aas, Fish allergy and the codfish allergen model. In: Brostoff, J. and Challacombe, S.J. (eds.). Food Allergy and Intolerance. Bailliere Tindall, London, UK. (1987) 356.
[178] T. Van Do, I. Hordvik, C. Endresen and S. Elsayed, Expression and analysis of recombinant salmon parvalbumin, the major allergen in Atlantic salmon (Salmo salar), Scand. J. Immunol. 50 (1999) 619.
[179] J. Bernhisel-Broadbent, D. Strause, H. A. Sampson, Fish hypersensitivity. II: Clinical relevance of altered fish allergenicity caused by various preparation methods, J. Allergy Clin. Immunol. 90 (1992) 622.
[180] J. Bernhisel-Broadbent, S. M. Scanlon, and H. A. Sampson, Fish hypersensitivity I. In vitro and oral challenge results in fish-allergic patients, J. Allergy Clin. Immunol. 89 (1992a) 730.
[181] R. Haydel, J. El-Dahr, M. McCants, A. Helbling, J. Musmand and S. Lehrer, Food allergy: challenge studies of fish allergic subjects, J. Allergy Clin. Immunol. 91 (1993) 344.
[182] A. Bugajska-Schretter, M. Grote, L. Vangelista, P. Valent, W. R. Sperr, H. Rumpold, A. Pastore, R. Reichelt, R. Valenta and S. Spitzauer, Purification, biochemical, and immunological characterization of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumi,.Gut 46 (2000) 661.
[183] H. Y. Lin, S. D. Shyur, J. L. Fu, Y. C. Lai and J. S. Lin, Fish induced anaphylactic reaction: report of one case, Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi 39 (1998) 200-202.
[184] Y. Lu, T. Ohshima and H. Ushio, Rapid detection of fish major allergen parvalbumin by surface plasmon resonance biosensor, J. Food Sci. 69 (2004) 652.
[185] http://www.efsa.eu.int/science/nda/nda_opinions/1296_en.html
[186] M. Radcliffe, G. Scadding and H. M. Brown, Lupin flour anaphylaxis, The Lancet 365 (2005) 1360.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 8

Sampling, detection, identification and quantification of genetically modified organisms (GMOs)

Arne Holst-Jensen

National Veterinary Institute, Ullevaalsveien 68, P.O.box 8156 Dep., 0033 Oslo, Norway

1. Introduction

1.1. Genetically modified organisms (GMOs)

Genetically modified organisms (GMOs) can be defined as organisms whose genetic constitution has been modified by gene technology. Although there are a range of slightly non-congruent definitions, the core part of the definitions remain the same; GMOs are living organisms with a genetic constitution that could not have been created by a natural route, and they have intentionally been modified to alter one or more traits. In other words, what identifies a particular GMO and makes it different from any other GMO or non-GMO is a particular genetic constitution, i.e. one or more unique (combinations of) DNA sequence(s). The process of transforming a plant into a GMO can be explained simplified as taking DNA elements of interest, putting them together in a specific order to make them functional (yielding a genetic construct), and then introduce the new genetic construct into a plant cell and stably integrate it into the plant cell's own DNA (its genome). The DNA elements of interest may be taken from practically any source of DNA. For example the cauliflower mosaic virus (CaMV) is the source of a widely used P35S promoter and T35S terminator, the soil bacterium *Agrobacterium tumefaciens* is the source of the widely used 3'-nos terminator, the plant *Petunia hybrida* is the source of the widely used EPSPS gene encoding tolerance to the herbicide Roundup, and the soil bacterium *Bacillus thuringiensis* is the source of several widely used *cry*-genes encoding various insecticidal toxins. The resulting plant cell may receive one or more complete and/or partial/rearranged copies of the genetic construct, and the transformation process may also involve rearrangements of parts of the recipient plant's genome. With current plant transformation technology it is not possible to direct the integration to a specific location in the recipient genome. In effect this means that integration (if repeated) will always take place in a unique location, creating a unique sequence at the junction between the inserted DNA and the recipient genome [1].

Traditional breeding programmes are limited by the availability of selectable genetic variation, because genetic features can not be transferred across natural species barriers.

Various ways of increasing the availability of selectable genetic variation have been explored, e.g. chemical or physical mutagenesis (exposure to chemicals or radiation that increases the mutation rate). However, the drawback of these methods is that they create random genetic variation, and that desired features co-exist with many other genetic changes. These latter changes may be neutral, undesirable or in some cases even harmful. Gene technology has made it possible to take only a short piece of genetic information and transfer this across almost any species barrier. Even though there are still some uncontrolled mechanisms involved that may result in co-transfer of undesired DNA sequence, rearrangements, duplications and substitutions, gene technology generally results in much lower undesired genetic change than mutagenesis technologies.

Gene technology has mainly been used to produce agriculturally improved plant varieties, i.e. varieties with higher yields. The majority of GM plants commercialised are either herbicide tolerant, produce their own insecticide, or both. There are also some virus resistant GM plants, and varieties with altered nutritional content (e.g. vitamin A enriched "golden" rice) and improved shelf life (e.g. FlavrSavr tomato) have also been developed. So far the impact on the consumer is therefore limited, although it is claimed that insect resistant maize (corn) has lower mycotoxin levels than conventional maize grown under similar conditions [2]. This secondary effect may be explained by a reduction in fungal infections through holes produced by insects on the plants' protective tissues. So far there are no genetically modified livestock animals commercialised, but several are in the pipeline or late in developmental stages [3]. For example have genes been inserted that speed up the growth rate or lead to synthesis of novel proteins. Both plants and animals have been genetically modified to act as biofactories, e.g. to produce substantial quantities of pharmaceuticals [4,5]. Genetically modified ornamental crops and pets with desirable features (particular colours or patterns, and non-allergenic fur) have also been developed.

For the food industry, the main impacts from gene technology have been the availability of GM microorganisms that are used to produce particular enzymes [6], but despite recent expectations so far not as starter cultures for fermentation processes. However, this use is limited to contained environments, i.e. the living GM microorganism shall not be released into the environment or final product. Furthermore, widespread opposition to gene technology in many countries, in particular in Europe, has lead to very limited use of GM starter cultures. Consequently, many food industries are primarily faced with the gene technology in relation to plant based ingredients. For these industries the technology may be seen as a problem because the industries need to control for presence of GM material (authorised or not) and label their products if the concentration of authorised GM material exceeds a legal or contracted threshold. For organic farmers and retailers the presence of GM material in their products is sometimes devastating, if zero tolerance is implemented. This, in combination with globally increased focus on food safety and traceability, has lead to a growing interest in GM detection and monitoring methods.

1.2. GMOs on the world market

GMOs are regulated by diverse legislation, all concerned with aspects of safety and consumers' protection. In the USA, the worlds largest developer, grower and exporter of GMOs, the authorisation procedure is simple, focus on human and animal (consumer) and environmental safety, and there is no requirement for traceability or labelling of de-regulated (approved) GMOs [7]. Their position is that product tracing should only be considered where necessary for food safety reasons, i.e. to protect against or manage identified risks for consumers. In practice this is done by governmental establishment of food safety performance standards for food producers and processers. The producers and processers are regularly monitored through inspections, and failure to follow the standards may lead to regulatory action. De-regulation also covers breeding of the GMO with other GMOs (gene stacking). In contrast, the current EU based GM legislation is more complex, but core elements include pre-authorisation safety assessments [8] by the European Food Safety Authority (EFSA) [9], availability of validated detection methods, reference materials, and thresholds for labelling [10,11], post-market monitoring and post-marketing traceability requirements [12,13], as well as more emphasis on co-existence [14,15]. Gene stacked GMOs require separate authorisation in the EU. Consequently there is a need for methods to sample, detect, identify, quantify and trace GMOs and derived products with global trade. The Cartagena protocol further enforces the need for identification and traceability of GMOs worldwide [16].

Approximately 60% of all soya beans on the world market are genetically modified (GM), and almost one fourth of all maize is GM, with the numbers increasing every year. GM varieties of several other major food crops are marketed, e.g. rice and rape seed, and more are expected to follow [17]. Table 1 is giving a few examples of traits introduced to plants. For examples of plant derived pharmaceutical proteins that have reached clinical development, see e.g. [4].

GMOs and conventional crops are often grown side by side, and the harvested crops are transported along the same routes. Pollen may flow between fields; grains may incidentally overwinter in the fields and grow the following year; harvest and transport vessels, as well as storage units may be insufficiently cleaned; processing equipment may be contaminated with dust, etc. This poses a challenge in relation to co-existence between conventional and GM crops, in particular when tolerance levels for contamination of non-GM products with GM-material are low such as e.g. implemented in current EU regulations (threshold = 0.9% for authorised events, transitional threshold until April 2007 = 0.5% for events having received a favourable risk evaluation by the EFSA scientific committee, and 0% for all other events [5]).

Table 1. Examples of traits introduced to genetically modified plants[a]

Plant species	Event name	Company	Trait	Gene
Soybean	GTS 40-3-2	Monsanto Company	Tolerance to glyphosate herbicide	*epsps*
	G94-1, G94-19, G168	DuPont Canada Agricultural Products	High in oleic fatty acid	*GmFad2-1*
Maize (corn)	Event 176	Syngenta Seeds Inc.	Tolerance to phosphinotricin herbicide	*pat*
			Resistance to European corn borer	*cry1A(b)*
Rape seed (Argentine canola)	Ms8xRf3	Bayer Crop Science	Male sterility	*barnase*
			Fertility restored	*barstar*
			Tolerance to gluphosinate ammonium herbicide	*bar*
Papaya	55-1, 63-1	Cornell University	Resistance to papaya ringspot virus (PRSV)	CP (viral coat protein) gene
Potato	RBMT21-129, RBNT21-350, RBMT22-082	Monsanto Company	Resistance to colorado beetle	*cry3A*
			Resistance to potato leaf roll virus (PLRV)	*rep* + *hel* gene from PLRV

[a] The examples are meant to exemplify the diversity of traits introduced. While GTS 40-3-2 is the most widely distributed GM event at the present time, other listed events may not even be commercially available. For a full record of genetically modified plants, traits, etc., the reader is referred to e.g. [18].

1.3. The analytical procedure

Independent of the specific field of application, GMO detection methods generally constitute a part of a long series of steps that jointly may be referred to as the analytical procedure [19] which start with sampling and ends with the interpretation of the analytical result, see Figure 1.

The first step in the analytical procedure is sampling (I) of seed or in the field (a), from harvested crops during loading, transport, offloading or storage (b), from processed crops such as flour (c), from pre-packed foods (d) or from the consumer or livestock (e) to study possible healt effects. The second step in the procedure is processing of the sample into a laboratory sample (II). The third step is purification of the analyte (III), followed by laboratory analysis (IV). The final step (V) is the interpretation of the results of the laboratory analysis. Depending on the analyte and the purpose of the analysis, the analyst may apply protein based methods (e.g. lateral flow strips (a), metabolite profiling by e.g. GC-MS (b), qualitative nucleic acid detection e.g. by gel electrophoresis (c), identification through nucleic acid sequencing (d) or quantitation e.g. by real-time PCR (e). The specific sampling protocol (I) will depend on the matrix to be sampled. The major challenge is to find a balance between sample representativity and sample size. Reducing the sample size increases the sampling uncertainty, while increasing the sample size increases costs and may produce samples that are difficult to handle for the analytical laboratory. Where the lot is consisting of discrete units, e.g. seeds or grains, sampling may be fairly simple, (seed lots may be more homogeneous than grain lots). In the field, each individual plant may not have the same probability of being sampled, exposure to pollen from neighboring fields is not even across the field, and growth conditions differ locally within a field, e.g. due to wind, shade, ground water and herbivore predation.

A flour produced from a discrete batch of grain is likely to be homogeneous. Whether pre-packed foods are likely to be homogeneous depend on the ingredients. A blend of ingredient batches will most likely result in heterogeneity. Studies of effects on humans and animals are particularly challenging since the whole individual can not be sampled and analysed, and since effects may be limited to a particular tissue or organ. Most studies therefore have been limited to blood, feces and milk samples. The dilution effect is extremely high and sampling uncertainty therefore does not allow for drawing reliable quantitative conclusions. A batch or lot sample often consist of relatively large sample units (e.g. kernels or biscuits). Producing a laboratory sample (II) from a batch or lot sample primarily involves transformation of the sample into many (small) units, homogenize the sample and successively resample, to obtain a much smaller and yet representative sample. It may be particularly challenging to process the whole batch or lot sample, and to ensure that the processed material is truly homogeneous, e.g. with respect to particle size and distribution. Dry kernels consist of a hard seedcoat, a softer endosperm and a lipid rich embryo, and these will not respond equally to e.g. a blender with rotating knifes. Purification of the analyte (III) is probably the most critical step in the

analytical laboratory. The choice of purification protocol depends on the specific type of analyte, the matrix from which the analyte shall be extracted, and the yield and purity requirements of the analytical method to be applied. Unfortunately, apparently similar matrixes (e.g. maize gluten) may differ significantly in chemical and nucleic acid composition, and a purification protocol may not always be as optimal as expected. The choice of analytical method (IV) is usually a trade off between costs (time and money), level of detail (identity and quantity) and relevance of information. Protein based methods may be applied to screen grain for presence/absence of a particular protein, and are typically used on farms and for bulk testing because they are fast and cheap. Metabolite based methods may e.g. be used in relation to safety testing. Gel based methods are commonly used for screening purposes, but are more and more often replaced by semi-automated fragment analysis, quantitative real-time PCR methods, or sequencing, because this reduces the work-load, risk of carry-over contamination, and yields more detailed information.

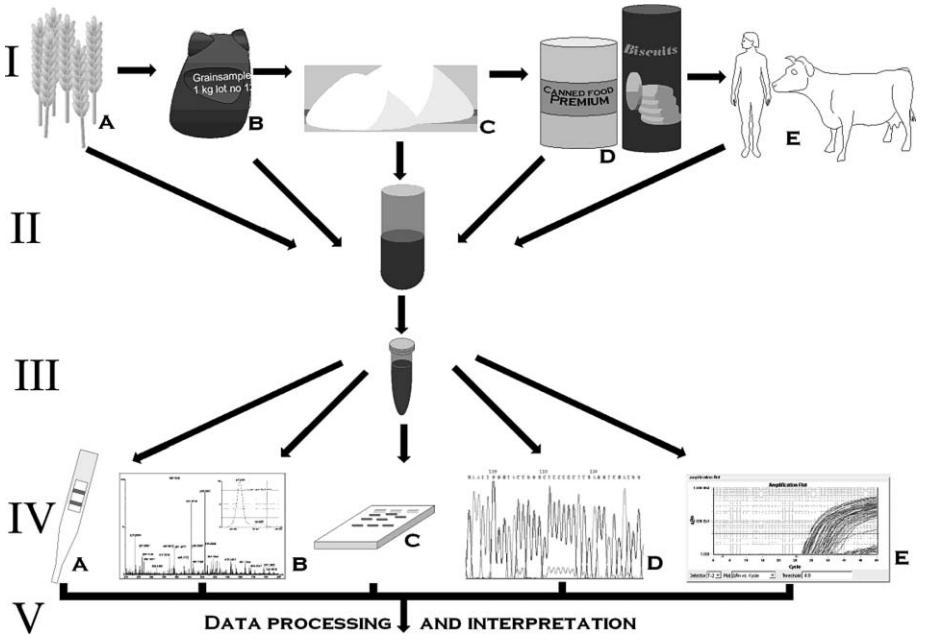


Figure 1. The analytical procedure and major challenges associated.

This chapter will outline the basic components of the entire analytical procedure, describe how each step can be performed, and discuss some of the major challenges associated with each step and the complete analytical procedure for a number of applications.

1.4. Analyte relationships and associated characteristics

A genetic modification is by definition a modification of DNA, which is also the subject to authorisation. A modified gene encodes the transcription of a messenger RNA (mRNA) sequence, and this transcription is regulated by a series of transcription factors including several proteins. Proteins are the products of translation of mRNA sequences into chains of amino acids, and the translation is regulated by multiple factors including availability of corresponding tRNA molecules and amino acids and the concentration of the final protein itself. The protein is the functional product of the genetic modification. More than one DNA sequence can give rise to the same mRNA sequence, and more than one mRNA sequence can give rise to the same protein. Sometimes the mRNA is degraded before it is translated to protein and sometimes DNA is not transcribed. Consequently, while there is a linear quantitative relationship between the copy numbers of any two given DNA sequences in all cells of an organism, independently on the tissue, season, climate, soil, etc., the same is not true for the relationship between any DNA and mRNA sequence, DNA sequence and protein, or mRNA sequence and protein. Lack of linear relationship may result in inconsistent measurements of GM content, e.g. between seeds sown and harvest produced or between results from protein and DNA based methods, with possible serious consequences for stakeholders. This can be avoided if a coherent definition of the unit of measurement is established [1].

DNA is very stable, due to its double stranded helix structure. The mRNA is a considerably less stable molecule which is typically degraded within minutes after synthesis in the cell. Proteins, on the other hand may be stable or unstable, depending on the type of protein as well as a range of biotic and abiotic conditions. Often, particular parts of proteins are more stable than others, and protein based detection methods may preferably be directed towards detection of these more stable epitopes, provided that they show sufficient specificity. A stable analyte is more resistant to degradation and therefore more likely to be detectable through the food production chain over time and in quantifiable amounts than unstable analytes which may be degraded or damaged after short time or as a result of processing. Methods that target stable analytes consequently have a wider application range than methods that target unstable analytes.

A detailed discussion about units of measurements and expression of GMO quantities, analyte relationships and factors that may affect the reliability of quantitative measurements may be found in [1]. DNA is inherited according to Mendelian laws, and the authors claim that gene (allele) ratios therefore can be predicted from seed to harvest and derived products. A GM insert can be present in homozygous (from both parents) or heterozygous (only from one of the parents) condition. Furthermore, a GM plant may contain inserts from more than one GM event, i.e. crosses between two different GM events result in gene stacking. Plants can be polyploid, and in some fruit tissues (endosperm and seed coat) the gene contribution

from the mother and father may not be the same. With DNA based methods, none of these factors would affect the predictable GM quantity in a product, provided that it is known what the DNA content was in the upstream material (e.g. seed) and that it is known what the resulting product is produced from (e.g. flour is usually produced only from endosperm, not from embryo or seed coat). The authors claim that no other alternative unit of measurement or expression of GM content would allow for such a predictability and coherence throughout the food production chain. The authors, however, admit that so called botanical impurities (e.g. presence of 1% soybeans that is 100% GM in a maize grain lot) may create a problem even with DNA based methods (e.g. if the maize is later mixed with a non-GM soybean ingredient), unless the botanical impurity itself is also measured. The authors also point out that the definition of "haploid genome" (one complete set of chromosomes) is critical, since a haploid genome may refer to at least two different things in polyploid plant species, including many food plants.

2. Sampling

The purpose of sampling is to obtain a representative sample. This can only be achieved on the basis of certain statistical assumptions, e.g. about the homogeneity of the original matrix, introducing a degree of uncertainty [20,21]. Generally, the larger the sample, the more likely it is to be representative, and it is often considered more appropriate to take many small samples, than few large samples. Sampling can be done systematically, e.g. taking every 100th unit or one unit every two minutes on a belt, or it can be done randomly, e.g. by dividing a lot into a three dimensional grid and sample e.g. 1% of the resulting subunits according to a random sampling plan (drawing a sufficient number of subunits randomly without replacement). For matrices with clearly identifiable units such as grains, potatoes or pre-packed foods, this may be feasible. However, if the matrix is e.g. pollen dispersed from one particular field to another, representative sampling becomes much more complex because pollen movement and concentration is affected by local climatic conditions (wind, temperature, humidity, air pressure), season (relative to pollen release from the plants) and pollen dispersal mechanisms (wind, insects, birds). Design of sampling plans may therefore become very difficult, and the choice of sampling strategy will have to be determined on a case-by-case basis, taking into consideration the desirable/acceptable level of sampling uncertainty as well as the cost of implementing various sampling plans. Normally, a highly reliable sample for GMO testing of food commodities can only be obtained with very costly sampling plans. In effect, this means that GMO sampling is often less reliable than desirable, because otherwise the sampling costs would be prohibitive.

Sampling to test for the presence of GMO or derived material is often compared with sampling to test for the presence of mycotoxins because the distribution of both mycotoxins and GMO derived materials may be extremely heterogeneous and overall contamination

levels low. Unfortunately, application of available sampling standards [e.g. 22,23] will produce samples that are associated with a very high degree of sampling uncertainty. It is therefore critical prior to implementing these standards that the user is aware of the associated sampling uncertainty, and decides whether or not the uncertainty is acceptable. An *ad hoc* working group within the European Committee for Normalisation (CEN), has produced a draft technical specification (TS) [24] that provides guidance on how sampling can be performed to obtain a pre-specified level of sampling uncertainty. Much of the data material that formed the basis for the TS is derived from two EU initiated research projects, "Kernel Sampling Technique Evaluation" (KeSTE) [21] and "Kernel Lot Distribution Assessment" (KeLDA) [25]. The former of these is a software that can be used to simulate kernel lots, and may be used to establish confidence intervals in relation to different sample sizes and strategies. This tool may therefore help the stakeholder in designing a sampling plan with *a priori* knowledge about the sampling uncertainty.

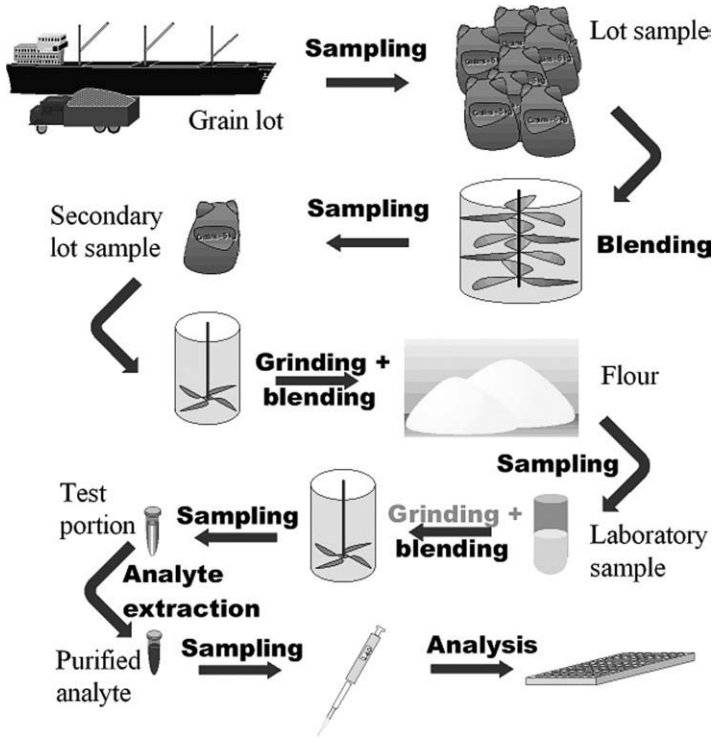


Figure 2. Sampling process from lot to test portion.

The process of obtaining an analytical statement about a product usually requires several sampling steps, involving measures to reduce heterogeneity (blending) of the material and various processing to facilitate sample size reductions (grinding) and purification and concentration of the analyte, see Figure 2. Before a lot of e.g. 50 tons of grains can be analysed, the original lot must be sampled and the sample reduced to a size that can be analysed in the test laboratory. To ensure that the final test portion is as representative of the original lot as possible, a series of steps and corresponding measures to reduce heterogeneity are performed. The first step is sampling of the lot. This may lead e.g. to the creation of a lot sample of 100 bags of 5 kg of grains. This lot sample is successively blended to produce a homogeneous distribution of grains from the original 100 bags. Then, a subsample is taken from the lot sample. This secondary lot sample may e.g. consist of a single bag of 5 kg of grains. This secondary lot sample may then be ground into a flour and simultaneously homogenised in the grinder. From this a laboratory sample is taken, e.g. consisting of 50 g of flour. The laboratory sample is then taken to the laboratory, homogenised again, and one or more test portions (of e.g. 500 mg) are then sampled and subjected to analyte extraction. Finally, the purified analyte is subject to analysis, e.g. by PCR, cf. Figure 1. This step too usually involves subsampling, since only rarely the entire quantity of purified analyte is used in the analytical reaction. Each of these steps have the potential to increase the sampling uncertainty.

3. Analyte purification

Purification helps to concentrate the analyte, so that it may be more detectable and possibly even quantifiable. This step typically removes cell debris and releases the analyte from any chemically or physically attached compounds that may, otherwise, interfere with the analytical reaction, usually by inhibiting detection of the analyte. It is critical that the purification protocol applied does not affect the representativity of the resulting matrix compared to the original matrix, i.e. that the relative ratio of GM and reference analyte is not biased by the purification step. For analytical GMO testing, there are primarily three types of analytes that could be studied: 1) the genetic modification itself, which is by definition a modified DNA sequence; 2) the transcript from the modified DNA sequence, i.e. an mRNA sequence; or 3) the translation product of the transcript, i.e. the functional product of the modified sequence, viz. a protein. The properties of each of these analytes differ, e.g. with respect to stability. Consequently, the analyte purification protocol should be selected accordingly. In particular, the use or incidental presence of analyte degrading enzymes such as DNases, RNases and proteinases must be under control.

The analytes are generally intact in fresh plant tissues and unprocessed grains, but once the material is stored and processed, the analyte may be severely affected. This has several

consequences. The commutability of a reference material and the product of interest is a critical factor, and in many cases processing and storage means that a truly commuting reference material is not available for the product. Application of purification and detection methods inappropriate for the material in question may lead to erroneous results. For example, if processing has removed all nucleic acids, then the use of methods to determine GMO content by DNA or RNA content is bound to fail. Depending on the processing a sample may contain compounds that will interfere with the analyte purification and/or the analytical method. The results may be low yield, low purity and inhibition of the analytical reaction, and consequently that the analyte concentration may be too low to be determined (on the basis of absolute quantity) or erroneous measurements and interpretations of the GMO content. Typical industrial food and feed processing shears DNA into short fragments that can not be detected with methods targeting lengthy fragments (>> 100 basepairs [bp]). Similarly, mRNA is often completely degraded and proteins are affected to various degrees.

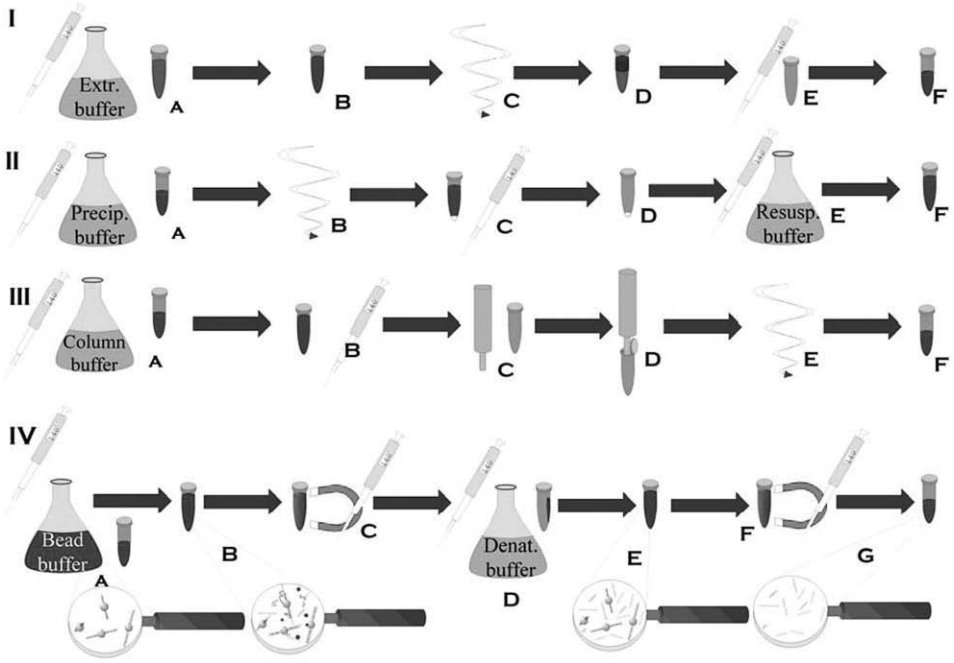


Figure 3. Analyte purification.

Ideally, the purification protocol will have a high recovery of the analyte while removing all other compounds that may interfere with the detection method that will be applied to the purified analyte. However, the choice of protocol may also depend on time and resources available. Automation and/or use of kits are often preferred, but for plant derived products this may result in low recovery and presence of various inhibitors of the successive analytical reaction. Many laboratories prefer manual purification protocols because the yield and purity of the analyte ensures the necessary reliability of the analytical result, while automated or kit based purification may yield low quantities of analyte of low quality, resulting in unreliable results or limits of detection and quantification that do not satisfy the needs of the stakeholder. Examples of DNA purification protocols may be found in [26] and an example of a protein purification protocol may be found in [27]. Figure 3 shows some of the typical steps in analyte purification protocols. The most commonly applied DNA extraction protocols are probably various types of CTAB and SDS based protocols, and various types of kits applying spin columns. Magnetic particle based protocols are primarily applied for automation. The CTAB and SDS based protocols normally have a high yield of high quality DNA, but they are laborious and involve the use of organic solvents like chloroform. The kits normally have lower yield and the DNA quality may also be inferior. However, there are many examples of matrixes where CTAB or SDS protocols do not perform well, and where kits may perform much better. Notably, kits are usually designed for specific applications, e.g. particular plant matrices.

There are a wide variety of analyte purification protocols available, and no single scheme can be used to illustrate all of them. However, the following is a description of some of the most typical steps and a representative order in which they are performed. The optional use of enzymes, organic solvents, spin columns and magnetic capture beads is matrix and purpose dependent. For example, the use of RNase, DNase or proteinase is only relevant if the purpose is to remove RNA, DNA or protein, respectively. (I) The basic protocol, more or less common to all methods and applications. (A) The first step is addition of the extraction buffer, and optionally of enzyme(s) to the test portion, followed by mixing and incubation (B). Successively, the sample is centrifugated (C) precipitating the cell debris and other undesired components (D). The analyte suspension is then transferred to a clean tube by pipetting (E,F). Further purification may involve repetition or modification of the basic protocol (I) or by selecting alternative purification protocols (II,III,IV). (II) Purification by precipitation is commonly applied in many DNA extraction protocols. The analyte may be precipitated with an appropriate precipitation buffer (A), concentrated by centrifugation (B) and the supernatant removed (C,D), and finally the analyte is rinsed and resuspended (E,F). (III) Alternatively, the analyte may be mixed with a buffer (A) and the mixture (B) transferred to a column (C) containing a semipermeable matrix, placed into a collecting tube (D), purified by spinning (E) and collecting the liquid that passes through the column (E). (IV) Finally, the analyte may be purified by application of magnetic particles coated with appropriate capture

molecules (e.g. nucleic acid probes or antibodies, A) The capture molecules capture the target analyte (B), the beads are retained with a magnetic particle concentrator (C) while the liquid and impurities are removed. Successively, the beads are resuspended in a denaturing buffer (D), leading to release of the analyte from the capture molecules (E). The beads are retained with the magnetic particle concentrator (F) and the liquid containing the purified analyte is transferred to a clean tube and renatured (G) with an appropriate buffer. Protocols may combine or omit elements, and particular measures may be required e.g. to cope with particularly lipid rich or polysaccharide rich matrices. These may include the use of smaller test portions, addition of larger volumes of extraction buffer, special buffers, etc.

4. Characterising GMOs

Characterisation of GMOs is performed in order to obtain a description of particular (distinctive and discriminatory) features, and in relation to analytical detection, may focus on the same parameters as described for analyte purification; DNA, mRNA and proteins. Metabolites can also be added. Isolation of each of these analytes can be relatively easy. However, to obtain an unknown part of the full length genome (DNA) or products (RNA/protein/metabolite) can be considerably more difficult. One of the challenges of GMO characterisation and detection has been the availability of samples of known origin that may be used as reference materials.

4.1. DNA sequence based characterisation

For DNA based characterisation, several PCR derived methods have been applied [28-32], all having in common a starting point which is prior knowledge about a short DNA sequence adjacent to the sequence to be characterised. The sample DNA is digested with a restriction endonuclease and the resulting DNA fragments are either self ligated to form circular molecules, or they are ligated to some type of adaptor prior to further characterisation. The short known DNA sequence is used as a PCR sequencing primer, and is typically combined with its own reverse complementary primer or a primer designed to amplify adapter-DNA. In the Qpcrgmofood project this approach was used successfully to characterise 11 different transformation events [33], and additional events have been characterised by others applying similar techniques [e.g. 34,35, see also 36]. Notably, almost every second genetic map produced in the Qpcrgmofood project revealed errors in the data submitted by the biotechnology companies with their applications for approval of their GM events in the EU [26]. On the basis of the obtained sequence data, event specific PCR methods were developed for all these events. It has since 2004 been a legal requirement that the notifier prior to approval of new GM events in the European Union [10,11] must provide complete and verified sequence data on the insert, the insertion locus and the integration junction, and a

validated event specific detection method. This requirement is partly a result of the observations and developments made in the Qpcrgmofood project.

DNA based characterisation usually involves cloning of DNA fragments of interest, followed by sequencing of the cloned fragments. With current DNA sequencing technology whole genomes of ≥109 bp can be sequenced within reasonably short time (a few years) by the most advanced laboratories. Per March 2006, only a few fully sequenced plant genomes are published (Arabidopsis thaliana [model plant], Oryza sativa [rice]). Several others are in the process of being completed (e.g. Glycine max [soybean], Hordeum vulgare [barley], Triticum aestivum [wheat], Zea mays [maize]) [37,38]. For the majority of crop plants, however, sequence information is fragmented, and usually limited to particular genes of interest to evolutionary biologists, taxonomists, breeders and plant geneticists.

Availability of detailed sequence information for a given plant species may facilitate detection and characterisation of changes in the genome as a result of genetic modification. It is also helpful in relation to risk assessments to be able to provide detailed information on the nature and function of insertion loci of the integrated sequence.

4.2. RNA based characterisation

The DNA based sequence information, corresponding to the whole genome, is always complete in the nucleus of the cells of all tissues. On the contrary, only parts of the total spectrum of RNA sequences that can be produced by the plant may be found in a specific sample, because the transcription of mRNA from the DNA depend on a series of regulatory elements. For example, in Event 176 (Bt-176) maize, there are two genetic constructs that encode the synthesis of the CryIA(b) insecticidal protein. However, both are regulated by tissue specific promoters [18], and as a result, the corresponding mRNA sequence may not be transcribed or may be transcribed at concentrations below the limit of detection (LOD). The effect is that if the sample is derived from the wrong tissue, then the mRNA can not be isolated and consequently it can not be characterised. Isolation of mRNA sequences can easily be achieved by poly-dT capture, e.g. a solid matrix with an attached poly-dT chain can be used to capture mRNA molecules because mature mRNAs have poly-dA tails. Successive characterisation of the captured mRNA molecules may be facilitated e.g. by cDNA synthesis applying a reverse transcriptase and again a primer derived from prior knowledge about a short sequence (cf. DNA based characterisation). Another alternative is to convert mRNAs into cDNAs followed by cloning of the cDNAs into a cDNA library and screen the library with e.g. labelled hybridisation probes corresponding to the previously known short sequence. The clones of interest are successively sequenced [39].

For characterisation of the mRNA profile of a GMO a cDNA library can be spotted on a micro-array. Cell lines of interest are then successively studied with or without prior treatment (e.g. herbicide treatment or physiological stress), successively subjected to purification of total mRNA and the purified mRNA is then labeled and hybridised against the

cDNA array to study the tissue and treatment specific changes in expression levels of specific mRNAs.

4.3. Protein based characterisation

Protein or peptide characterisation depends on purification of the protein/peptide of interest, e.g. followed by protein sequencing. However, protein function is highly dependent upon the three dimensional conformation that may depend on e.g. pH, temperature, and often also upon the interaction between several similar or different polypeptides, each resulting from a particular gene transcript. Characterisation of proteins and protein function may therefore be much more complex than characterisation of nucleic acids. On the basis of the protein sequence it is possible to predict the folding structure and function by comparison with other proteins with similar sequence and known folding structure and function. However, even very similar protein sequences may have different folding structures and functions. The folding structure and function of proteins can therefore not be reliably determined without use of advanced techniques for which a description is outside the scope of this chapter. Protein profiling is an alternative where total protein is isolated and successively analysed by gel electrophoresis and staining of total protein. This approach may be further combined with the use of labelled antibodies with affinity to specific proteins. One of the problems with protein profiling is that resolution may be insufficient and patterns too complex to allow for identification of the target proteins.

For specific detection of proteins the by far most widely applied approach exploits the affinity between selective antibodies and the protein (as antigen). Antibody production requires availability of pure protein or of a specific epitope of the target protein.

To better understand the function of genes and derived proteins, regulation of gene expression, etc. comparison between the genome (encoding the proteins) and the corresponding proteins, e.g. between species, tissues or cells subject to specific treatments can be made. Proteomics primarily focus on the transcriptional level, i.e. on RNA, although its purpose is to understand what is going on at the protein level. For more detail see e.g. [40].

4.4. Metabolite based characterisation

The function of a protein is often the catalysation of a chemical reaction, and the product of the reaction is a metabolite. While genes or proteins are often useful and more or less specific indicators of the presence of a particular GMO, the metabolites resulting from active genes may be of particular interest in the context of safety assessments. Particular attention may be paid to unpredictable effects, i.e. effects on metabolites that are not known to be associated with the function of the gene from e.g. studies on other taxa, metabolites similar to known toxins and allergens. For more detail see e.g. [40]. Metabolite based characterisation is frequently used to screen for presence of biotoxins, and may e.g. be useful to examine if

mycotoxin levels in GM and non-GM crops grown under otherwise identical conditions differ, or to see if the production of certain metabolites in the plant is altered relative to the non-GM counterpart. In these cases, the availability of appropriate standards (metabolite reference materials) may be required. However, it is also possible to compare metabolite profiles directly, and look for significant changes between the GM and non-GM counterpart [41,42].

Metabolite profiling and characterisation usually applies sophisticated chemical analytical techniques such as GC-MS. However, description and discussion of metabolite profiling and characterisation is largely outside the scope of this chapter.

5. Detecting GMO derived analytes

Generally, detection of analytes is based on some kind of capture of or by the analyte of interest, usually achieved by application of specific molecular interactions between the analyte and another molecule, in combination with some type of reporter signal. The reporter signal may either stem from a capture molecule, e.g. a labelled antibody or hybridisation probe, or from the analyte itself, e.g. if it is radioactively labelled or contains an intercalating fluorescent dye.

5.1. Establishing the GM quantity

Within the EU and most other jurisdictions with labelling requirements, the quantity of GM material shall be expressed relative to the species or ingredient, not to the total product. The GM quantity of a product is established indirectly by comparison against a reference sample. The features of the reference sample may be assigned on the basis of mass, particle numbers or analyte copy numbers. For protein based methods, the sensitivity of the method may be calibrated to slightly less than the maximum tolerated contamination level, in which case a positive test result may be interpreted directly as presence of higher levels of GM material than tolerated. It may also be that the method is semi-quantitative within a limited concentration range, i.e. that the signal intensity on e.g. a lateral flow strip correspond more or less linearly with the concentration of GM protein within that concentration range (typically 0.1% to 1% in kernels). Generally, protein based methods are matrix dependent, i.e. the analyte purification and detection steps are closely integrated.

For DNA based methods, the GM concentration is usually established as the ratio between a GM sequence and a taxon (species) specific sequence which is also the required approach as defined by the European Commission [43]. However, some laboratories also report DNA based quantities by comparing signal intensities directly against the reference material (taking an equal quantity of DNA from the unknown sample and the reference material). This approach, unless applied strictly to commutable matrixes, may produce highly erroneous

results. This is evident if the reference material is derived from 100% soybean material with a GM content of 1%, while the unknown sample contain only 1% soybean which is 100% GM. The approach would lead to the conclusion that the unknown sample contained 1% GM soybean, while the correct result would be that 100% of the soybean is GM. The GM concentration determined from the ratio of GM to taxon specific sequence is usually expressed according to the equation:

$$\frac{PFU_{GM}}{PFU_{Taxon}} \times 100\% = GM\% \qquad (1)$$

where PFUGM is the estimated number of copies of the GM specific sequence and PFUTaxon is the estimated number of copies of the species specific sequence in the same amount of the sample. These two parameters are determined by comparison against calibrant materials. Such calibrant materials are typically serial dilutions of plasmids or genomic DNA where the copy numbers (PFU) are determined experimentally. Unfortunately, such calibrant materials are not yet commercially available, and consequently, the PFU numbers associated with the calibrant materials are uncertain.

A less commonly applied equation for determination of the GM concentration is:

$$\frac{1}{2^{\Delta Ct}} \times 100\% = GM\% \qquad (2)$$

where ΔCt is the difference in Ct-value for the taxon specific sequence CtTaxon and the Ct-value for the GM specific sequence CtGM:

$$\Delta Ct = Ct_{Taxon} - Ct_{GM} \qquad (3)$$

The Ct-value or cycle threshold value, is the number of cycles required for the amount of amplification product (and corresponding fluorescence) to reach a defined threshold (baseline). This must be determined for both target sequences from the same template DNA (volume and dilution). Unfortunately, Eq. 2 and 3 can only be used if the amplification efficiency for both target sequences is exactly the same, since otherwise the difference in amplification efficiency will bias the quantity estimate exponentially.

Figure 4 shows how copy numbers (PFU) or Ct-values can be determined for a specific target sequence in a real-time PCR reaction.

The figure exemplifies reactions targeting a single target sequence, e.g. the GM specific sequence. Separate amplification reactions (e.g. tubes or wells on a microtiter plate) are shown as a separate coloured graphs. The fluorescence released in response to synthesis of the target amplicon is measured in real-time. The increase in amplification products in the initial PCR cycles is too small to be detectable (dotted lines), but as the reaction proceeds it

enters a phase where the increase can be visualised as a linear increase in signal (the log linear phase, shown as unbroken lines). When the signal corresponds to a threshold level (the baseline, red horisontal line on figure), the corresponding cycle number for the reaction is scored (*Ct*-value). Quantitation can be done using a direct comparison of *Ct*-values (*ΔCt* method), or by comparison of copynumbers derived from a standardcurve (standardcurve method). For an unknown sample two reactions of the same concentration of the template DNA must be performed, one targeting the taxon, the other targeting the GM-specificsequence. A reaction representing the unknown sample is shown in the figure as a black stipled graph. While the use of *ΔCt* for quantitation is only valid if the two targets are amplified with the same efficiency, the use of the standardcurve method is not limited by such a requirement. With this method, a standardcurve based on amplification of a series of reactions with known initial target copy number is established for each of the two targets. A regression line (the standard curve) relates initial target copynumber to *Ct*-value, consequently allowing for conversion of the *Ct*-values of the unknown sample to initial target copynumbers. In the example shown the Ct-value is 23.76 because this is the number of cycles required for the fluorescence signal to equal the baseline signal. The corresponding number of PCR forming units (PFU = amplifiable template copies) is estimated to 4756 from the standard curve, because the *Ct*-value of the unknown sample (23.76) regressively correspond to 4756 PFU.

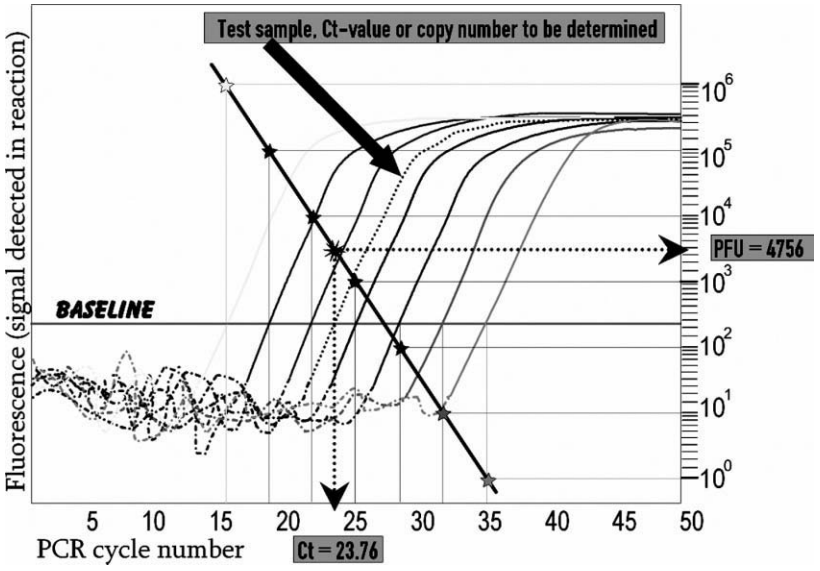


Figure 4. Real time PCR based quantity determination.

5.2. The unit of measurement and expression of GM content

Whether the GM content of a product is measured or expressed on the basis of mass, particle number (seeds, kernels), volume or analyte copies, is highly relevant. Unless clearly defined units are applied, the measurement or expressed content can be misinterpreted. Unfortunately, this is not sufficiently recognised by the majority of stakeholders, although the problem has been highlighted for at least half a decade [44,45]. This is discussed in extensive detail in [1]. For example, due to different paternal and maternal contribution of haploid genomes in maize endosperm, a GM maize flour produced from 1% GM grain (determined by mass or grains) may contain between 0.33% and 0.67% GM DNA, if only one of the parental maize plants was GM and from a single GM event. However, if both parents were GM and/or from two or more GM events (gene stacking) then the DNA based GM content may be >> 1%.

5.3. Protein based detection methods

Currently applied protein based detection methods are primarily derivatives of immunological techniques, i.e. techniques that exploit the affinity of specific antibodies to the antigen (the protein of interest). The Western blot technique is based on separation of total protein on a gel followed by staining of the target protein with a labelled antibody. Enzyme Linked Immuno-Sorbent Assay (ELISA) and derived lateral flow strip (LFS) techniques use two or more specific antibodies in a sandwich format. One antibody attached to a solid surface and specific to the target protein is used to capture the target protein, while another antibody labelled to allow for detection of the presence of the protein will bind to the captured protein. The development of antibodies for immunological methods requires access to the purified protein. The antibodies may be more or less specific to the protein and GMO of interest, depending on which part of the protein the antibody will target. Currently available LFSs may fail to distinguish between different GMO events expressing the same protein, even if the proteins are slightly different. Immunological methods may also fail to detect particular GMOs, e.g. Event 176 (Bt176) maize where the expression of the CryIA(b) gene is under control of two tissue specific promoters that confer expression in green tissues and pollen, respectively, but not in kernels.

Protein based methods are often considerably cheaper and faster than other analyte based GMO detection methods, and are consequently preferred over DNA based methods for practical screening of large unprocessed agricultural commodities in the field and during harvest, storage and transportation. The sensitivity of e.g. LFSs may be calibrated to determine whether the GMO content exceeds a particular threshold or not, but generally the protein based methods are inferior to DNA based methods for quantitation purposes, as well as for identification of specific transformation events.

Protein based detection methods may be divided into specific and non-specific methods, where the former rely on detection by immunological reactions involving the binding between the antigen (the protein or an epitope of the protein) and an antibody. Antibody production requires availability of the pure protein or its epitope. Although it may be possible to obtain a large and pure quantity of protein by e.g. cloning the gene encoding the protein into a bacterium, the resulting expressed protein may not correspond structurally and functionally to the protein in the genetically modified plant. This may have impact not only on the reliability of the protein based detection method, but also on e.g. feeding trials to study the safety of the protein, if the protein source is not commutable with the GM plant and its derived products.

Unspecific protein characterisation can be achieved by gel electrophoresis of total protein homogenates from the potentially genetically modified plant, where the proteins are successively stained and patterns compared with e.g. the pattern produced by the non-GM counterpart of the GM plant.

5.4. RNA based detection methods

A few RNA based detection methods deserve to be mentioned, although none of them have found wide application in relation to GMO detection. The oldest approach is the Northern blot, where total RNA is separated by gel electrophoresis followed by blotting of the RNA to a membrane and hybridisation with a labelled probe specific to the RNA of interest. The RNA may successively be purified and sequenced, e.g. via reverse transcription and cDNA cloning, to verify the identity of the RNA.

An amplification technique called Nucleic Acid Sequence Based Amplification (NASBA) [46] has also been proposed for GMO detection in connection with a European research project (DMIFGEN) [47], but no published data on the results of this are available, and the technique is unlikely to find widespread application due to the instability of mRNA in processed food- and feedstuffs.

5.5. Metabolite based detection methods

Generally, metabolite based detection methods also involve characterisation in conjunction with the detection. A profile is created for a reference which may be GM and/or non-GM, and the profile of the unknown sample is compared with the profile(s) of the reference(s). Published alternatives include the use of near infrared spectroscopy (NIR) [48], nuclear magnetic resonance spectroscopy (NMR) [49], surface plasmon resonance [50] and HPLC profiling [51]. Distinction between the GM and non-GM materials is reported to be simple, but in addition, at least some of these methods appear to provide additional resolution, e.g. between different non-GM materials as a result of differences in production of particular metabolites. While these techniques therefore may find applications for screening purposes, they may not suffice to reliably identify from which event the material is derived.

5.6. DNA based detection methods

5.6.1. Target specificity

With reference to current international standards for DNA based GMO detection [52-54], one may distinguish between four different levels of specificity of the analytical targets for DNA based detection methods (Table 2). The lowest level of specificity is characteristic of the taxon specific methods, i.e. methods that may be used to identify whether DNA of a particular taxon (usually a species, e.g. soybean or maize) is present.

The second level, i.e. the lowest level of specificity associated with specific detection of GM material may be classified as screening methods. These are methods targeting genetic elements that may be found in several different GMOs, e.g. common promoter or terminator elements, elements from cloning vectors or some genes that have a widespread use [62]. Unfortunately, these screening elements may also be found naturally, e.g. due to presence of viruses, bacteria or other natural sources of the target sequence.

Table 2. Examples of GM detection methods, ranked by specificity, with examples of their application

Method category	Target	Application in the food chain	Type of method	References
Protein based methods				
	EPSPS protein in soybeans	Qualitative or semiquantitative screening for Roundup tolerant GM soybeans	Lateral flow strip (LFS)	[27]
	CryIA(b) protein in maize	Qualitative or semiquantitative screening for insect resistant maize lines	LFS	Commercial kits
DNA based methods				
Species/taxon specific methods	Chloroplast trnL-trnF intron	Qualitative or semiquantitative screening for plant derived DNA	Conventional PCR and gel electrophoresis	[52]
	Chloroplast trnL-trnF intron	Qualitative or semiquantitative	Conventional PCR and array analysis	[55]

Method category	Target	Application in the food chain	Type of method	References
		screening for DNA from particular cereal plant species		
	Cauliflower mosaic virus (CaMV) ORF V and ORF VI	Detection of DNA from the CaMV as negative control for P35S based GM screening	Conventional PCR	[56]
	Maize *adh1* gene	Quantitation of DNA from maize	Real-time PCR	[53,57]
	Soybean *lec1* gene	Quantitation of DNA from soybean	Real-time PCR	[53]
GMO screening methods	CaMV 35S promoter (P35S)	Screening for and semiquantitation of the commonly used P35S promoter as indicator of presence of GM material	Conventional and real-time PCR	[52,53]
	Agrobacterium tumefaciens *nos* terminator	Screening for the commonly used *nos* terminator as indicator of presence of GM material	Conventional and real-time PCR	[52,58]
	Phosphinotricin acetyltransferase *pat* gene	Screening for the *pat* gene as indicator of presence of GM material	Conventional PCR	[58]
	CryIA(b) gene	Screening for the *CryIA(b)* gene as indicator of presence of GM material	Conventional PCR	[58]
	Multiple genes,	Screening for	Multiplex PCR and	[59]

Method category	Target	Application in the food chain	Type of method	References
	regulatory elements and construct specific junctions	presence of GM derived sequences to try to establish qualitative information about GM content	array based screening	
	Multiple genes, regulatory elements and construct specific junctions	Screening for presence of GM derived sequences to try to establish semiquantitative information about GM content	Multiplex PCR and array based semiquantitative screening/identification	[60]
GM construct specific methods	IVS6-*CryIA(b)* junction	Quantitation of genetic construct present in e.g. Bt11 and Bt10 maize	Real-time PCR	[53]
	P35S-CTP junction	Quantitation of genetic construct present in e.g. GTS 40-3-2 soybean	Real-time PCR	[53]
GM event specific methods	Junction between recipient plant genome and inserted construct in GTS 40-3-2 soybean	Specific detection and quantitation of DNA derived from GTS 40-3-2	Real-time PCR	[61]
	Junction between recipient plant genome and inserted construct in several GM events	Specific detection and quantitation of DNA derived from the GM events	Real-time PCR	[36]

The third level of specificity is often referred to as construct specific methods. These are methods targeting sequence motifs originating from the joining of genetic elements in the creation of the genetic construct, i.e. combined sequence motifs that can not be found naturally. Therefore detection with construct specific methods may be considered as evidence of presence of genetically modified DNA. Because the same construct is sometimes or potentially may be used to produce more than one GMO (event), cf. Figure 5, the specificity of construct specific methods is sometimes insufficient to identify the GMO [1, 62].

The final and highest level of specificity is associated with so called event specific methods. These are methods targeting the sequence motifs created by the integration of the genetic construct into the recipient genome, i.e. the junction motif spanning a part of the inserted DNA and a part of the recipient genome. This combined sequence motif is not only undisputable evidence of presence of genetically modified DNA, it is also undisputable evidence of the transformation event from which the DNA is derived. Furthermore, the event specific motif is always single copy, i.e. only one copy of the sequence motif is created during the transformation process. The newly transformed cell has two sets of haploid genomes, and only one of these contain the event specific sequence motif. Although, it is possible due to back crossing of successive generations of cells to produce plants where all haploid genomes contain the event specific sequence motif, it is still true that each haploid genome can only contain one copy of the motif. Therefore, event specific sequence motifs are perfectly suited for quantitation of genetically modified materials, although even this concept may be associated with some controversial implications [1], primarily how the haploid genome is defined.

5.6.2. Technologies applied to DNA based GMO detection

Although there are a number of alternative technologies that could be applied to DNA based GMO detection, practically all published methods rely on amplification techniques, mainly the polymerase chain reaction (PCR). Direct analysis of genomic DNA, e.g. by direct hybridisation, is only possible if a large quantity of DNA from the same species and eventually GMO is available, because otherwise detection limits associated with current technologies will render the target undetectable. The main part of this chapter will therefore focus on PCR based detection methods. At this point it may also be appropriate to point out that the quality of the DNA has considerable impact on its amplifiability. DNA damaged by strand breaks or physically linked to other compounds such as proteins, may not be amplifiable. Therefore, by definition PCR can only amplify PCR forming units (PFU). This is important because DNA based GMO quantification is based on establishing the relative ratio between PFU representing the species (ingredient; PFUTaxon) and the GMO of interest (PFUGM), respectively (see section 5.1).

5.6.3. Qualitative detection methods

Qualitative detection methods are methods applied to assess whether or not a particular target DNA sequence is present. These methods are typically applied for screening purposes, such as screening of maize samples for presence of P35S which is known to be present in most presently authorised GM maize events. More sophisticated approaches could involve combinatorial analyses for presence/absence of a series of specific genes or other genetic elements, followed by deduction of which GMO(s) that were used to produce the analysed sample [59]. Multiplex screening assays may also be used to identify species specific DNA sequences [55], to determine if DNA from species that include GM varieties is present in the sample. This may facilitate the successive choice of more specific detection methods, since it may exclude further analysis to detect GM varieties belonging to species not found in the sample. Interestingly, the latter type of method may also find applications in the context of verifying ingredient declarations, detecting potential allergens and hosts of particular mycotoxin producers.

A number of multiplex GMO screening assays have been developed. The majority of these are conventional multiplex PCR assays, i.e. methods where several pairs of PCR primers are combined and where the PCR amplification of target sequences in the unknown sample will occur under more or less competitive conditions [63-65]. Such assays usually require extensive optimisation to achieve as similar amplification efficiency as possible for all the targets. Most often, some of the target sequences will amplify with lower efficiency than others, and the impact of different starting concentrations of the different target sequences may severely affect the final result. A number of different approaches for analysis of the resulting pool of amplification products have been proposed, ranging from conventional gel electrophoresis via capillary electrophoresis to microarray analysis [55,59,60,66]. Direct array based hybridisation analysis has also been proposed [67], although this may be more complex due to the significantly reduced sensitivity resulting from fewer target copies in a high background of non-target genomic DNA. An advantage may be that the risk of false positives resulting from amplification of minute DNA contamination is eliminated.

A highly sophisticated approach exploiting the growing amount of detailed genome sequences was recently proposed [68]. This approach involves a strong bioinformatics component and the use of a very high number of random probes and full length genome hybridisation to high-density arrays to detect and eventually profile a suspected unknown GM material (pure single specimen derived DNA). The positive hybridisation signals are then used to construct PCR primers that may successively allow for sequencing of unknown genetic constructs. This and other approaches are currently being investigated in the EU funded research project Co-Extra [69].

5.6.4. Quantitative detection methods

Quantitative detection methods may be divided into semi-quantitative and fully quantitative methods. Semiquantitative methods may e.g. be screening methods applied to assess whether a full quantitation is required or not. An example could be quantitative PCR based P35S analysis, where 1) a very low quantity detected in e.g. maize (preferably together with a similar result for each of the GM maize that do not contain the P35S) may lead the analyst to conclude that the GM DNA content of the maize is certain to be below a contractually or regulatory specified threshold (e.g. for labeling); or 2) a very high quantity detected in e.g. maize may lead the analyst to conclude that the GM DNA content of the maize is certain to be above a specified threshold. Then, only samples falling outside the two described examples might require the analyst to proceed with specific testing and quantitation.

A multiplex semiquantitative approach has been published [66] where a DNA-array based approach was combined with a PCR derived approach, and specific probe labeling prior to hybridisation.

Fully quantitative methods are typically applied to determine the quantity of single GMOs, often followed by evaluation of the additive contamination relative to a specified threshold (regulatory or contractual). The majority of published and currently applied detection methods fall into this category. Notably, however, as the number of events approved in a global perspective is constantly increasing, so does the number of separate quantitative tests needed to establish the additive GM DNA quantity. At some point this will result in extremely resource costly analyses, and other alternatives are therefore urgently needed. Ongoing efforts, e.g. in the frame of the Co-Extra project [69] include application of DNA-arrays, multiplex-PCR methods, alternative amplification techniques and direct hybridisation approaches.

PCR based quantification methods may be divided into three types: the competitive PCR approach [70-73], the most probable number (MPN) statistics approach [74-76], and the most widely applied approach which is the real-time PCR [53,77,78].

Competitive PCR is based on the idea that two similar but distinguishable sequences (amplicons) can be co-amplified with the same efficiency, and that the final relative ratio of their quantities is the same as their initial relative ratio of quantities. Competitive PCR is set up with one amplicon representing the target sequence, i.e. the GMO derived sequence or the corresponding species specific reference gene, while the other is a so called competitor, i.e. a sequence almost identical to the target sequence, but modified to be distinguishable e.g. by size. By setting up a series of dilutions, and determination of the dilution level at which the two competing amplicons are present in equal amount (always knowing how much of the competitor is added), is it possible to assess the quantity of an amplicon of interest. This is done for both of the unknown sequences, and their relative ratio can then be calculated on the basis of the dilution factors and the amount of competitor in the PCR reactions. Assessment of the point of equilibrium is done by gel electrophoresis and visual inspection. This

technique is largely replaced by less labour intensive and error prone quantitation techniques, and the interested reader is therefore referred to [62] for more detail.

MPN statistics is based on the idea that if a volume (V) containing X units of an attribute (e.g. a GMO derived DNA sequence) is divided into a set of N equally sized subvolumes (v), then the distribution of the units will follow a Poisson distribution model, in which each v may contain any number between zero and X with an associated probability. If X = N, then the nominal value, i.e. the expected number of attributes in any sampled v is one. However, the Poisson distribution model also tell us that for X = N the probability of absence of the attribute in any v is approximately 0.3. In effect this means that whenever the average number of attributes per v is one, approximately 30% of the N subsamples will not contain the attribute. If X is increased without increasing N, this probability is reduced, while reducing X without reducing N increases the probability of negative subsamples. These probabilities associated with the ratio of X:N can be tabulated. To use MPN methodology, it is necessary to dilute the attribute concentration to a nominal value close to one per v. By taking a sufficient number of subsamples and analyse for the presence of the attribute using a highly sensitive method such as PCR, the true X/N ratio can be estimated with a high degree of reliability by comparison with the tabulated probabilities. Quantitation of the GMO concentration is then done by comparing the estimated PFU numbers from dilutions, taking into consideration the degree of dilution.

Real-time PCR is based on quantitation by measurements of fluorescence in real-time, where the fluorescence signal is corresponding to the increase in amplification product synthesis. Two alternative approaches have been described, one applies a DNA intercalating dye, the other applies specific probes. Amplification of the target sequence results in the synthesis of double stranded DNA (dsDNA), and the dye emits fluorescent light when it is intercalated in the dsDNA. The dye does not discriminate between the specific target and any co-amplified non-target sequence. However, it is possible to verify the identity of the amplified dsDNA by melting curve analysis, i.e. by subjecting the dsDNA to denaturing temperature (95C) followed by a gradual cooling. The reassociation of the dsDNA will result in the creation of a peak signal, and the specific temperature profile of the peak is characteristic of a specific sequence. Unfortunately, it may be difficult to quantify each specific dsDNA sequence if more than one amplicon is present. With probes, the reliability of real-time PCR increases significantly. However, probe design can be difficult, and probe chemistries render probes highly expensive. Probes for real-time PCR may be divided into different categories; e.g. hydrolysis probes and hybridisation probes, or activation and quenching probes. Hydrolysis probes emit fluorescent light upon degradation, while hybridisation probes emit fluorescence upon specific hybridisation to the target amplicon sequence. All involve two types of fluorophores and their function is linked with the spatial location of the fluorophores relative to each other. To emit light, activation probes require that the two fluorophores are brought into close proximity by hybridisation and/or altered

configuration of the probe(s), while quenching probes require that the two fluorophores are sufficiently separated in space by hydrolysis and/or altered configuration of the probe(s). The most commonly applied real-time PCR probe chemistry is TaqMan chemistry, a type of hydrolysis probe where a reporter and a quencher fluorophore are located on the same probe, within a relatively short distance apart, and where the hydrolysis of the probe results in the release of the reporter from the quencher.

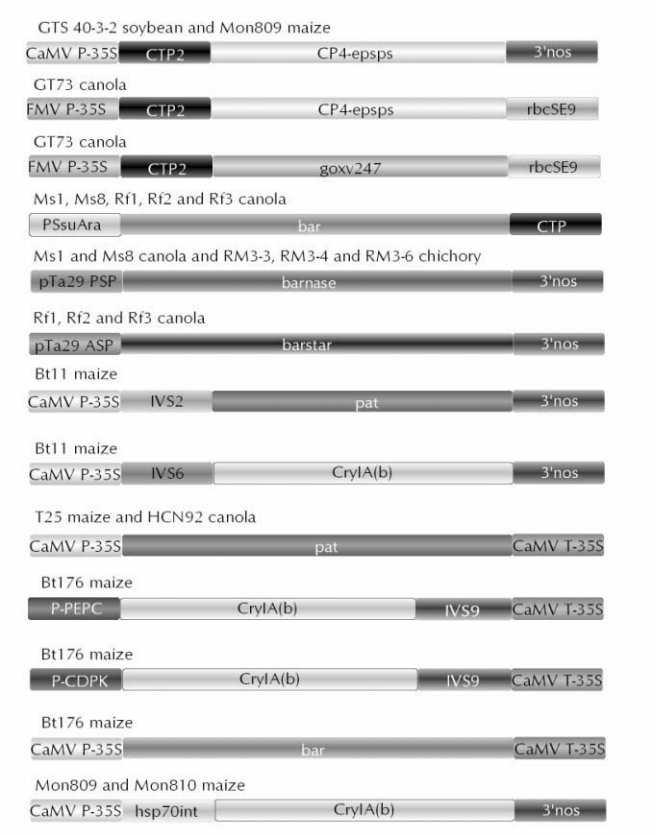


Figure 5. Schematic structure of the genetic constructs used to transform some of the GMOs authorised on the world market, source: [18]. If the same promoter was used in different gene constructs, this will be seen as promotors with the same colour. Similar for enhancers, genes and terminators. Note that although the same elements may appear in different constructs, they may differ at the sequence level.

Recently, Rudi et al. [79] proposed to use quencher extension to measure sequence amplification in real-time. With their approach, named QEXT, amplification is associated with a decrease in fluorescence, in contrast to conventional approaches using hydrolysis probes. QEXT may therefore be described as an inverse TaqMan approach.

A number of alternative approaches for detecting target DNA sequences have been described for GMOs. The majority of these deal with detection of captured target sequences on probes attached to a solid phase e.g. arrays [80-82].

5.7. Detection and quantification limits

The limits of detection (LOD) and quantification (LOQ) are defined as the lowest concentration of the analyte that can be reliably detected and quantified, respectively. Usually the LOD and LOQ refer to the limits associated with 95% probability of obtaining a correct result. For quantitative analyses this also needs to be associated with an acceptable uncertainty range, e.g. the obtained analytical estimate E must not deviate more than E/2 from the true value.

The LOD and LOQ of analytical methods may refer to absolute and relative values, depending on the type of methodology and attribute [61,62]. For conventional chemical analyses, relative values apply because the detectability/quantifiability is related to the number of attributes and the analytical volume. Notably, however, with PCR methodology in principle every PFU will form the basis for amplification, i.e. every PFU is by definition detectable/quantifiable. Therefore, for PCR based methods, the LOD/LOQ is more correctly referred to as absolute values, i.e. the lowest nominal number of attributes per analysed subvolume v that can be reliably detected and quantified, respectively. As described above in section 5.6.4, the nominal value one is associated with approximately 30% negative rate. From a Poisson distribution model, the nominal value associated with less than 5% negative rate is approximately five [61]. This implies that the LOD of PCR based methods can not be less than five copies of the attribute (the target sequence). The LOQ is more difficult to estimate, because it is dependent on the acceptable uncertainty range. Data from validation studies suggest that the LOQ of PCR methods may be approximately 100 copies of the attribute, if the maximum uncertainty range is 25% to 50% with a 95% confidence range.

Most stakeholders refer to relative quantities rather than absolute quantities. Therefore, it is desirable to be able to provide relative LOD and LOQ estimates in addition to absolute values. This can be done for any purified DNA solution by establishing the absolute concentration of haploid genomes of the relevant ingredient species PFUTaxon (cf. section 5.1) and divide the absolute LOD/LOQ of the GM derived attribute by PFUTaxon. For more detail, the reader is referred to [62].

5.8. Reference materials

Reference materials serve several functions. They may be used to verify the specificity of the analytical method, to calibrate the detection system, or they may be used to verify the ability of the applied method to properly purify and/or detect the attribute. A wide range of reference materials are therefore produced, each associated with a particular purpose in mind.

The most widely applied reference materials for GMO testing purposes are matrix matched certified reference materials (CRMs) produced from ground kernels by the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium. These CRMs have been produced by blending of GM and non-GM kernels on the basis of mass ratios, and per March 1st 2006 the available CRMs include GTS 40-3-2 soybeans (RoundupReady, ERM-BF410 series) and Event 176 (Bt-176, ERM-BF411), Bt11 (ERM-BF412), Mon810 (ERM-BF413), GA21 (ERM-BF414), NK603 (ERM-BF415), Mon863 (ERM-BF416), Mon863 x Mon810 (gene stacked, ERM-BF 417) and 1507 (ERM-BF418) GM maize. The rationale behind production of these IRMM-CRMs was that they would correspond to the matrix of interest. Notably, however, the analytical methods applied to GM testing do not measure mass ratios, but measure analyte concentrations either as absolute or as relative values. Furthermore, the matrices of interest are not limited to ground kernel materials, and consequently, the IRMM-CRMs often do not commute to the matrix being subject to analysis. While it has been proposed that the lack of commutability may be solved by adjusting the quantitative measurements by applying a correction factor *Cf* to correct for the bias introduced by differences in ploidy or zygosity, DNA content per mass unit, etc. [annexes C4-C9 in 53,83], the correction factor would still only apply correctly if the unknown sample material and the reference material used to determine the *Cf* commute. The problem then is that the unknown sample material by definition is unknown, and consequently that assuming commutability is not justified.

Another widely applied type of reference material is cloned plasmids, i.e. solutions with known concentrations of cloned target DNA sequences. Such reference materials are an integral part of the current national standards of Japan [84,85] and some of the quantitative methods published in ISO 21570 [annexes C4-C9 in 53], and have also been produced by several European laboratories [86]. The European Network of GMO Laboratories (ENGL) [87] is currently establishing a series of plasmid based reference materials that should serve as an integral part of the European Communities GMO Reference Laboratory (GMO-CRL) [88] system, i.e. for enforcement purposes by the national competent authorities.

A less widely applied but highly relevant type of reference material is purified genomic DNA, i.e. DNA isolated from the plant material [e.g. 89]. The main reason why these materials have a more narrow application is that it may be difficult to produce it in sufficient quantity and to assess the target sequence copy number, taking into consideration whether the copies are amplifiable or not. One possible means of doing this is by application of MPN statistics. This approach is currently being explored by several European laboratories.

For the time being, protein based methods mostly rely on the existing IRMM-CRMs, although some commercial kits may include other sample material. It is also possible to develop protein based reference materials, but no such material is presently available.

For RNA based methods, the existing IRMM-CRMs may not be fit for purpose because the RNA is unlikely to survive the relatively harsh processing associated with the production of the CRMs. No other appropriate reference material is presently available for RNA based methods, although whole kernels of freshly sampled plant materials could serve.

The currently available CRMs are only certified with respect to the attribute(s) of interest, i.e. they are not always pure in the sense that they are not certified for absence of other GM derived sequences. Consequently, they may be fit for verification of presence and quantity of the attribute, but not for specificity testing, i.e. demonstration of the absence of cross reactivity. For example, the ERM-BF414 (attribute GM maize GA21) also contain a small quantity of GM maize Mon810 [90].

5.9. Method validation and performance reliability

The traditional GM detection method validation approach is referred to as the global approach, and includes analyte extraction and detection/quantitation. Alternatively the modular approach [19] introduces more flexibility and potentially reduces costs of validation and application of the detection methods. For example, the global approach implies that every time a method is validated, a DNA extraction module and two PCR modules need to be validated. For more details on the potential benefits of applying the modular approach, one may consider the following examples:

Example 1:

To provide a typical GMO related scenario, where modules related to detection of GM maize are considered, imagine that the module collection for the final two steps of the analytical procedure include the following six modules:

extraction of DNA from maize flour
extraction of DNA from maize grits
real-time PCR for *adh* gene to quantify maize derived DNA in a pure DNA solution
real-time PCR for *hmg* gene to quantify maize derived DNA in a pure DNA solution
real-time PCR for genetically modified maize *S* derived DNA in a pure DNA solution
real-time PCR for genetically modified maize *T* derived DNA in a pure DNA solution

When method validation follows a global approach in which validation is done for a combination of modules, then only the complete combination (constituting the method) can be applied (no flexibility in the application of modules). Imagine that the following methods have been validated applying the global approach:

A + *C* + *E* (to test for GM maize *S* in maize flour)
B + *D* + *F* (to test for GM maize *T* in maize grits)
B + *C* + *E* (to test for GM maize *S* in maize grits)

Validation of the method requires successful performance of all three modules, and some modules may be included in the validation of several methods. To validly apply the modules according to the global approach, they can only be used in the combination that has been validated. The global approach would therefore not allow the analyst to validly perform a test for presence of GM maize *T* in maize flour (method = *A* + *C* + *F* or *A* + *D* + *F*), nor would it allow the analyst to substitute the use of the *adh* (module *C*) with the hgm (module *D*) to rationalise the analysis (implying running only one reference gene PCR system to establish the quantity of maize derived DNA in a sample of maize grits).

With the modular approach, the analyst may select appropriate modules, provided that each module has been validated for the relevant input material. Therefore all combinations of modules *A* or *B*, *C* or *D*, and *E* or *F* would be allowed (yielding 2 x 2 x 2 = 8 methods) in stead of 3 methods defined by the global approach. Furthermore, four methods for a new GMO could be made available just by validating a single new module *G* = real-time PCR for genetically modified maize *U* derived DNA in a pure DNA solution, i.e. because module *G* could be combined with both module *A* and *B*, and with module *C* and *D*. Modular validation may require fewer resources since each module is only validated once, instead of every time it is part of the method as it is done with the global approach.

Example 2:
The following example extends the use of modules to include also other food/feed related applications. For the final two steps of the analytical procedure the module collection could e.g. comprise modules for:

extraction of DNA from soya bean flour
extraction of DNA from wheat flour
real-time PCR to quantify soya bean derived DNA in a pure DNA solution
real-time PCR to quantify genetically modified soya bean *X* in a pure DNA solution
real-time PCR to quantify a mycotoxin producing fungus *Y* in a pure DNA solution.
Imagine then that the following two methods have been validated globally:

IV) *H* + *J* + *L* (to test for fungus *Y* in soya bean flour)
V) *I* + *J* + *K* (to test for GM soya bean *X* contamination in wheat flour).

Under the global approach, the analyst can not apply the modules to perform a valid test for GM *X* in soya bean flour. Such a test will require the use of modules *H* + *J* + *K*. Although, each module is part of one of the validated methods IV and V, the combination (the method) has not been validated. In the absence of a validated module for extraction of DNA from texturised soya bean protein, modules *J*, *K* and *L* can not be validly applied to pure DNA solutions extracted from texturised soya bean protein. However, with the modular approach, validation of a new module for extraction of DNA from texturised soya bean protein may be performed as a stand alone validation. This new module may then be combined with modules *J* and *K* to provide a complete method to test for GM soya bean in texturised soya bean protein.

6. Concluding remarks

Characterisation, detection, identification and quantification of GMOs have been performed routinely for almost a decade. However, we are still far from an international consensus on what the characterisation shall cover and how GMOs shall be detected, identified and quantified. This is partly linked with the position and level of education of the various stakeholders involved. While the large producers see GMOs as equivalent to conventional crops, others like many European consumers are more sceptical and demand labeling to be able to choose between GM and non-GM products. The costs of GM testing are highly dependent on the level of detailed information that shall result from the testing. Different testing methods most likely do not yield the same results, and even the same testing method may sometimes produce significantly different results in different laboratories. While harmonisation of the methods of choice is the objective of international standardisation, the lack of international agreement is reflected in the fact that the only currently available GMO testing standards [26,27,52-54] are all informative, simply because it has not been possible to agree to whether e.g. protein or DNA is the prevailing analyte. Furthermore, the law-makers do not seem to appreciate the analytical realities, i.e. that it is impossible to establish the GM content of a product on the basis of mass, volume or particles except in some very particular cases. Consequently, the lack of coherence between the regulatory and the analytical world is a major obstacle to international harmonisation [1].

Availability of appropriate reference materials has been a major challenge since the first critics of GM foods sounded, and most likely will continue to be a problem in the foreseeable future. However, the scientific community may contribute to reduce the problem if cloned plasmid DNA can be accepted as appropriate and researchers focus more on cloning the relevant sequences and sharing the plasmids. Notably, however, the plasmids should then be subject to the necessary quality assurance and control measures, e.g. by being deposited in

high quality plasmid banks [91]. Plasmid based reference materials also fit well with the concept of modularity in method validation and application [19].

The major challenges in relation to detectability are primarily linked with two factors. The first is the increasing number of GMOs on the world market, implying that testing has to be done for more and more targets, and consequently increasing the costs. Rationalising the analytical work is a must, unless the requirements for testing are lowered. The second factor is the highly diverse authorisation and control regimes established and implemented in different parts of the world, which combined with increasing global trade may bring unauthorised and potentially unhealthy and harmful products on the market. Availability of methods to be able to detect and characterise, and eventually trace and retract such products may be more relevant to safe foods than all the current testing for compliance with labeling thresholds. Within the Co-Extra project [70], researchers have started efforts to cope with this challenge, [see e.g. 69].

References

[1] A. Holst-Jensen, M. De Loose, and G.J. Van den Eede. Agric. Food Chem. 54 (2006) 2799.
[2] B. Bakan, D. Melcion, R. Molard and B. Cahagnie. J. Agric. Food Chem. 50 (2002) 728.
[3] E.A. Maga. Trends Biotechnol. 23 (2005) 533.
[4] J.K.C. Ma, R. Chikwamba, P. Sparrow, R. Fischer, R. Mahoney and R.M. Twyman. Trends Plant Sci. 10 (2005) 580.
[5] R. Ivarie. Trends Biotechnol. 24 (2006) 99.
[6] Z.W.S. Olempska-Beer, R.I. Merker, M.D. Ditto and M.J. DiNovi. Regul. Toxicol. Pharmacol. 45 (2006) 144.
[7] USDA-APHIS. US Department of Agriculture – Animal and Plant Health Inspection Service. Factsheet. USDA's biotechnology deregulation process. http://www.aphis.usda.gov/lpa/pubs/fsheet_faq_notice/fs_biodereg.pdf (2005)
[8] European Commission. Directive 2001/18/EC of the European Parliament and of the Council of 17 April 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Off. J. Eur. Commun. L 106 (2001) 1-38
[9] European Commission. Regulation (EC) 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Off. J. Eur. Commun. L 31 (2002) 1-24.
[10] European Commission. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Off. J. Eur. Union L 268 (2003) 1-23
[11] European Commission. Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation. Off. J. Eur. Union L 102 (2004) 14-25.

[12] European Commission. Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Off. J. Eur. Union L 268 (2003) 24-28.
[13] European Commission. Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. Off. J. Eur. Union L 10 (2004) 5-10.
[14] European Commission. Commission recommendation 2003/556/EC of 23 July 2003 on guidelines for the development of national strategies and best practices to ensure the co-existence of genetically modified crops with conventional and organic farming. Off. J. Eur. Union L 189 (2003) 36-47
[15] European Commission. Commission decision 2005/463/EC of 21 June 2005 establishing a network group for the exchange and coordination of information concerning coexistence of genetically modified, conventional and organic crops. Off. J. Eur. Union L 164 (2005) 50-51
[16] Cartagena protocol on biosafety to the convention on biological diversity. Montreal, Quebec, Canada. ISBN: 92-807-1924-6. http://www.biodiv.org/doc/legal/cartagena-protocol-en.pdf (2000)
[17] C. James. Executive Summary of Global Status of Commercialized Biotech/GM Crops: 2005. *ISAAA Briefs* No. 34 (2005). ISAAA: Ithaca, NY.
[18] Agbios. Agriculture and Biotechnology Strategies Canada inc. GMO database. http://www.agbios.com (2006)
[19] A. Holst-Jensen and K.G. Berdal. J. AOAC Int. 87 (2004) 927.
[20] C. Paoletti, M. Donatelli, S. Kay and G. Van den Eede. Seed Sci. Technol. 31 (2003) 629.
[21] G.W. Cochran. Sampling techniques. 3rd edition. J Wiley & sons Inc., NY, USA (1977).
[22] International Organization for Standardization. ISO 542:1990. Oilseed sampling. ISO, Geneva, Switzerland (1990)
[23] International Organization for Standardization. ISO 13690:1999. Cereals, pulses and milled products -- Sampling of static batches. ISO, Geneva, Switzerland (1999)
[24] Committee Europeen de Normalisation. Draft CEN/Technical specification. Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products. Sampling strategies (2006).
[25] C. Paoletti, A. Heissenberger, M. Mazzara, S. Larcher, E. Grazioli, P. Corbisier, N. Hess, G. Berben, P.S. Lübeck, M. De Loose, G. Moran, C. Henry, C. Brera, I. Folch, J. Ovesna and Van G. den Eede. Eur. Food Res. Technol. (2006) Published online April 12, DOI: 10.1007/s00217-006-0299-8.
[26] International Organization for Standardization. ISO 21571:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – nucleic acid extraction. ISO, Geneva, Switzerland (2005)
[27] International Organization for Standardization. ISO 21572:2004 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – protein based methods. ISO, Geneva, Switzerland (2004)
[28] J. Becker, P. Vos, M. Kui, F. Salamini and M. Heun. Mol. Gen. Genet. 249 (1995) 65.
[29] Y.G. Liu, N. Mitukawa, T. Oosumi and R.F. Whittier. The Plant Journal 8 (1995) 457.
[30] P.D. Siebert, A. Chenchik, D.E. Kellog, K.A. Lukyanov and S.A. Lukyanov. Nucleic Acids Res. 23 (1995) 1087.
[31] Hartl D, and Ochman H. Meth. Mol. Biol. 58 (1996) 293.
[32] Theuns I, Windels P, De Buck S, Depicker A, Van Bockstaele E, and De Loose M. Euphytica 123 (2002) 75.

[33] Qpcrgmofood. Reliable, standardised, specific, quantitative detection of genetically modified food. European research project, contract QLK1-1999-01301. Final report – workpackage 3, http://www.vetinst.no/Qpcrgmofood/Deliverable3.pdf (2004)
[34] Nielsen, C.R., Berdal, K.G. and Holst-Jensen, A. Eur. Food Res. Technol. 219 (2004) 421.
[35] Côté, M.-J., Meldrum, A.J., Raymond, P. and Dollard, C. J. Agric. Food Chem. 53 (2005) 6691.
[36] GMO-CRL. Community Reference Laboratory for GMO. Published methods submitted under article 47 of Commission Regulation EC 1829/2003. http://gmo-crl.jrc.it/statusofdoss.htm (2006)
[37] Kyoto Encyclopedia of Genes and Genomes - KEGG. http://www.genome.ad.jp/kegg/java/org_list.html (2006)
[38] The institute for Genome Research - TIGR. http://www.tigr.org/tdb/euk/ (2006)
[39] Sterky, F. and Lundeberg, J. J. Biotechnol. 76 (2000) 1.
[40] Cellini, F., Chesson, A., Colquhoun, I., Constable, A., Davies, H.V., Engel, K.H., Gatehouse, A.M.R., Kärenlampi, S., Kok, E.J., Leguay, J.-J., Lehesranta, S., Noteborn, H.P.J.M., Pedersen, J. and Smith, M. Food Chem. Toxicol. 42 (2004) 1089.
[41] Frenzel T, Miller A and Engel KH. Cereal Chem. 79 (2002) 215.
[42] Kuiper HA, Kok E and Engel KH. Curr. Opin. Biotechnol. 14 (2003) 238.
[43] European Commission. Commission recommendation 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. Off. J. Eur. Union L 348 (2004) 18-26.
[44] Holst-Jensen, A. CHAPTER 2. GMO detection methods and validation. 2.2 DNA-based methods. A review, with notes on future needs and directions. Version June 22nd 2001. http://www.entransfood.nl/workinggroups/wg4TQA/GMO detection methods using PCR review.htm (2001)
[45] Miraglia, M., Berdal, K.G., Brera, C., Corbisier, P., Holst-Jensen, A., Kok, E., Marvin, H.J.P., Schimmel, H., Rentsch, J., van Rie, J.P. and Zagon, J. Food Chem. Toxicol. 42 (2004) 1157.
[46] Compton, J. Nature 350 (1991) 91-92
[47] DMIFGEN. Development of methods to identify foods produced by means of genetic engineering. EU project no. SMT4-CT96-2072. http://europa.eu.int/comm/research/quality-of-life/gmo/04-food/04-03-project.html (2000)
[48] Roussel SA, Hardy CL, Hurburgh CR and Rippke GR. Appl. Spectrosc. 55 (2001) 1425.
[49] Le Gall G, Colquhoun IJ, Davis AL, Collins GJ and Verhoeyen ME. J. Agric. Food Chem. 51 (2003) 2447.
[50] Gardenghi S, Finotti A, Gambari R and Feriotto G. Minerva Biotecnologica 16 (2004) 203.
[51] Defernez M, Gunning YM, Parr AJ, Shepherd LVT, Davies HV and Colquhoun IJ. J. Agric. Food Chem. 52 (2004) 6075-6085.
[52] International Organization for Standardization. ISO 21569:2005. Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – qualitative nucleic acid based methods. ISO, Geneva, Switzerland (2005)
[53] International Organization for Standardization. ISO 21570:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – quantitative nucleic acid based methods. ISO, Geneva, Switzerland (2005)
[54] International Organization for Standardization. ISO 24276:2006 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – general requirements and definitions. ISO, Geneva, Switzerland (2006)

[55] Rønning, S.B., Rudi, K., Berdal, K.G. and Holst-Jensen, A. J Agric. Food Chem. 53 (2005) 8874.
[56] Wolf C, Scherzinger M, Wurz A, Pauli U, Hübner P and Lüthy J. Eur. Food Res. Technol. 210 (2000) 367.
[57] Hernandez M, Duplan MN, Berthier G, Vaïtilingom M, Hauser W, Freyer R, Pla M and Bertheau Y. J. Agric. Food Chem. 52 (2004) 4632.
[58] Permingeat HR, Reggiardo MI and Vallejos RH. J. Agric. Food Chem. 50 (2002) 4431.
[59] Leimanis S, Hernandez M, Fernandez S, Boyer F, Burns M, Bruderer S, Glouden T, Harris N, Kaeppeli O, Philipp P, Pla M, Puigdomenech P, Vaïtilingom M, Bertheau Y and Remacle J. Plant Mol. Biol. 61 (2006) 123.
[60] Rudi, K., Rud, I., and Holck, A. Nucleic Acids Res. 31 (2003) e62.
[61] Berdal KG and Holst-Jensen A. R. Eur. Food Res. Technol. 213 (2001) 432.
[62] Holst-Jensen, A.; Rønning, S.B.; Løvseth, A.; and Berdal, K.G. PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal. Bioanal. Chem. 375 (2003) 985-993.
[63] Matsuoka T, Kuribara H, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, and Hino A. J. Food Hyg. Soc. Japan 42 (2001) 24.
[64] Forte, V.T., Di Pinto, A., Martino, C., Tantillo, G.M., Grasso, G. and Schena, F.P. Food Control 16 (2005) 535.
[65] M. Hernandez, D. Rodriguez-Lazaro, T. Esteve, S. Prat and M. Pla. Anal Biochem. 323 (2003)164.
[66] A. Germini, S. Rossi, A. Zanetti, R. Corradini, C. Fogher and R. Marchelli. J. Agric. Food Chem. 53 (2005) 3958.
[67] M. M. Nagarajan and S.H. De Boer. lant Mol. Biol. Rep. 21 (2003) 259.
[68] H. Nesvold, A.B. Kristoffersen, A. Holst-Jensen and K.G. Berdal. Bioinformatics 21 (2005) 1917.
[69] Co-Extra. GM and non-GM supply chains. Their co-existence and traceability. EU-funded integrated project, contract FOOD-2005-CT-007158. http://www.coextra.org (2006)
[70] Borkhart, A., Repp, R., Harbott, J., Kreuder, J. and Lampert, F. Q Brit. J. Haemathology 83 (1993) 39-44.
[71] Studer E, Rhyner C, Lüthy J, and Hübner P. Z. Lebensm. Unters. Forsch. 207 (1998) 207-213
[72] Hardegger M, Brodmann P, and Herrmann A. Eur. Food Res. Technol. 209: 83-87
[73] Van den Eede G, Lipp M, Eyquem F, and Anklam E. Validation of a double competitive polymerase chain reaction method for the quantification of GMOs in raw materials. European Commission, Joint Research Centre, IHCP, EUR 19676 EN (2000).
[74] Sykes PJ, S-H. Neoh, M.J. Brisco, E. Hughes, J. Condon, and A.A. Morley. Bio Techniques 13 (1992) 444–449.
[75] NMKL. Measurement of uncertainty in microbiological examination in foods. Nordic Committee on Food Analysis. NMKL-procedure No 8, 2nd edn (2002)
[76] Nielsen, C.R., Holst-Jensen, A., Løvseth, A. and Berdal, K.G. Eur. Food Res. Technol. 222 (2006) 258–265
[77] Lee, LG, Connell, CR, and Bloch, W. Nucleic Acids Res. 21 (1993) 3761-3766
[78] Hernandez, M., Rodriguez-Lazaro, D. and Ferrano, A. Curr. Anal. Chem. 1 (2005) 203-221.
[79] Rudi, K., and Holck, A.L. (2003). Real-time closed tube single nucleotide polymorphism (SNP) quantification in pooled samples by quencher extension (QEXT). Nucleic Acids Res. Vol 31 (2003) e117
[80] Mannelli I, Minunni M, Tombelli S and Mascini M. IEEE Sensors Journal 3 (2003) 369-375

[81] Meric B, Kerman K, Marrazza G, Palchetti E, Mascini M and Ozsoz M. Food Control 15 (2004) 621-626
[82] D.P. Kalogianni, T. Koraki, T.K. Christopoulos and P.C. Ioanou. Biosens. Bioelectron. 21 (2006) 1069.
[83] H. Kuribara, T. Matsuoka, K. Takubo, S. Futo, N. Aoki, T. Hirao, H. Akiqama, Y. Goda, M. Toyoda and A. Hino. J. AOAC Int. 85 (2002) 1077.
[84] Centre for Food Quality, Labelling and Consumers Services, Saitama, Japan. Instruction Manual for Testing and Analysing Genetically Modified Food .Quantitative PCR-, Japanese Agricultural Standard Testing and Analysis Handbook Series (2002).
[85] Ministry of Health, Labour and Welfare, Japan. Testing for Foods Produced by Recombinant DNA Techniques by Ministry of Health, Labor and Welfare (2002).
[86] I. Taverniers, P. Windels, M. Vaïtilingom, A. Milcamps, E. Van Bockstaele, G. Van den Eede and M. De Loose. J. Agric. Food Chem. 53 (2005) 3041.
[87] European Network of GMO Laboratories (ENGL). http://engl.jrc.it (2006).
[88] GMO-CRL. Community Reference Laboratory for GMO. http://gmo-crl.jrc.it (2006)
[89] FLUKA. Powdered genomic maize DNA containing approximately 1% of transgenic NK603, GA21 and CBH-351 maize (prod. ref. 69407) (2003).
[90] IRMM. Institute for Reference Materials and Measurements, GA21 maize reference material certification report. http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/attachements/ERM-BF414_report.pdf (2004)
[91] European Network of GMO Laboratories. Activity report 2002-2004. 56pp. http://ihcp.jrc.cec.eu.int/docs/b&gmos/engl.pdf (2004)

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 9

Extraction procedures

Sara Bogialli, Antonio Di Corcia, Manuela Nazzari

Dipartimento di Chimica, Università "La Sapienza", Piazza Aldo Moro 5, 00185 Roma, Italy

1. Introduction

Qualitative and quantitative analysis concludes a procedure of sample preparation. The extraction step is the least evolved part of most analytical procedures. As a matter of fact, still today Soxhlet extraction (developed by F. Soxhlet in 1879) and conventional liquid-liquid extraction (LLE) are routinely used in many regulatory laboratories for extracting toxicants from respectively solid and liquid foodstuffs. In the last 15 years there has been an increasing demand for new extraction techniques, amenable to automation, with shortened extraction times and reduced organic solvent consumption. This could prevent pollution in analytical laboratories and reducing sample preparation costs [1, 2]. Driven by these purposes, advances in sample preparation for analyzing contaminants in solid matrices have resulted in a number of techniques such as microwave-assisted extraction (MAE) [3-5], supercritical fluid extraction (SFE) [6-8], pressurized liquid extraction (PLE, Dionex trade name ASE, which stands for accelerated solvent extraction) [9], matrix solid-phase dispersion (MSPD) [10-11]. Solid-phase extraction (SPE), solid-phase microextraction (SPME) [12], stir bar sorptive extraction (SBSE) [13] and supported liquid membranes (SLM) [14] are progressively replacing LLE for extracting analytes from liquid foodstuff matrices, such as milk and fruit juices.

2. Analyte extraction from liquid foodstuff

Methods for the extraction of toxicants in liquid foodstuffs exploit the partitioning of analytes between the aqueous phase and a water-immiscible solvent or a sorbent material. Conventional LLE is still the most diffused in many environmental laboratories. However, SPE and, to a lesser extent, SPME are constantly increasing in popularity in regulatory laboratories because of numerous advantages over LLE which will be later illustrated.

2.1. Liquid-liquid extraction

Among the solvents used for analyte extraction, ethyl acetate and dichloromethane are the most preferred, owing to their effectiveness in extracting compounds having a broad range of polarity. [15]. When extracting lipophilic compounds, for example polybrominated biphenyls (PBBs), polychlorinated biphenyls (PCBs), dioxins, non polar solvents, such as hexane and

petroleum ether, can also be used advantageously [16, 17]. Solvent extraction is usually carried out in a separatory funnel, which is vigorously shaken to increase the contact area between the two liquids. This operation enhances extraction rate and yield.

Factors determining analyte recovery are the affinity of the analyte for the solvent, the solvent to sample volume ratio, and the number of extraction steps [15-25]. When extracting analytes acidic or basic in nature by LLE, the water pH has to be suitably adjusted to suppress analyte ionization, in this way favoring partition into the organic solvent.

Drawbacks of this technique are that is labor intensive and time-consuming. When performing trace analysis of contaminants, the extensive use of glassware may result in cumulative loss by adsorption on glass of hydrophobic analytes, such as DDT, polycyclic aromatic hydrocarbons (PAHs), dioxins, PBBs and PCBs. LLE requires the use of relatively large amounts of highly purified solvents that are expensive as well as flammable and toxic. Vigorous shaking of solvent and liquid matrix may create serious problems of emulsions. Emulsions can be eliminated only by additional time-consuming operations. Last but not least, extraction of contaminants from milk may require a pretreatment step to eliminate milk proteins by addition of trichloro acetic acid [18] or sodium tungstate [19] to the sample..

Table 1 shows selected extraction procedures for analyzing contaminants in liquid foods by liquid-liquid extraction.

Table 1. Selected extraction procedures for analyzing contaminants in liquid foods by liquid-liquid extraction

Analyte(s)	Matrix	Extractant	Ref.
Benzimidazoles[a]	Milk	Ethyl acetate	[15]
PAHs[b]	Milk	Hexane after saponification	[16]
PCBs[c], PCDDs[d] and PCDFs[e]	Milk and oil	Diethyl ether/hexane	[17]
β-Lactams[f]	Milk	Acetonitrile	[20]
β-Lactams	Milk	Acetonitrile	[21]
Macrolides[f]	Milk	Tris buffer	[22]
Sulfonamides[g]	Eggs	Acetonitrile	[23]
Coccidiostats	Eggs	Acetone/Tetrahydrofuran	[24]
Mycotoxins	Milk	Hexane/acetonitrile	[25]

[a] Anthelmintic agents. [b] PAHs=polycyclic aromatic hydrocarbons. [c] PCBs= polychlorinated biphenyls. [d] PCDDs= polychlorinated dibenzo-p-dioxins [e] PCDFs= polychlorinated dibenzofurans. [f] Antibiotics. [g] Antibacterials.

2.2. Solid-phase extraction

Since the 1970's, as an alternative to LLE, the method of combined extraction and preconcentration of organic compounds in liquid matrices by passing the sample through a short column filled with an adsorbing medium followed by desorption with a small quantity of an organic solvent (Figure 1) has attracted the attention of many researchers. The advantages of SPE over LLE are that it is faster, more reproducible, emulsion creation is avoided and smaller sample sizes are needed. Additionally, SPE can be easily incorporated into automated analytical procedures, which can lead to greater accuracy and precision, as well as greater laboratory productivity [26].The availability of small-size particle (ca 40 μm) sorbents in inexpensive cartridges has largely contributed to the dramatic expansion of the SPE technique. This technique appears especially appealing to researchers and analysts and it is rapidly replacing LLE in official methods.

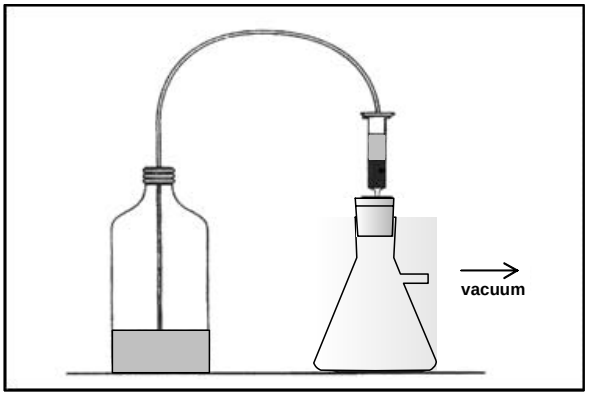


Figure 1. Schematic of apparatus for solid-phase extraction

Typical sorbents for SPE are silica chemically modified with an octadecyl alkyl chain, commonly referred to as C-18 [27-31]; highly cross-linked styrene-divinylbenzene (SDVB) copolymers, commonly referred to as PRP-1, Envichrom P or Lichrolut [32, 33]; another copolymer sorbent named Oasis-HBL; graphitized Carbon Blacks (GCBs), commonly referred to as Carbopack, Carbograph or Envicarb [34-37]. All these materials are commercially available in medical-grade polypropylene housing and polyethylene frits. At the present, C-18 is by far the most commonly used material and it has been considered for introduction in official methods by European and American Health agencies.

SDVB copolymers are a family of sorbents with high surface areas (800-1400 m^2/g). This characteristic enables them to extract efficiently polar analytes from liquid matrices. A

copolymeric sorbent more and more used for the extraction of analytes from biological liquid samples is Oasis-HLB. This is a hydrophilic-lipophilic balanced copolymer (HLB) of N-vinylpyrrolidone and divinylbenzene. The hydrophilic N-vinylpyrrolidone increases the water wettability, while the lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes.

Carbograph (or Carbopack) 1 and 4 are two materials pertaining to the GCB sorbent family and having surface areas of about 100 and 200 m^2/g, respectively. Their peculiarity is that of having essentially non porous surfaces. This enables intimate contact between the adsorbent and the adsorbate, which results in high adsorption heats and thus strong retention of hydrophilic compounds dissolved in aqueous solutions. A Carbograph 1 cartridge has shown to be much more efficient than a C-18 cartridge for extracting very polar analytes from aqueous samples [38].

Alternatively, membrane extraction disks typically containing SDVB or C-18 materials with particle sizes finer than those used with SPE cartridges and imbedded in a Teflon matrix have been used. The specific advantages claimed for disk design over cartridge design are shorter sample processing and decreased plugging by particulate matter due to the large cross-sectional are, reduced channeling effects, and cleaner background interferences. The latter advantage derives from the fact that, unlike SPE with cartridges, the extraction apparatus with disks consists of glass. The use of an extraction disk is simple. The membrane is placed in a filtration apparatus connected to a vacuum source by a water pump. After the disk has been washed and conditioned by 10 ml of methanol followed by 10 ml of distilled water, the liquid sample is passed through the disk. After eliminating part of the sample by vacuum, the assembly supporting the disk is transferred to a second vacuum flask containing a vial. Then, 5-10 ml of the eluent phase, usually, methanol or acetonitrile, is slowly drawn onto the membrane by moderate vacuum. The vacuum is interrupted for 2-4 min to allow the solvent to soak the membrane. Thereafter, analytes are eluted and collected into the vial. This operation is repeated by applying another 5-ml aliquot of the eluent phase to the top of the disk. A sketch of an extraction apparatus employing membranes is shown in Figure 2.

Molecular imprinting is a technique for preparing synthetic polymers with recognition sites specific for a target molecule [39]. These artificially generated recognition sites have their shapes, sizes and functionalities complementary to the target molecule, and are capable of rebinding the target molecule in preference to other closely related structures. In a typical molecularly imprinted polymer (MIP) synthesis, the target (or template) molecule is first allowed to interact with a functional monomer in a predetermined orientation. The monomer-template interaction can be reversible covalent bonding, non-covalent or metal ion coordination. This monomer-template complex is then copolymerized with a crosslinker, leading to a highly cross-linked macroporous polymer with the imprint molecules in a sterically fixed arrangement. After removal of the template molecules, recognition sites that bind specifically to the target molecules are established. These peculiar sorbents packed in

mini-columns have been used mainly for purification of extracts containing one or few target compounds [40, 41].

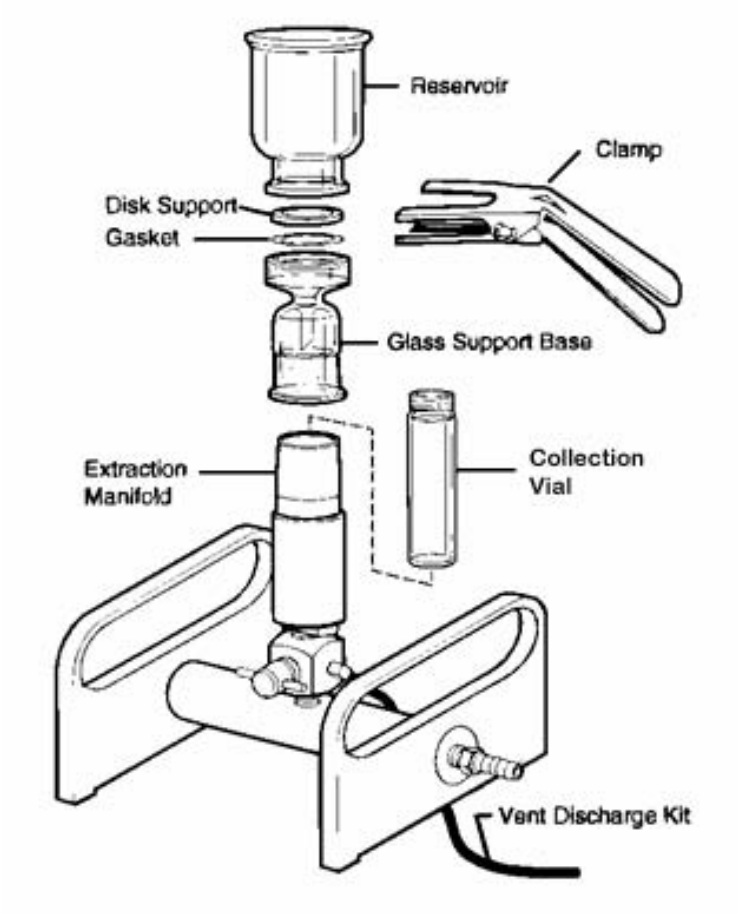


Figure 2. Schematic of apparatus for membrane extraction disks

Table 2 shows selected extraction procedures for analyzing contaminants in liquid foods by solid-phase extraction.

Table 2. Selected extraction procedures for analyzing contaminants in liquid foods by solid-phase extraction

Analyte(s)	Matrix	Sorbent	Format	Ref.
Penicillins[a]	Milk	C-18[b]	Cartridge	[27]
Pesticides	Vegetable juices	C-18	Tube	[28]
PCBs[c] PCDFs[d], PCDDs[e]	milk	C-18	Cartridge	[29]
Nitrosamines	Sausages	C-18	Cartridge	[30]
Ochratoxin A[f]	Wine and beer	C-18	Mini-column	[31]
Atrazine	Orange Juice	SDVB[g]	Cartridge	[32]
Phenylurea Herbicides	Milk	SDVB	Cartridge	[33]
Triazine Herbicides	Milk	Carbograph 1[h]	Cartridge	[34]
β-Lactams[a]	Milk	Carbograph 4[h]	Cartridge	[35]
Sulfonamides[i]	Milk And Eggs	Carbograph 4	Cartridge	[36]
Tetracyclines[a]	Milk	Carbograph 4	Cartridge	[37]
Atrazine	Apple Extract	MIP[j]	Cartridge	[40]
Deoxynivalenol and zearalenone[f]	Beverages	MIP	Cartridge	[41]

[a] Antibiotics. [b] Octadecyl-modified silica. [c] PCBs= polychlorinated biphenyls. [d] PCDFs= polychlorinated dibenzofurans. [e] PCDDs= polychlorinated dibenzo-p-dioxins. [f] Mycotoxins. [g] SDVB= Styrene-divynylbenzene copolymer. [h] Types of graphitized carbon blacks. [i]. Antibacterial agents. [j] MIP= molecularly imprinted polymers

2.3. Techniques based on sorptive extraction

In the past analytical chemists paid a lot of attention to solventless sample preparation technique based on sorptive extraction. These techniques include solid-phase microextraction (SPME) [12] and stir-bar sorptive extraction (SBSE) [13]. Sorptive extraction has proven to be an interesting and environmentally friendly alternative to liquid extraction. In sorptive extraction, the analytes are extracted from the liquid matrix into a non-miscible liquid phase. In contrast to extraction with adsorbents in which the analytes are bound to surface active centers, the total amount of extraction phase is important in sorptive extraction, not the surface only. The most widely used sorptive extraction phase is polydimethylsiloxane. This phase is well known as a stationary phase in gas-chromatography, is thermostable, can be used in a broad temperature range (220-320 °C) and has interesting diffusion properties. Extraction with polydimethylsiloxane can therefore be compared to a micro-liquid-liquid extraction. After extraction, the solutes can be introduced quantitatively into the analytical system by thermal desorption. This process provides high sensitivity because the complete

extract can be analyzed. The main difference between SPME and SBSE is the much higher mass of polydimethylsiloxane available with the latter technique, which results in higher analyte recovery and higher sample capacity.

2.3.1. Solid-phase microextraction.

Solid-phase microextraction is a technique for extracting analytes from liquid matrices and was introduced by Pawliszyn in 1990 [12]. Figure 3 shows a typical SPME device. A 0.05-1 mm ID uncoated or polydimethylsiloxane-coated fiber (in the latter case it would be more nearly to call it liquid-phase microextraction) is immersed into a continuously stirred liquid sample [43-49]. Analytes are then partitioned between the liquid matrix and the liquid phase or sorbent phase. Alternatively, for analyzing volatile contaminants, the fiber can be located above the surface of the liquid so that analyte vapors are captured. This technique is known as Head Space Solid-Phase Microextraction (HSSPME) [50]. To increase the extraction yield, the liquid matrix can be salted with NaCl, so weakening water-analyte interactions. After equilibrium is reached (a good exposure time takes 15-25 min), the fiber is introduced into the injection port of a gas chromatography (GC) instrument, where analytes are thermally extracted and analyzed. Factors influencing the extraction step include fiber type, extraction time, ionic strength, sample pH, extraction temperature and sample agitation. Variables affecting the desorption step include temperature, desorption time, focusing oven temperature. An important factor in SPME of food samples, especially in fruit and fruit juices, is matrix effect. Negative matrix effects can be reduced by diluting the sample 50-100 fold with distilled water.

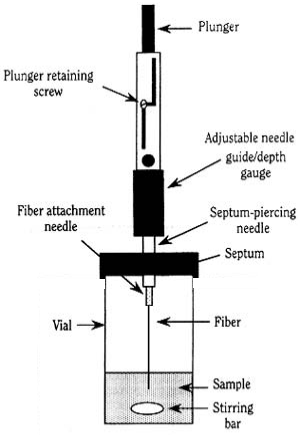


Figure 3. Solid-phase microextraction device

In the last years, the original concept of the SPME technique has changed in that several recently published SPME-based methods propose the use of organic solvents for re-extracting analytes from the fiber instead of thermal desorption. In this way, fractionation techniques other than GC, such as liquid chromatography (LC) or capillary electrophoresis (CE), can be used for analyzing final extracts [43, 44].

A variant of the SPME technique performed with a fiber is in-tube SPME [44]. The latter technique employs a piece of capillary GC column coated with a suitable non volatile liquid (typically, 50-60 cm, 0.25 μm i.d., 0.24 μm film thickness) for the extraction of the analytes from aqueous samples. Figure 4 shows a sketch of an automated in-tube SPME-LC-tandem mass-spectrometry system. The in-tube SPME can be used with various coated capillaries for different analytes, and there is a larger range of coatings available for the GC capillary columns than for the SPME fibers. There are two methods for analyte re-extraction from the capillary column. In the dynamic mode, analyte re-extraction is performed into the flow of the appropriate mobile phase by switching the six-port injection valve from the load to inject position for transport to the analytical column. Static re-extraction is used when the analytes are more strongly retained by the capillary coating, and performed into the solvent aspirated from the outside.

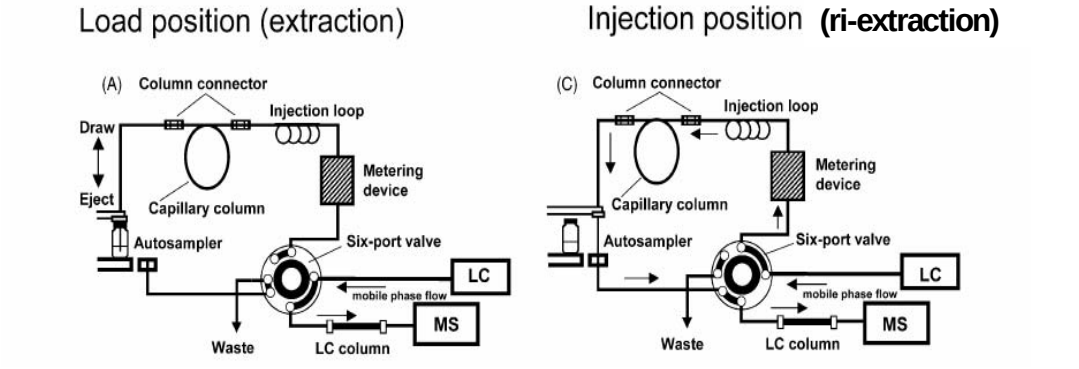


Figure 4. Schematic diagram of in-tube SPME-LC device. A) Load position. B) Inject position (Reproduced (modified) from [42] with permission from Elsevier© 2003).

Table 3 shows selected extraction procedures for analyzing contaminants in liquid foods by fiber and in-tube solid-phase microextraction.

Table 3. Selected extraction procedures for analyzing contaminants in liquid foods by solid-phase microextraction

Analyte(s)	Matrix	Pretreatment	Extractant	Ref.
Tetracyclines[a]	Milk	None	Coated Fiber	[43]
Pesticides	Wine	None	In-tube	[44]
Furan	Heated Foodstuff	None	Uncoated Fiber	[45]
Methanol	Sweeteners	None	Uncoated Fiber	[46]
Butyltin[b]	Wine	pH adjustment	Uncoated Fiber	[47]
Pesticides	Vegetables	Homogenization	Coated Fiber	[48]
Pesticides	Potato, Honey	Homogenization	Coated Fiber	[49]
Nitrosamines	Sausages	None	Coated Fiber	[50]

[a] Antibiotics. [b] Fungicide.

2.3.2. Stir-bar sorptive extraction

SBSE of a liquid sample is performed by placing a suitable amount of sample in a headspace vial or other container. A polydimethylsiloxane-coated magnetic stir bar is added and the sample is stirred for 30-240 min. The extraction time is determined by the sorption kinetic, sample volume, stirring speed and stir bar dimensions, and should be optimized for any given application [51-55]. By using a control liquid matrix to which analytes are added prior to extraction, optimization is normally performed by measuring the analyte recovery as a function of the extraction time.

After extraction, the stir bar is removed, dipped on a clean paper tissue to remove liquid sample droplets and introduced to an empty glass thermal desorption tube. Desorption temperature are application dependent, primarily determined by the volatility of the solutes, and typically between 150 and 300 °C. Desorption can be accomplished in 5-15 min under a Helium flow.

In contrast to conventional SPME, in which desorption is performed in the inlet of the gas chromatograph, SBSE is used in combination with a thermal-desorption device. Because more extraction phase is used as compared to SPME, desorption process is slower, and thus desorption combined with cold trapping and re-concentration is required.

Like SPME, several recently proposed methods have replaced thermal desorption of the analytes with analyte re-extraction with suitable organic solvents to combine SBSE with LC or CE. Analogously to the other techniques based on Sorptive Extraction, SBSE can be carried out in the head space modality.

However, both SPME and SBSE are limited by the nature of the solvent phase available, thus restricting their applications. In addition, extractions are rarely complete due to the low volume of the sorbent phase.

Table 4 shows selected extraction procedures for analyzing contaminants in liquid foods by stir-bar sorptive extraction.

Table 4. Selected extraction procedures for analyzing contaminants in liquid foods by stir bar sorptive extraction

Analyte(s)	Matrix	Pretreatment	Extractant	Ref.
Pesticides	Pear pulp	Ultrasonic bath	polydimethylsiloxane	[51]
Pesticides	Wine	none	polydimethylsiloxane	[52]
Fungicides	Wine	none	polydimethylsiloxane	[53]
Pesticides	Vegetables, fruits	ultrasonic bath	polydimethylsiloxane	[54]
Preservatives	Beverages, vinegar	none	polydimethylsiloxane	[55]

2.4. Supported liquid membranes

For analytes that are ionizable under suitable conditions, the supported liquid membrane (SLM) can be an attractive alternative to other liquid sample extraction methods [14]. The advantages of using SLM over conventional LLE include low organic reagent volume, the simplicity of the system, low operating cost and suitability for automation [56-60]. SLM coupled to flow systems can be used for the selective and efficient extraction of various types of analytes with the efficient removal of interfering matrix constituents. The basic principle is a double extraction in which the analytes are extracted from an aqueous donor phase, i.e. the liquid sample, into the organic sample, immobilized in a porous hydrophobic membrane, followed by continuous back-extraction into a second aqueous phase, the acceptor. This process is driven by differences in pH between the two aqueous phases. The selectivity of the extraction process depends primarily on the ability to transfer the compounds of interest between active and inactive forms in the required sequence, without making the same transfers for interfering compounds. By careful selection of the pH of the donor and acceptor solutions, compounds can be selectively extracted. Figure 5 shows a schematic diagram of the manifold used for analyte extraction by the SLM-flow system.

In order to be extracted into the immobilized hydrophobic membrane liquid, the analytes must be uncharged. The analytes are then in the extractable form and are extracted into the membrane liquid. They then diffuse across the membrane and are back-extracted into the acceptor when they are in a charged, non-extractable form, and thereby trapped in the acceptor.

The main inherent drawback of this technique is that it can be applied only to extraction of compounds that are weakly basic or acidic in nature.

Table 5 shows selected extraction procedures for analyzing contaminants in liquid foods by supported liquid membrane extraction.

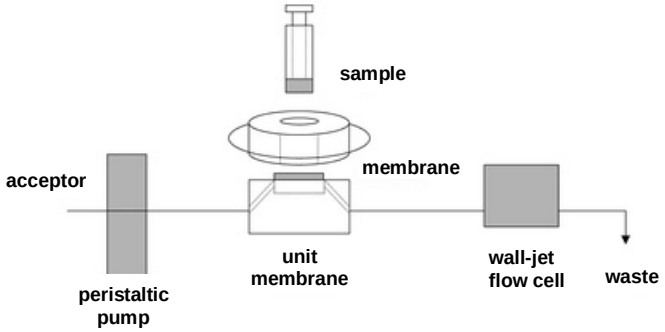


Figure 5. Schematic diagram of the manifold used for extraction by the supported liquid membrane (Reproduced from [56] with permission from Royal Society of Chemistry© 2000)

Table 5. Selected extraction procedures for analyzing contaminants in liquid foods by supported liquid membrane extraction

Analyte(s)	Matrix	Extractant	Ref.
Atrazine	Fruit juices	Dihexylether	[57]
Macrolides[a]	Milk	Decanol/n-undecane (1:1)	[58]
Biogenic amines	Wine	di-2-ethylhexyl phosphoric acid	[59]
Pesticides	Wine	toluene	[60]

[a] Antibiotics

3. Analyte extraction from solid foodstuff

The extraction and recovery of trace organic material from semi-solid and solid matrices is often the slowest and most error-prone step of an analytical method. The conventional liquid extraction techniques for solids and semi-solids materials (Soxhlet) have two main disadvantages. The first, large volumes of organic solvent are required, which can lead to sample contamination and "losses" due to volatilization during concentration steps. The second, to achieve an exhaustive extraction can require several hours to days. With the development of sophisticated instrumentation with detection limits in the picogram and femtogram levels, pressure is finally felt within the analytical community to develop and validate sample preparation procedures which can be used to rapidly isolate trace level organics from complex matrices.

3.1. Soxhlet extraction.

In the Soxhlet device, a solid matrix is placed in a cavity that is gradually filled with the extractant by condensation of vapors from a distillation flask. When the liquid reaches a certain level, a siphon aspirates the whole content of the cavity and unloads it back into the distillation flask, carrying the extracted analytes in the bulk liquid. This operation is repeated many times until virtually extraction is obtained and the analytes are all in the flask. Inasmuch as the solvent acts stepwise the assembly can be considered a batch system; however, since the solvent is recirculated through the sample, the system also bears a continuous character. The most salient advantages of Soxhlet are that the sample is repeatedly brought into contact with fresh portion of the solvent, thereby aiding displacement of the distribution equilibrium, and that no filtration is needed. The drawbacks involved in the use of this traditional extraction technique are the inability to provide agitation, which would help process acceleration, and the constant heat applied to the leaching cavity. This heat is dependent on the solvent boiling point and could be insufficient to break some matrix-analyte bonds. A microwave-assisted Soxhlet extractor (Soxtec) has been proposed and commercialized without noticeable success. Nevertheless, the literature quotes applications in which a saving time (1 instead of 4 h) is achieved using Soxtec instead of conventional Soxhlet [61].

While both Soxhlet and Soxtec extraction techniques are still of large use in environmental applications, only few works describing the use of these two techniques for extracting contaminants from food is quoted in the recent literature [62-66].

Table 6 shows selected extraction procedures for analyzing contaminants in solid foods by Soxhlet extraction.

Table 6. Selected extraction procedures for analyzing contaminants in solid foods by Soxhlet extraction.

Analyte(s)	Matrix	Extractant	Ref.
PAHs[a], PCBs[b]	Vegetables	Hexane/Acetone (1:1)	[62]
PAHs	Palm dates, tuna fish	Hexane	[63]
PAHs	Fish tissues	Hexane/Acetone (1:1)	[64]
Acrylamide	Potato chips	Methanol	[65]
Fenitrothion[c]	Beans	Dichloromethane	[66]

[a] PAHs=polycyclic aromatic hydrocarbons. [b] PCBs= polychlorinated biphenyls.[c] Insecticide

3.2. Liquid-phase extraction

Although manually shaking a finely dispersed solid sample with a suitable solvent can be effective in many cases, blending the sample in the presence of the solvent in high-speed

homogenizer machines or ultrasonication baths ensures complete sample disruption and a better analyte extraction. This technique is called liquid-phase extraction (LPE) or liquid-solid extraction (LSE). So far, this technique is the most popular for extracting contaminants in animal tissues, eggs, fruits and vegetables [67-90]. As an example, about 30% of the proposed procedures for analyzing veterinary drug residues in food-producing animal tissues employ LPE [67-78]. Water-miscible solvents, such as acetone, acetonitrile, and methanol, are now widely used as they are effective in extracting both polar and non polar toxicants. Ethyl acetate with added anhydrous sodium sulfate is an alternative extractant [90]. Its use offers advantages in that no subsequent partition step is required and the extract can be used directly in gel permeation chromatographic cleanup. Each extraction system offers well distinct advantages, some of which depend on the way in which the extraction/partition steps are integrated into the cleanup/determination steps of the analytical method. Other factors which may influence the choice of the solvent over another one is solvent consumption which is related to costs, health, and disposal problems.

Table 7 shows selected extraction procedures for analyzing contaminants in solid foods by liquid phase extraction.

3.3. Supercritical fluid extraction

On an industrial scale, SFE has been applied for many years and only recently has been deployed in the analytical field for extracting a variety of pesticide toxicants from solid matrices, such as soil, sediments, vegetables, animal tissues and foodstuffs. Carbon dioxide has been by far the most used fluid. Besides offering advantages mentioned below, this fluid has a low critical temperature (31°C). Over sonication, blending and Soxhlet extraction, definite advantages of SFE are listed here below:

1. A non toxic, non flammable, inexpensive fluid, such as CO_2, is used.
2. SFE can be automated and coupled to both gas chromatography and liquid chromatography.
3. Selective extraction can be performed by suitably modifying the density of the supercritical fluid. Increasing the density of the fluid increases the extraction yield of high-molecular weight compounds. The density of the fluid can be varied by varying its temperature and pressure.
4. Faster extraction. Extraction by SFE is a matter of minutes, instead of hours. Compared to conventional solvents, the low viscosity of the supercritical fluid helps rapid penetration into the core of the solid matrix and extraction of analytes. In addition, the high solute diffusivities into the supercritical fluid results in rapid removal of the analytes from the matrix by a decreased mass transfer resistance.

Table 7. Selected extraction procedures for analyzing contaminants in solid foods by liquid-phase extraction.

Analyte(s)	Matrix	Extractant	Ref.
Anthelmintics	Bovine Liver	Acetone	[67]
Gentamicin[a]	Animal Tissues	KH_2PO_4, 2% TCA[b]	[68]
β-Lactams[a]	Kidney	Acetonitrile	[69]
Macrolides[a]	Poultry Muscle	HPO_3/Methanol (70:30)	[70]
Sulfonamides[c]	Kidney	Methanol	[71]
Tetracyclines[a]	Animal Tissues	Sodium succinate	[72]
Quinolones[c]	Fish	Acetonitrile	[73]
Chloramphenicol[a]	Shrimps	Phosphate buffer	[74]
Coccidiostats	Eggs	Acetonitrile	[75]
Anabolic Steroids	Bovine Muscle	HPO_3/acetonitrile (60:40)	[76]
Thyreostats	Thyroid Glands	Methanol	[77]
Tranquillizers	Muscle, Kidney	Sodium acetate	[78]
Pesticides	Vegetables	Methanol	[79]
Heterocyclic amines	Eggs	Acetonitrile/water (90:10)	[80]
Heterocyclic amines	Meat extract	NaOH	[81]
Heterocyclic amines	Chicken	Hydrochloric acid	[82]
Mycotoxins	Corn	Acetonitrile/water (75:25)	[83]
Toxins	Shellfish	Methanol/water (80:20)	[84]
Toxins	Shellfish	Acetone	[85]
PAHs[d]	Potato, toasted bread	Ethyl ether/methylene chloride	[86]
PAHs	Smoked cheese	Cyclohexane, after defatting	[87]
Aflatoxins[e]	Peanut	Sulphuric acid/potassium chloride/acetonitrile (10:1:89)	[88]
Aflatoxin M1[e]	Cheese	Chloroform saturated sodium chloride solution	[89]
Pesticides	Nectarines	Ethyl acetate and sodium sulfate	[90]

[a] Antibiotics. [b] TCA= trichloroacetic acid. [c] Antibacterials. [d] PAHs=polycyclic aromatic hydrocarbons. [e] Mycotoxins.

SFE is conceptually simple to perform (Figure 6). A pump is used to supply a known pressure of the supercritical fluid to an extraction vessel which is thermostated at a temperature above the critical temperature of the supercritical fluid. During the extraction, the analytes are removed from the bulk sample matrix into the fluid and swept into a

decompressing region. Here, the supercritical fluid becomes a gas and is vented, while analytes abandoning the gas are collected in a vial containing a small volume of a suitable solvent [92, 93] such as methanol [92]. A variation of this scheme is that of substituting the collecting liquid at the outlet of the extractor with a sorbent cartridge [94-104] such as C-18.

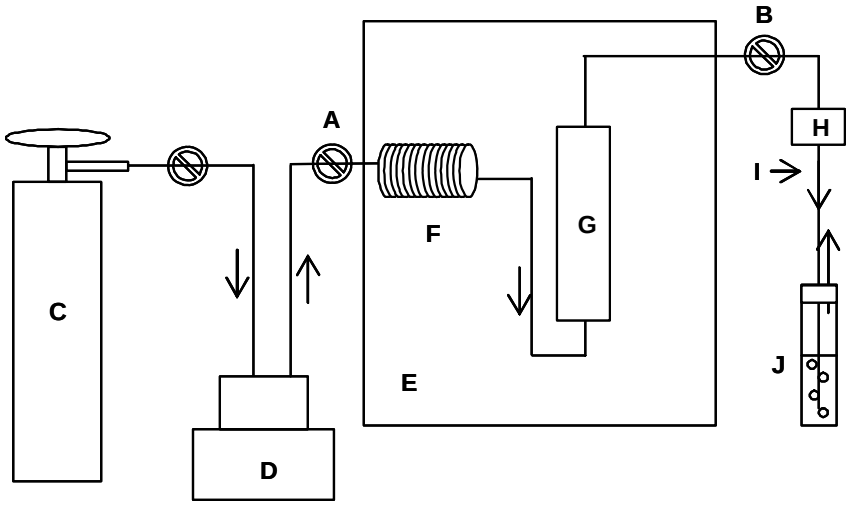


Figure 6. Schematic of a supercritical fluid extraction device (A-B: valve, C: gas cylinder, D: pump, E: oven, F: thermal equilibration coil, G: extraction cell, H: thermostat, I: restrictor, J: collection vial), (Reproduced (modified) from [91] with permission from Royal Chemistry Society© 2004).

The extraction of hydrophobic compounds from complex matrices containing sugar, proteins, and fat can be achieved almost quantitatively, but polar molecules give poor recovery rates. The recovery of these compounds can be improved significantly by the addition of a modifying solvent, such as methanol or acetonitrile. There are two possibilities when adding a modifier: static addition of the modifier directly into the extraction thimble, or addition of the solvent by a modifier pump. The pump delivers a constant flow rate and improves extraction efficiency and reproducibility.

In spite of its unique elevated selectivity, the interest in SFE has substantially decreases in the last years. This is basically due to the high cost of the instrumentation and the high dependence of the extraction conditions on the sample, leading to fastidious optimization procedures and difficulty in using this technique routinely.

Table 8 shows selected extraction procedures for analyzing contaminants in solid foods by supercritical fluid extraction.

Table 8. Selected extraction procedures for analyzing contaminants in solid foods by supercritical fluid (CO_2) extraction

Analyte(s)	Matrix	Modifier	Collection mode	Ref.
Enrofloxacin[a]	Chicken muscle	Methanol	Solvent	[92]
Mycotoxins	Corn meal and rolled oat	Methanol	Solvent	[93]
PAHs[b]	Smoked meat	None	Sorbent	[94]
Chloramphenicol[a]	egg	None	Sorbent	[95]
Pesticides	Cereals, vegetables	None	Sorbent	[96]
Pesticides	Rice, vegetables	None	Sorbent	[97]
Pesticides	Wheat, maize	None	Sorbent	[98]
Pesticides	Fatty foods	Acetonitrile	Sorbent	[99]
Pesticides	Meat	None	Sorbent	[100]
Pesticides	Strawberry	Sodium Sulfate	Sorbent	[101]
Pesticides	Orange	Various	Sorbent	[102]
Benzimidazoles[c]	Liver	Methanol/water (60:40)	Sorbent	[103]
Pesticides, PCBs[d], PBBs[e] and PBDEs[f]	Fish feed	None	Sorbent	[104]

[a] Antibiotics. [b] PAHs=polycyclic aromatic hydrocarbons. [c] Anthelmintic agents [d] PCBs= polychlorinated biphenyls. [e] PBBs= polybrominated biphenyls. [f] PBDEs= polybrominated biphenyl ethers.

3.4. Microwave-assisted extraction.

In the 1980's, Ganzler et al. [105] conducted early experiments using household microwave ovens to aid in extraction conducted at atmospheric pressure. Since then, the process has been further investigated, refined and patented. As a result, the technique has been successfully applied to extraction of organic compounds from solid matrices. That early development has resulted in specialized microwave instruments designed to provide temperature-controlled, closed-system operation [106] (Figure 7). These modern units permit heating of many samples simultaneously in closed, pressurized vessels which allow for heating of polar solvents to temperature of approximately 100 °C above their standard conditions boiling points. Inertness of extraction vessel surfaces in contact with the sample is assured through the use of perfluoroalkoxy liners. Newer developments in vessel liner materials now include both Teflon and glass.

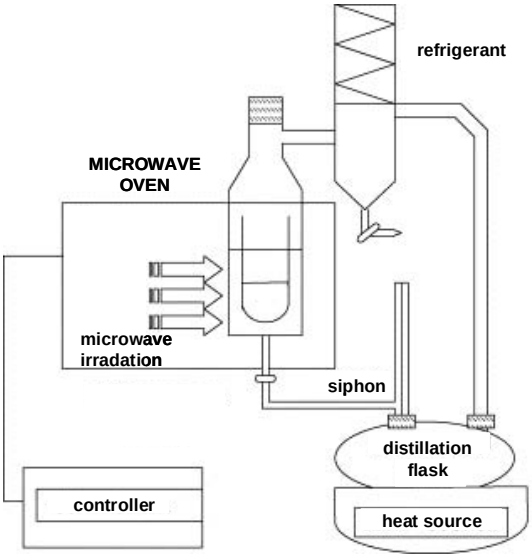


Figure 7. Schematic of a microwave assisted extraction apparatus (Reproduced (modified) from [107] with permission from Elsevier© 2004)

The microwave part of the electromagnetic spectrum is defined as energy of wavelength between 3-300 mm corresponding to a frequency range of 1-100 GHz. MAE is the process that use this energy to heat solid sample-solvent mixture resulting in the partitioning of the compounds of interest from the solid matrix into the solvent. Typically, extraction is done in a sealed vessel under temperature-controlled conditions. This generates extraction temperatures above atmospheric condition boiling points for the solvent selected, thereby making more effective and accelerating the extraction process. The closed-vessel microwave heating system suited for analyte extraction from solid matrices usually are outfitted with a turntable to rotate the sample within the cavity, thus ensuring even homogeneous distribution of thermal energy.

Microwave heating is significantly different from conductively heating methods. Conductive heating is sample independent. All samples placed inside a conduction heating oven will equilibrate to the pre-selected temperature quite slowly. Microwave heating is much more solute-to-solvent ratio dependent. The rate of increase in the temperature depends on the microwave-absorbing characteristics of the sample. Microwave energy does not heat non polar solvents, while low polarity solvents are heated at very slow rates. In the past, this shortcoming has been overcome by some addition of a more polar solvent to the non polar one. This, in turn, usually resulted in other complications regarding analyte selectivity of the extraction process. The problem is now circumvented with Carboflon vessel inserts which permit the direct heating of non polar solvents via convection heat transfer. Analysts may now

transfer the solvent-extraction scheme from their traditional-methods approach to MAE without changing their solvent system.

The MAE technique has been largely used for extracting contaminants in environmental solid samples but has received limited attention by researchers involved in developing analytical procedures for detecting toxicants in solid foodstuff [108-115]. This is so because diffusion of the solvent and the analytes is inhibited with samples containing more than 30% of water (this is the case of food samples), this resulting in low analyte recovery. This problem can be circumvented only by eliminating water from the sample by lyophilization.

Table 9 shows selected extraction procedures for analyzing contaminants in solid foods by microwave-assisted extraction.

Table 9. Selected extraction procedures for analyzing contaminants in solid foods by microwave-assisted extraction

Analyte(s)	Matrix	Extractant	Ref.
Pesticides	Vegetables	Acetone	[108]
Trichlorobenzenes	Fish	Pentane	[109]
Zearalenone[a]	Wheat, Corn	Methanol/Acetonitrile	[110]
Pesticides	Fish	Ethyl acetate/Cyclohexane	[111]
Dichlorvos[b]	Vegetables	Ethylene Glycol/H_2O	[112]
Pesticides	Oyster	Acetone	[113]
Flavor Ingredients	Potato Chips	Water	[114]
Salinomycin[c]	Eggs, Tissues	Ethanol/2-propanol (88:12)	[115]

[a] Mycotoxin. [b] Insecticide. [c] Coccidiostat.

3.5. Pressurized Liquid Extraction

Pressurized fluid extraction (PLE) is also known as pressurized solvent extraction (PSE), accelerated solvent extraction (ASE), pressurized accelerated solvent extraction (PASE) and enhanced solvent extraction (ESE).

In PLE (Figure 8), a solid sample is packed into the extraction cell and analytes are extracted from the matrix with conventional low-boiling solvents or solvent mixtures at elevated temperatures up to 200 °C and pressure (30-200 atm) to maintain the solvent in the liquid state. Under these conditions, analyte recoveries equivalent to or better than those obtained from Soxhlet extraction are achieved using less solvent and remarkably less time than Soxhlet procedure. Increasing temperature results in increased solubility, diffusion rate and mass transfer, while solvent viscosity and surface tension decrease. At elevated temperatures, the activation energy associated with desorption is more easily achieved and then the kinetics of desorption and solubilization become more favorable. The sample is

mixed with an inert diluent, such as sand or clay, to ensure that no dead space in the high-pressure stainless-steel cell used for the extraction is present. Following an initial heating period, the analyte is allowed to interact statically with the pressurized solvent for a predetermined time, after which the pressurized solvent is purged from the cell by a nitrogen stream and the extract is collected in a vial. The PLE extraction can be performed in a static mode, a dynamic mode, in this case the solvent flows continuously through the device, or in a combination of both modes. A very interesting feature of this technique is the possibility of full automation and many samples can be extracted sequentially. Most of the applications reported were performed on a Dionex ASE 200, until recently the only commercially available PLE system [116-124]. With the Dionex ASE 200, one can reach temperatures up to 200 °C and pressures up to 21 MPa in extraction cells having volumes between 1 and 33 ml. Up to 24 samples can be placed in the carrousel and the extracts collected in 26 vials of 40 or 60 ml.

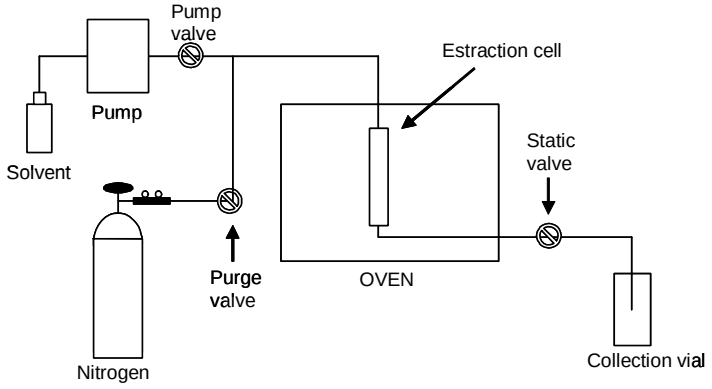


Figure 8 Schematic of a pressurized fluid extraction apparatus.

In general, the extraction efficiency of ASE is influenced by both extraction pressure and temperature, which are the operation parameters of ASE. In addition, sample matrix effects also affect the extraction efficiency. Therefore, the extraction behaviour of ASE is not plain and the optimization of operating conditions is laborious. Another weakness of ASE is that, when using hydrophobic organic solvents, the presence of relatively high water percentages in the sample strongly decreases analyte extraction efficiency, as water hinders contact between the solvent and the analyte. When extracting food samples, a remedy adopted by many searchers is that of adding anhydrous sodium sulphate in the extraction cell or to adopt a preliminary lyophilization step.

Table 10 shows selected extraction procedures for analyzing contaminants in solid foods by pressurized liquid extraction.

Table 10. Selected extraction procedures for analyzing contaminants in solid foods by pressurized liquid extraction

Analyte(s)	Matrix	Extractant	T, °C	Ref.
Acaricides	Honey	Hexane/Propanol (1:3)	95	[116]
Pesticides	Strawberry	H_2O/Acetonitrile (9:1)	120	[117]
Pesticides	Vegetables	Acetone/dichloromethane (3:1)	110	[118]
Glucocorticoids	Bovine Liver	Hexane/ethyl acetate (1:1)	50	[119]
Pesticides	Fish Tissue	Ethyl acetate/Cyclohexane	125	[120]
PAHs[a]	Smoked Food	Methylene chloride/Acetonitrile (9:1)	100	[121]
Pesticides	Foods	Acetonitrile	100	[122]
PCBs[b], Pesticides	Fish Tissue	Hexane/ dichloromethane (1:1)	90-120	[123]
Zearalenone[c]	Wheat, Corn	Methanol/Acetonitrile	80	[124]

[a] PAHs= polycyclic aromatic hydrocarbons. [b] PCBs= polychlorinated biphenyls. [c] Mycotoxin.

3.6. Matrix solid-phase dispersion

Usually, solid samples are prepared for subsequent extraction and cleanup by a stepwise process that begins with disruption of the gross architecture of the sample. The process of sample disruption is intended to divide the sample into smaller and smaller pieces, providing an overall greater surface that may subsequently be exposed to extraction. This may involve mincing or dicing of the sample, followed by homogenization in the presence of water or organic solvents of various compositions. Similarly, samples may be frozen in liquid nitrogen or by exposure to dry-ice or they may be freeze-dried to produce a material that can be mechanically pulverized. These processes produce a finely divided powder that may then be extracted with suitable solvents.

The procedures offered for disruption and extraction ideally isolate the target analytes with high efficiency and a fair degree of chemical specificity. However, many efforts to isolate the compound(s) of interest require repeated extraction of the homogenized matrix, replacing the solvent with a fresh one each time, remixing, centrifuging and pooling the supernatant fractions in the end, in order to obtain adequate analyte recovery. This approach can require the use of large solvent volumes and the subsequent need to evaporate and dispose of the solvent so employed. In many cases, the sample and solvent combination lead to formation of emulsions, which further complicates the efficiency of the extraction and adds greatly to the time required for the analyst to complete the protocol.

Another approach involves the use of abrasives, such as sand or chemically modified silica sorbents, blended with the sample by means of a mortar and pestle or by a related mechanical device. The shearing forces generated by the blending process disrupt the sample architecture and provide a more finely divided material for extraction. The material can either be mixed directly with solvents or packed into a column to perform a more classic chromatographic elution. Some procedures use abrasives that also possess the properties of a drying agent, such as sodium sulfate or silica, producing a material that is finely divided but also quite dry for subsequent extraction as described. Such sample preparation also permits extraction by continuous reflux techniques (Soxhlet devices). Blending samples with a drying agent is also commonly used today in Supercritical Fluid Extraction protocols, wherein the presence of water otherwise compromises the extraction results.

It has to be pointed out that, according to the definition given by Barker [11], only those procedures making use of chemically bonded silica sorbents (C18 or C8) as a mean for dispersing a solid or even a liquid matrix (such as milk) should be referred to as the MSPD technique [125-130]. Anyway, many authors in recent years have adopted the acronym MSPD even when materials other than chemically bonded silica sorbents were used [131-141].

Once the MSPD process is complete, the material is transferred to a column constructed from a syringe barrel, or some other appropriate device, containing a frit that retains the entire sample. The sample is then compressed to form a column packing by using a modified syringe plunger. A second frit may be placed on top of the material before compression. The principles of performing good chromatography always apply: one should avoid channels in the column and not over-compress or compact the material.

Many MSPD procedures also employ the use of co-columns to obtain further fractionation and to assist in extract cleanup (Figure 9). A co-column material (Florisil, silica, alumina, for example) can be packed in the bottom of the same cartridge containing the dispersant/matrix blend or used as an external column. Such columns may be literally stacked so as to collect and fractionate the sample as it elutes from the MSPD column.

Addition of eluting solvent to the column may be preceded by use of some or all of the solvent to backwash the mortar and pestle. Most applications have utilized 8 ml of solvent to perform analyte extraction. Evidence from some studies indicates that most target analytes are eluted in the first 4 ml of extractant, provided that 0.5 g of the sample is mixed with 2 g of the solid support.

The analytical protocol is drastically simplified and shortened; (2) the possibility of emulsion formation is eliminated; (3) solvent consumption is substantially reduced; (4) last but not least, the extraction efficiency of the analytes is enhanced as the entire sample is exposed to the extractant. An interesting feature of the MSPD technique is that it can be used for extracting analytes from both solid and liquid foods.

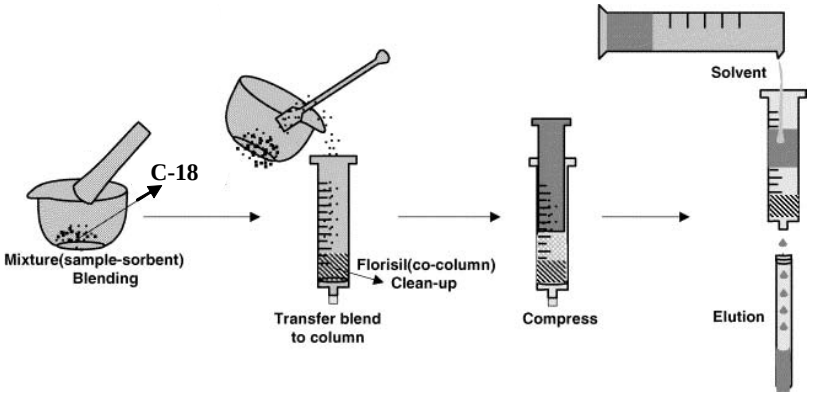


Figure 9. Schematic representation of a typical MSPD extraction procedure (Reproduced (modified) from [142] with permission from Elsevier© 1999).

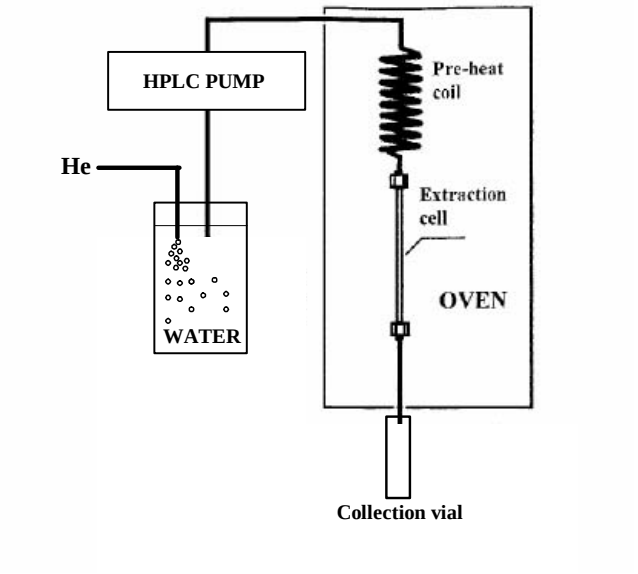


Figure10. Schematic view of the laboratory-made extraction device for matrix solid-phase dispersion technique with hot water as extractant

Table 11. Selected extraction procedures for analyzing contaminants in solid and liquid foods by matrix solid-phase dispersion

Analyte(s)	Matrix	Extractant	Support	T, °C	Ref.
Sulfonamides[a]	Meat, Baby Food	Water	C-18[b]	160	[125]
Pesticides	Vegetables	DCM[c]/AcN[d]	C-8[e]	room	[126]
Abamectin[f]	Orange	DCM	C-18	room	[127]
Chloramphenicol[g]	Muscle Tissue	AcN/water	C-18	room	[128]
Clenbuterol[h]	Liver	AcN, 1% AcH	C-18	room	[129]
Mycotoxins	Peanut	AcN	C-18 +sand	room	[130]
Aminoglycosides[g]	Bovine muscle	Water	Cyanopropyl[i]	room	[131]
Sulfonamides	Bovine/fish muscles	Water	Crystobalite[j]	80	[132]
Pesticides	Vegetables	DCM	Florisil[k]	room	[133]
Pesticides	Milk	Ethanol-LP[l]	Hydromatrix[m]	room	[134]
Sulfonamides	Milk	Water	Crystobalite	75	[135]
Sulfonamides	Eggs	Water	Crystobalite	100	[135]
Sulfonamides	Liver and Kidney	Water	Crystobalite	80	[136]
Pesticides	Milk	Water	Crystobalite	90	[137]
Aminoglycosides	Milk	Water	Crystobalite	70	[138]
Microcystins[n]	Fish tissue	Water (pH 2)	Crystobalite	80	[139]
PCBs[o], PCDDs[p] and PCDFs[q]	Yoghurt	Acetone/ hexane (1:1)	silica	room	[140]
PAHs[r]	Honey	Hexane/ethyl acetate (9:1)	Florisil	room	[141]

[a] Antibacterial agents. [b] octadecyl-modified silica. [c] DCM= dichloromethane. [d] AcN= acetonitrile. [e] Octyl-modified silica. [f] Insecticide. [g] Antibiotics. [h] Antiasthmatic. [i] Cyanopropyl-modified silica. [j] Low-surface area siliceous material. [k] Magnesium silicate. [l] LP= light petroleum. [m] Hydromatrix is a diatomaceous material. [n] Algal toxins. [o] PCBs= polychlorinated biphenyls. [p] PCDDs= polychlorinated dibenzo-p-dioxins. [q] PCDFs= polychlorinated dibenzofurans. [r] PAHs=polycyclic aromatic hydrocarbons.

Over classical sample treatment procedures, MSPD offers distinct advantages in that: (1) With the exception of a work proposing water at ambient temperature for extracting the highly hydrophilic aminoglycoside antibiotics from bovine kidney [131], all other methods based on MSPD have used moderate amounts of organic solvents as extractants. This means that problems associated to the use of organic solvents are minimized by MSPD, but not completely removed. Moreover, since no organic solvent is capable of selectively extracting target compounds from complex biological matrices, a sample cleanup step is often included

in protocols involving analyte extraction by the MSPD technique. Finally, the use of an organic solvent precludes direct introduction of the extract into a reversed-phase LC column.

An extraction scheme first introduced by Hawthorne et al. [143] which has recently received a considerable interest, involves the use of hot water as an effective extractant for a large number of compounds having a broad spectrum of polarity in solid environmental samples [144-150]. Like CO_2 used in supercritical fluid extraction, water is an environmentally acceptable solvent, it is cost effective and hot water conditions are easily achieved with commercial laboratory equipment (Figure 10). The polarity of water decreases as the temperature is increased. This means that selective extraction of polar and medium polar compounds can be performed by suitably adjusting the water temperature.

Very recently, the MSPD technique with hot water as extractant has been successfully used for developing LC-MS methods capable of detecting contaminants in various foodstuffs, such as animal tissues, milk, eggs, vegetables, baby food. Besides to the advantages mentioned above, heated water provides sufficiently clean extracts needing little manipulation (pH adjustment and filtration) before injection. When using a reversed-phase LC column (this occurs virtually in all analytical procedures concerning analysis of toxicants in food), volumes as large as 1 ml of the aqueous extract can be injected into the LC apparatus without provoking any peak distortion. By suitably adjusting chromatographic conditions, it was estimated that the concentration level of a targeted compound in an incurred animal muscle tissue could be determined in less than 1 h after receiving the sample [132].

Table 11 shows selected extraction procedures for analyzing contaminants in solid and liquid foods by solid-phase matrix dispersion extraction.

Some general conclusions regarding state-of-the-art of extraction procedures of contaminants in food and future trends are reported below.

A. In a large majority of all quoted studies, LPE (or, with liquid samples, LLE) is used for analyte isolation, with subsequent cleanup and analyte enrichment by means of SPE cartridges filled with C-18.
B. For solid, semi-solid and liquid foodstuffs, MSPD with cold or heated extractants is gradually replacing the above two mentioned extraction techniques.
C. Future challenges in the field of toxicant analysis in food will be replacement of current methods dependent on hazardous solvents and replacement of methods which are very time and/or analyst intensive.
D. Developing analytical approaches for “multi-compound class” analysis, with a typical example being veterinary drugs and pesticides.

References

[1] H. B. Wan, M. K. Wong, J. Chromatogr. A, 754 (1996) 43.
[2] C. F. Poole, S. K. Poole, Anal. Comm. 33 (1996) 11H.
[3] V. Lopez-Avila, Crit. Rev. Anal. Chem. 29 (1999) 195.

[4] M. Leterrier, H. Budzinski, Analusis 27 (1999) 259.
[5] V. Camel, TRAC-Trends Anal. Chem. 19 (2000) 229.
[6] S. B. Hawthorn, Anal. Chem. 62 (1990) 633A.
[7] S. Bøwadt, S. B. Hawthorne, J. Chromatogr. A 703 (1995) 549.
[8] R. M. Smith, J. Chromatogr. A 856 (1999) 83.
[9] E. Björklund, S. Bøwadt, T. Nilsson, TRAC-Trends Anal. Chem., 19 (2000) 434.
[10] S. A. Barker, A. R. Long, C. R. Short, J. Chromatogr. A, 475 (1989) 353.
[11] S. A. Barker, J. Chromatogr A., 885 (2000) 115.
[12] C. L. Arthur, J. Pawliszyn, Anal Chem. 62 (1990) 2145.
[13] E. Baltussen et al., J. Microcol. Sep. 11 (1999) 737.
[14] G. Audunsson, Anal. Chem. 58 (1986) 2714.
[15] H. de Ruyck, E. Daeseliere, H. de Ridder, R. van Renterghem, J. Chromatogr. A, 976 (2002) 181.
[16] N. Kishikawaa, M.Wadaa, N. Kurodaa , S. Akiyamab, K. Nakashima, J. Chromatogr. B, 789 (2003) 257.
[17] C. Danielsson, K. Wiberg, P. Korytar S. Bergek,U. A.Th. Brinkman, P. Haglund, J. Chromatogr. A, 1086 (2005) 61.
[18] C. Y. W. Ang, W. Luo, J. AOAC Int., 80 (1997) 25.
[19] L. K. Sørensen, B. M. Rasmussen, J. O. Boisen, L. Keng, J. Chromatogr. B, 694 (1997) 383
[20] L.K. Sørensen, L.K. Snor, J. Chromatogr. A 882 (2000) 145.
[21] S. Ghidini, E. Zanardi, G. Varisco, R. Chizzolini, Food Addit. Contam. 20 (2003) 528.
[22] D. M. Holstege, B. Puschner, G. Whitehead, F. D. Galey, J. Agric. Food Chem., 50 (2002) 406.
[23] M. Dubois, D. Fluchard, E. Sior, Ph. Delahaut, J. Chromatogr. B, 753 (2001) 189.
[24] D. N. Heller, M. A. Ngoh, D. Donoghue, L. Podhorniak, H. Righter, M. H. Thomas, J. Chromatogr. B, 774 (2002) 39.
[25] L. K. Sørensen, T. H.Elbæk, J. Chromatogr. B, 820 (2005) 183.
[26] V. Hormazábal, M. Yndestad, O. Ostensvik, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 1585.
[27] K. Terada, J. Sakabe, .J. Chromatogr. 348 (1985) 379.
[28] Albero, C. Sánchez-Brunete, J.L. Tadeo, Talanta, 66 (2005) 917.
[29] J.-F. Focant, E. De Pauw, J. Chromatogr. B, 776 (2002) 199.
[30] S. Raoul, E. Gremaud, H. Biaudet, R. J. Turesky, J. Agric. Food Chem., 45 (1997) 4706.
[31] A. Bacaloni, C. Cavaliere, A. Faberi, E. Pastorini, R. Samperi, A.Laganà, J. Agric. Food Chem., 53 (2005) 5518.
[32] J. Dallüge, T. Hankemeier, R. J. J. Vrelus, U. A. Th. Brinkman, J. Chromatogr. A 830 (1999) 377.
[33] Laganà, A. Marino, G. Fago, Chromatographia 41 (1995) 178.
[34] Laganà, G. Fago, A. Marino, B. Pardo-Martinez, Chromatographia 38 (1994) 88.
[35] F. Bruno, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, J. Agric. Food Chem. 49 (2001) 3463.
[36] Cavaliere, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, J. Agric. Food Chem., 51 (2003) 558.
[37] F. Bruno, R. Curini, A. Di Corcia, M. Nazzari, M. Pallagrosi, Rapid Commun. Mass Spectrom., 16 (2002) 1365.
[38] A. Di Corcia, R. Samperi, Anal. Chem., 65 (1993) 907.

[39] M. Yan, J. Clin. Ligand Assay, 25 (2002) 234.
[40] B. Bjarnason, L. Chimuka, O. Ramström, Anal. Chem. 71 (1999) 2152
[41] R. Weiss,., M. Freudenschuss, R. Krska, B. Mizaikoff, Food Addit. Contam. 20 (2003) 386.
[42] T. Kumazawa, X.-P. Lee, K. Sato, O. Suzuki, Anal. Chim. Acta, 492 (2003) 49.
[43] C. M. Lock, L. Chen, D. A. Volmer, Rapid Commun. Mass Spectrom. 13 (1999) 1744.
[44] J. Wu, C. Tragas, H. Lord, J. Pawliszyn, J. Chromatogr. A, 976 (2002) 357.
[45] T. Goldmann, A. Perisset, F. Scanlan, R. H. Stadle, Analyst, 130 (2005) 878.
[46] J. A. Sales, Z. De Lourdes Cardeal, Food Addit. Contam., 20 (2003) 519.
[47] M. Azenha, M. T. Vasconcelos, Anal. Chim. Acta, 458 (2002) 231.
[48] M. Volante, M. Pontello, L. Valoti, M. Cattaneo, Pest. Manag. Sci. 56 (2000) 618.
[49] M. Volante, M. Cattaneo, M. Bianchi, G. Zoccola, J. Environ. Sci. Health, B33 (1998) 279
[50] R. Andrade, F. G.R. Reyes, S. Rath, Food Chem., 91 (2005) 173.
[51] C. Bicchi, C. Cordero, P. Rubiolo, P. Sandra, Eur. Food Res. Technol., 216 (2003) 449.
[52] F. David, A. Tredoux, E. Baltussen, A. Hoffmann, P. Sandra, in P. Sandra (Ed) proceedings of the 23rd International Symposium on Capillary Chromatography, IOPMS, Kortriik Belgium 2000.
[53] P. Sandra, B. Tienpoint, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A, 928 (2001) 117.
[54] P. Sandra, B. Tienpont, F. David, J. Chromatogr. A, 1000 (2003) 299.
[55] N. Ochiai, K. Sasamoto, M. T. S. Yamashita, S. Daishima, A. C. Heiden, A. Hoffmann, Anal. Bioanal. Chem. 373 (2002) 56.
[56] M. Luque, A. Rios, M. Valcarcel, Analyst, 125 (2000) 1805.
[57] M. Khrolenko, P. Dzygiel, P. Wieczorek, J. Chromatogr. A, 975 (2002) 219.
[58] T A. Msagati, M. Nindi. Microchim. Acta, 148 (2002) 199
[59] R. Romero, J. A. Jonson, D. Gazquez, M. G. Bagur, M. Sanchez-Vinas, J. Sep. Sci., 25 (2002) 584.
[60] T. Hyoetylaeinen, K. Luethje, M Rautiainen-Raemae, M.-L. Riekkola, J. Chromatogr. A, 1056 (2004) 267.
[61] B. B. Sitholé, S. M. Nyarku, L. H. Allen, Analyst 120 (1995) 1163.
[62] A. Zohair, A. B. Salim, A. A. Soyibo, A. J. Beck, Chemosphere, 63 (2006) 541.
[63] A. Al-Omair, M. I. H. Helaleh, Chromatographia, 59 (2004) 715.
[64] M. Jánská, M. Tomaniová, J. Hajsl̆ová, V. Kocourek, Food Addit. Contam., 23 (2006) 309.
[65] J.R. Pedersen, J.O.Olsson, Analyst, 128 (2003) 332.
[66] R.G. Diagne, G.D. Foster, S.U.Khan, J. Agric. Food Chem., 50 (2002) 3204.
[67] M. S. Ali, T. Sun, G. E. McLeroy, E. T. Philippo, J. AOAC Int., 83 (2000) 39.
[68] M. Cherlet, S. De Baere, P. de Baker, J. Mass Spectrom., 35 (2000) 1342.
[69] C. K. Fagerquist, A. R. Lightfield, Rapid Commun. Mass Spectrom., 17 (2003) 660.
[70] R. Codony, R. Compañó, M. Granados, J. A. García-Regueiro, M. D. Prat, J. Chromatogr. A, 959 (2002) 131.
[71] N. van Eeckhout, J. C. Perez, C. van Peteghem, Rapid Commun. Mass Spectrom., 14 (2000) 2331.
[72] M. Cherlet, M. Schelkens, S. Croubels, P. de Backer, Anal. Chim. Acta, 492 (2003) 199.
[73] L. Johnston, L. Mackay, M. Croft, J. Chromatogr. A, 982 (2002) 97.
[74] M. Ramos, P. Muñoz, A. Aranda, I. Rodriguez, R. Diaz, J. Blanca, J. Chromatogr B, 791 (2003) 31.
[75] L. Mortier, E. Daeseleire, P. Delahaut, Anal. Chim. Acta, 483 (2003) 27.

[76] M. Horie, H. Nakazawa, J. Chromatogr. A, 882 (2000) 53.
[77] K. de Wasch, H. F. de Brabander, S. Impens, M. Vandewiele, D. Courtheyn, J. Chromatogr. A, 912 (2001) 311.
[78] B. Bocca, M. Di Mattia, C. Cartoni, M. Fiori, M. felli, B. neri, G. Brambilla, J. Chromatogr. B, 783 (2003) 141.
[79] Di Corcia, C. Crescenzi, A. Laganà, E. Sebastiani, J. Agric. Food Chem., 44 (1996) 1930.
[80] K.R. Grose, J.L. Grant, L.F.Bjeldanes, J. Agric. Food Chem., 34 (1986) 201.
[81] F. Toribio, L. Puignou, M.T.Galceran, J. Chromatogr. A, 836 (1999) 223.
[82] L. M. Tikkanen, K. J. Latva-Kala, R.-L. Heiniö, Food Chem. Toxicol., 34 (1996) 725.
[83] C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Laganà, Rapid Comm. Mass Spectrom., 19 (2005) 2085.
[84] L. A Stobo, J.-P. C. L. Lacaze, A. C. Scott, S. Gallacher, E. A. Smith, M. A.Quilliam, J. AOAC Int., 88 (2005) 1371.
[85] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, J. AOAC Int., 81 (1998) 441.
[86] M. J. Nieva-Cano, S. Rubio-Barroso, M. J. Santos-Delgado, Analyst, 126 (2001) 1326.
[87] M.D. Guillen, P. Sopelana, J. Dairy Sci., 87 (2004) 556.
[88] A. Cappiello, G. Famiglini, B. Tirillini, Chromatographia, 40 (1995) 411.
[89] A. Pietri, T. Bertuzzi, G. Piva, Food Addit. Contam., 14 (1997) 341.
[90] P. Pugliese, J. C. Molto, P. Damiani, R. Marin, L. Cossignani, J. Manes, J. Chromatogr. A, 1050 (2004) 185.
[91] C. Kong-Hwa, Y. Hwa Kwang, W. Joanna Shaofen, M. W. Chien, Green Chem., 6 (2004) 502
[92] J. H. Shim, J. Y. Shen, M. R. Kim, C. J. Lee, I. S. Kim, J. Agric. Food Chem., 51 (2003) 7528.
[93] R. P. Huopalahti, J. Hebel, J. D. Henion, J. Liq. Chromatogr. Relat. Technol., 20 (1997) 537.
[94] M. Y. Ali, R. B. Cole, J. Agric. Food Chem., 49 (2001) 4192.
[95] J. W. Pensabene, W. Fiddler, D. J. Donoghue, J. AOAC Int., 82 (1999) 1334.
[96] J. Poustka, K. Holadova, J. Hajslova, Eur. Food Res. Technol. 216 (2003) 68.
[97] A. Kaihara, K. Yoshii, Y. Tsumura, S. Ishimitsu, Y. Tonogai, J. Health Sci. 48 (2002) 173.
[98] K. N. T. Norman, S. H. W. Panton, J. Chromatogr. A, 907 (2001) 247.
[99] M. L. Hopper, J. Chrmatogr. A, 840 (1999) 93.
[100] R. K. Juhler, Analyst, 123 (1998) 1551.
[101] C. Nerin, R. Batlle, J. Cacho, J. Chromatogr. A, 795 (1998) 117.
[102] M. T. Tena, A. Rios, M. Varcarcel, M. Sanchez-Alarcon, Chromatographia, 46 (1997) 524.
[103] M. Danaher, M. O'Keeffe, J. D: Glennon, Anal. Chim. Acta, 483 (2003) 313.
[104] R. Rodil, A. M. Carro, R. A. Lorenzo, R. Cela Torrijos, Anal. Chem., 77 (2005) 2259.
[105] K. Ganzler, A. Salgo, K. Valko, J. Chromatogr., 371 (1986) 299.
[106] H. M. Kingston, L. B. Jassie, Introduction to microwave Sample Preparation, Theory and practice. American Chemical Society, Washington, D. C., 1986.
[107] J. L. Luque-Garcia, M.D. Luque de Castro, Talanta, 64 (2004) 571.
[108] S. Singh, G. D. Foster, S. U. Khan, J. Agric. Food Chem., 52 (2004) 105.
[109] G. Wittmann, T. Huyvrechts, H. Van Langenhove, J. Dewulf, H. Nollet, J. Chromatogr. A, 993 (2003) 71.
[110] L. Pallaroni, C. von Holst, C. S. Eskilsson, E. Bjoerklund, Anal. Bioanal. Chem., 374 (2002) 161.
[111] M. Weichbrodt, W. Vetter, B. Luckas, J. AOAC Int., 83 (2000) 1334.
[112] Yi-I, Chen, Y-S, Su, J-F, Jen, J. Chromatogr. A, 976 (2002) 349.

[113] N. Carro, I. Garcia, M.-C. Ignacio, M. Llompart, M.-C. Yebra, A. Mouteira, Anal. Bioanal. Chem., 374 (2002) 547.
[114] Y. Wang, M. Bonilla, H. M. Mcnair, M. Khaled, J. High Resol. Chromatogr., 20 (1997) 213.
[115] M. H. Aktar, J. Environ. Sci. Health, B39 (2004) 835.
[116] E. Korta, A. Bakkali, L. A. Berrueta, B. Gallo, F. Vicente, J. Food Protection, 65 (2002) 161.
[117] L. Wennrich, P. Popp, G. Koller, J. Breuste, J. AOAC Int., 84 (2001) 1194.
[118] K. Adou, W. R. Bontoyan, P. J. Sweeney, J. Agric. Food Chem., 49 (2001) 4153.
[119] R. Draisci, C. Marchiafava, L. Palleschi, P. Cammarata, S. Cavalli, J. Chromatogr. B, 753 (2001) 217.
[120] M. Weichbrodt, W. Vetter, B. Luckas, J. AOAC Int., 83 (2000) 1334.
[121] G. Wang, A. S. Lee, M. Lewis, B. Kamath, R. K. Archer, J. Agric. Food Chem., 47 (1999) 1062.
[122] M. Okihashi, H. Obana, Analyst, 123 (1998) 711.
[123] P. Suchan, J. Pulkrabova, J. Hajslova, V. Kocourek, Anal. Chim. Acta, 520 (2004) 193.
[124] L. Pallaroni, , C. von Holst, J. Chromatogr. A, 993 (2003) 39.
[125] A. Gentili, D. Perret, S. Marchese, M. Sergi, C. Olmi, R. Curini, J. Agric. Food Chem., 52 (2004) 4614.
[126] M. Fernandez, Y. Pico, J. Mañes, J. Chromatogr. A, 871 (2000) 43.
[127] A. I. Valenzuela, M. J. Redondo, Y. Pico, G. Font, J. Chromatogr. A, 871 (2000) 57.
[128] H. Kubala-Drincic, D. Bazulic, J. Sapunar-Postruznik, M. Grubelic, G. Stuhne, J. Agric. Food Chem., 51 (2003) 871.
[129] Crescenzi, S. Bayoudh, P. A. G. Cormack, T. Klein, K. Ensing, Anal. Chem., 73 (2001) 2171.
[130] J. Blesa, J. M. Soriano, J. C. Molto, R. Marin, J. Manes, J. Chromatogr. A, 1011 (2003) 49.
[131] L. G. McLaughlin, J. D. Henion, P. J. Kijak, Biol. Mass Spectrom., 23 **(**1994) 417.
[132] S.Bogialli, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, Anal. Chem., 75 (2003) 1798**.**
[133] E. Viana, J. C. Moltó, G. Fouty, J. Chromatogr. A, 754 (1996) 437.
[134] Di Muccio, P. Pelosi, I. Camoni, D. A. Barbini, R. Dommarco, T. Generali, A. Ausili, J. Chromatogr. A, 754 (1996) 497.
[135] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, M.L. Polci, J. Agric. Food Chem., 51 (2003) 4225.
[136] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, M. Sergi, Rapid Comm. Mass Spectrom., 17 (2003) 1146.
[137] S. Bogialli, R. Curini, A. Di Corcia, A. Laganà, M. Nazzari, M. Tonci. J. Chromatogr. A, 1054 (2004) 351.
[138] S. Bogialli, R. Curini, A. Di Corcia, A. Laganà, M. Mele, M. Nazzari, J. Chromatogr. A, 1067 (2005) 93.
[139] S. Bogialli, M. Bruno, R. Curini, A. Di Corcia, A. Laganà, B. Mari, J. Agric. Food Chem., 53 (2005) 6586.
[140] L. Ramos, E. Eljarrat, L. M. Hernandez, J. Rivera, M. J. Gonzalez, Anal. Chim. Acta, 402 (1999) 241.
[141] B. Albero, C. Sanchez-Brunete, J. L. Tadeo, J. AOAC Int., 86 (2003) 576.
[142] J. F. Garcia-Reyes, I. Ferrer, E. M. Thurman, A. R Fernandez-Alba, , J. Chromatogr. A, 1069 (2005) 183.
[143] S. B. Hawthorne, Y. Yang, D. J. Miller, Anal. Chem., 66 (1994) 2912.
[144] Y. Yang, S. Bøwadt, S. B. Hawthorne, D. J. Miller, D. J. Anal. Chem., 67 (1995**)** 4571.
[145] Y. Yang, S. B. Hawthorne, D. J. Miller, Environ. Sci. Technol., 31 (1997**)** 430.

[146] M. M. Jiménez-Carmona, J. J. Manclús, A. Montoya, M. D. Luque de Castro, J. Chromatogr. A, 785 (1997) 329.
[147] J. A. Field, K. Monohan, R.Reed, Environ. Sci. Technol.,.33 (1999) 2782.
[148] C. Crescenzi, G. D'Ascenzo, A. Di Corcia, M. Nazzari, S. Marchese, R. Samperi, Anal. Chem., 71 (1999) 2157.
[149] C. Crescenzi, A. Di Corcia, M. Nazzari, R. Samperi, Anal. Chem., 72 (2000) 3050.
[150] F. Bruno, R. Curini, A. Di Corcia, I. Fochi, M. Nazzari, R. Samperi, Environ. Sci. Technol., 36 (2002) 4156.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 10

Clean-up and fractionation methods

Dimitrios J. Fletouris

Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Aristotle University, Thessaloniki 54124, Greece

1. Introduction

Despite advances in the sensitivity of analytical instrumentation for the end-point determination of analytes in food samples, a pre-treatment is usually required to extract and isolate the target analytes from the food matrix, thus facilitating their determination. In most cases, although the analytes of interest are isolated from the bulk matrix, several contaminants may also be co-extracted, as well as part of the matrix, which could interfere in the determination step of the analysis. Consequently, further purification of the extract is required before the analytes determination. This step is called the clean-up step and aims at the isolation of the target analytes from potential interfering co-extractives as well as discarding the extraction solvent and preparing the target analytes in a chemical form appropriate for its characterization and quantification.

Several options are available for sample clean-up depending upon the analytes of interest, the matrix and the potential interfering co-extractives. Conventional liquid-liquid partitioning techniques are still very popular and will remain as such in the near future. These techniques are time tested, and analysts are familiar with the relevant processes and procedures. However, they are often time-consuming and labour-intensive, and usually require large volumes of organic solvents.

During the last two decades, regulatory pressure towards increased productivity and reduction of the organic solvents used for extraction has created an increasing demand for alternative faster and more automated clean-up procedures. Some of the newer procedures use the same basic principles as the older ones but provide fast and easy-to-use options and generally consume less organic solvents. However, for the most part, they have higher initial purchase price than the traditional methods.

The research in this challenging topic has been stimulated and there has been more activity in the past 10 years than at any other time in history. New sample preparation technology that holds promise for increasing accuracy and throughput in sample preparation has been introduced. We have seen considerable advances in this area, in particular with the introduction of sorbents with high affinity and/or selectivity for solid-phase extraction (*e.g.* immunoaffinity columns and molecular imprinted polymers) of an ever-expanding list of analytes from various matrices. Nevertheless, the demand for quicker, more generic extraction

procedures is still on the rise. This is mainly because the time available to develop new assay extraction methods, as well as the time to process a batch of samples once the method has been validated, has been significantly compressed.

2. Nature of interferences

An inherent difficulty in the extraction of food samples is the co-extraction of matrix components that are also soluble in the extraction solvent. The presence of matrix interferences in sample extracts can result in a multitude of problems, including the generation of emulsions, sample turbidity, contamination or plugging of equipment, and, perhaps most importantly, the masking of the analytical signal for the target analyte and the consequent increase in the method limit of detection.

Matrix interferences may be caused by endogenous compounds and contaminants that are present in the sample. Peptides, aminoacids, carboxylic acids, phospholipids, pigments and many other food components may interfere with the analysis and their occurrence should be carefully evaluated in the sample preparation step. The extent of matrix interferences varies considerably from source to source, depending upon the nature of the food sample.

Spectral, physical, chemical or memory interferences can be observed when analysing a food sample using conventional detection systems. Spectral interferences may be observed when an overlap of a spectral line from another element or background contribution occurs; physical interferences may occur from effects associated with sample transport processes on instruments; chemical interferences are characterised by compound formation, ionisation or vaporisation effects; memory interferences occur from the contribution of signal from a previous sample to the sample being analysed.

Separation techniques such as liquid chromatography (LC) and gas chromatography (GC) are well suited for the analysis of complex multi-component samples. However, the analysis of food matrices requires well-designed sample preparation procedures since they often cause the largest variability in analytical results. In recent years the rapid adoption of mass spectrometry (MS) detectors to replace the conventional detectors has had the most significant impact on sample preparation demands.

In routine control of food toxicants, liquid chromatography obviously represents the method of choice whenever analysis of thermally unstable, polar or non-volatile toxicants is required. However, the demand for extensive clean-up when using this technique as a determinative step is urgent provided the most common fluorescence or ultraviolet detectors are used. These detectors are neither sensitive nor selective enough to enable unbiased determination of low levels of multiple residues of very different chemical structures in food matrices. The lack of a relevant detection technique has been overcome by combining liquid chromatography with mass spectrometric detection [1].

Nowadays, LC-MS instruments employing atmospheric pressure ionisation (API) are probably the most commonly used in trace analysis, whereas electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the most often applied ionisation techniques. To achieve better sensitivity and selectivity of target analytes detection, tandem mass spectrometry (MS/MS) employing either tandem-in-time MS/MS (ion trap analysers) or tandem-in-space MS/MS (e.g. triple stage quadrupoles) is a preferred option by most experts working in the field of trace analysis [1]. However, co-extracted components of the sample matrix can have adverse effects (matrix effects) on the results from an LC-API-MS(/MS) analysis. These matrix effects can be broadly categorized as spectral interference, adduct formation and ion suppression/enhancement [2-4], resulting in either suppression or enhancement of the analyte signal.

Although the exact mechanism and the origin of matrix suppression/enhancement phenomena are not fully understood, it is assumed [2] that, ions that appear at the same or nearly the same *m/z* value as the component of interest can cause spectral interference. Adduction of another ion with the component of interest shifts the *m/z* value at which the component of interest appears in the spectrum. Adduct ions such as sodium, potassium and ammonium can be picked up from the sample itself, from reagents used, or the container holding the sample. Ion suppression or enhancement is the result of components that suppress or enhance the ionisation of, or compete in the ionisation process with the component of interest. High levels of non-volatile substances may affect the transfer of ionised analyte into the gas phase by preventing the radius and surface charge of the droplets from reaching the levels necessary for ion emission [5]. Although both ionisation types can experience matrix effects [6,7], these effects are more pronounced with ESI than with APCI [8-10]. It is hypothesized that APCI is associated with a smaller degree of ion suppression because analytes are already in the gas phase when molecular reactions occur and therefore the smaller degree of suppression observed with APCI is due to analyte precipitation [5,7]. Ion suppression or enhancement caused by the matrix co-extractives is the most critical of these interferences because it is often the most difficult to determine. It has been demonstrated that even components of the sample that do not appear in the mass spectrum can cause ion suppression [11].

To address matrix effects in LC-MS(/MS), several approaches have been discussed in the literature. The possible strategies leading to elimination or reduction of matrix effects might include: (a) reduction of the amount of matrix components in injected sample by employing a more selective extraction procedure or a more extensive sample clean-up [11-13]; (b) decrease of the amount of injected sample [7,14]; (c) improvement of liquid chromatographic separation by modifying the mobile phase composition and/or changing the stationary phase [7]. If matrix suppression/enhancement phenomena cannot be eliminated by one of the above-mentioned ways, appropriate calibration technique (external matrix-matched standards, internal standard, and echo-peak technique) compensating as much as possible for matrix

effects might be used [1,7,15]. In addition, derivatisation is sometimes another interesting approach to enhance ionisation of low-mass (<150 g/mol) analytes and/or to induce more compound-specific fragmentation for multiple reaction monitoring detection [16].

In performing analyses of complex food samples using gas chromatography, miscellaneous problems can be also encountered, which are caused by the matrix unavoidably present in the sample injected into the gas chromatographic system. Such problems may occur both at the detector and the injector site. As far as conventional-element, structure or functional group selective detectors are used (electron capture, nitrogen phosphorus and flame photometric detectors are the most common in residue analysis), elution of volatile impurities with an identical retention time as the analyte may result in various unfavourable consequences, the most pronounced being [1]: (a) the peak of the analyte is obscured; (b) the impurity is falsely identified as analyte; (c) detector signal is increased; (d) quenching of the flame photometric detector signal. False positive and false negative results can both be hardly avoided if confirmation of the results by an independent alternative analytical procedure is not carried out. Mass spectrometric detectors obviously provide higher specificity based on a more detailed reflection of the molecular structure of a particular compound. However, adding a further dimension to the chromatogram does not necessarily guarantee positive handling of the co-elution problem. Common mass spectrometric detectors employing quadrupole and/or ion trap analysers can under some circumstances fail to provide unbiased confirmation of analyte identity. This might be a case especially for compounds yielding non-specific ions of low *m/z* values by electron impact ionisation process.

Apart from the above mentioned detection difficulties occurring in a particular run due to analyte-analyte and/or analyte-volatile matrix components co-elution, other severe problems might be encountered because of co-injection of non-volatile matrix constituents. Considering common sample preparation procedures, lipids (waxes, phospholipids, fatty acids etc), various pigments (chlorophylls, carotenoids, melanoidines etc) and other higher molecular mass components (plant resins etc) soluble in solvent used for extraction represent typical bulk co-extracts, part of which can be contained even in purified samples [1]. Depending on an employed injection technique, their deposits in the gas chromatography inlet and often also in the front part of the separation column may lead to loss of analytes, tailing of their peaks (integration problems) and impaired detectability (increased limits of detection). These phenomena, which are called “matrix-induced chromatographic response enhancement”, can be originated by the presence of various active sites in the injection port and the separation column that can be responsible for irreversible adsorption and/or catalytic (thermo)decomposition of susceptible analytes. Nevertheless, the sample components may compete for the active sites in the glass liner, decreasing the interaction between the active sites and the analyte, and thus allowing a larger amount of analyte to be transferred to the chromatographic column [17]. Compounds prone to matrix-induced chromatographic

enhancement effects are either thermolabile or rather polar and they are typically capable of hydrogen bonding [1].

To overcome matrix-induced chromatographic enhancement effects in gas chromatography applications five alternative approaches can be considered: (a) elimination of their primary causes by using a gas chromatographic system free of active sites or employing extensive clean-up of the extracts [1,18]; (b) calibration employing masking of active sites by preparing the calibration standards in blank extracts or using analyte protectants [17-21]; (c) use of an alternative injection technique, such as pulsed splitless injection or programmable temperature vaporizer injection [22,23], to avoid the effects of active sites; (d) correction function on biased results generated by calibration based on standards in pure solvents [24,25]; (e) standard additions or use of deuterated internal standards to compensate for matrix effects [1].

Undoubtedly, the assessment of an influence of sample matrix on the quality of generated data as a part of the validation procedure is a critical issue. Regardless of using gas or liquid chromatography are used for quantitation, the in-depth understanding of the nature of adverse matrix effects is a basic prerequisite of taking effective measures to prevent/compensate the occurrence of encountered problems.

Another source of interferences may be the possible carryover whenever high-concentration and low-concentration samples are sequentially analysed. To reduce carryover, the sample syringe must be thoroughly rinsed with solvent between samples. Whenever a sample with an unusually high analyte(s) concentration is encountered, it should be followed by the analysis of solvent to check for cross contamination.

Interference may also be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artefacts and/or elevated baselines in the chromatograms. The use of high purity reagents and solvents, and thoroughly cleaned glassware helps to minimise the above mentioned interference problems.

A conceivable strategy might be based on avoiding sample matrix to be injected into the chromatographic system. Unfortunately, the presence of residual sample components in sample can be hardly eliminated by common clean-up procedures such as liquid-liquid partitioning or (often in combination with) fractionation employing gel permeation, adsorption, partition and/or ion-exchange chromatography [19]. As far as sufficiently high recoveries for target analytes are required, at least part of co-extractives, possessing similar properties like analytes of interest, penetrate into their fraction. In a recent study addressing the accuracy issue in pesticide residue analysis, Schenk and Lehotay [26] have shown that even extensive clean-up of crude extracts obtained from various vegetables, fruit and cereals carried out by a combination of three types of solid-phase extraction sorbents (cartridges filled with graphitised carbon, primary/secondary amine and strong anion exchanger were used in series) cannot assure that no recoveries exceeding 100% will be found for any organophosphorus pesticides/matrix combination, although in many cases substantially reduced matrix enhancement effects were observed in purified samples. Nevertheless, the

results of another recent study [3] indicate that molecular imprinted polymer-based clean-up is effective in reducing ion suppression phenomena in ion trap LC-MS/MS multi-residue analysis of β-agonists at levels below 10%, with no detectable loss of sensitivity after 20 runs.

Moreover, the determination of multiple residues of food toxicants with a wide range of physicochemical properties within a single run can be a difficult analytical task to achieve sufficiently low limits of quantitation, good precision as well as relevant trueness of results for all the target analytes. In general terms, the wider the range of physicochemical properties of target analytes, the more complicated is the efficient removing of co-isolated matrix components from a particular crude extract. Depending on their nature (molecular size, polarity, thermal stability, volatility etc.), matrix components, which are unavoidably present in analysed samples (even after the thorough clean-up step), may interfere in various stages of the determinative step.

In any case, the importance of efficient clean-up should not be underestimated, since not only matrix effects but also the overall performance of an analytical system is unfavourably affected by non-target matrix components.

3. Methods based on partitioning

3.1. Conventional liquid-liquid partitioning

Conventional liquid-liquid partitioning constitutes one of the more popular clean-up techniques in food toxicants analysis. It involves the separation of analytes from interferences by partitioning the sample between two immiscible solvents. In most cases, one of the liquids is an aqueous solvent and the other is a water-immiscible organic solvent. The selectivity and efficiency of the extraction process is governed by the choice of the solvents pair. In aqueous and organic solvent pairs, the more hydrophilic compounds prefer the aqueous phase and the more hydrophobic compounds will be found in the organic phase. For successful liquid-liquid partitioning, the analyte should be extracted quantitatively in one of the two immiscible solvents; the extraction efficiency or the recovery should be close to 100%.

The water-immiscible organic solvents are suitable for liquid-liquid partitioning if they are volatile enough, show compatibility with the detection system, and posses polarity and hydrogen-bonding properties that enhance partitioning of the analytes in the organic phase. Among these provisions, polarity is usually the most important factor in choosing the extraction solvent [27]. The closer the solvent polarity is to the analyte polarity, the higher the extraction efficiency will be. The best solvent for a selective extraction procedure is the most apolar solvent with which the analyte can be extracted with sufficient efficiency. Various water-immiscible and volatile organic solvents are used, including dichloromethane, ethyl acetate, chloroform, diethyl ether, hexane and pentane. An increase in selectivity can also be

attained by the addition of inorganic salts that favour the transfer of the analyte from the aqueous to the organic phase through a salting-out effect.

Apart from polarity, extraction efficiency is also determined by the intensity and duration of mixing. A thorough mixing may generate an enormous interfacial area that ensures intimate contact between the two phases, which aids mass transfer [28]. Due to this vigorous mixing, emulsion formation is always a possibility in liquid-liquid partitioning, particularly for samples containing surfactants or fatty matrices. Emulsification can be avoided by using larger volumes of the extracting solvent or by less vigorous mixing of the samples. Methods that may be used for breaking up emulsions include centrifugation, addition of salts to the aqueous phase, addition of a small amount of a different organic solvent, and filtration of the emulsion through glass wool or phase-separation filter paper [27].

For compounds that are either acidic or basic, pH adjustment is normally of high importance in order to ensure high partition coefficients. It is the undissociated molecule of the analyte that is soluble in a non-polar organic solvent. Thus, prior to liquid-liquid partitioning of a basic compound, the pH of the aqueous sample solution has to be adjusted into the alkaline range, which favours the partition of basic compounds into water-immiscible organic solvents, due to suppression of their ionisation. For an efficient liquid-liquid partitioning clean-up, the extracted basic analyte can be back-extracted into an acidic aqueous solution that can in turn be basified to favour re-extraction of the analyte into the organic solvent. Acidic analytes have to be extracted at low pH taking advantage of their pK value and partitioning characteristics [29]. As far as amphoteric analytes, they show an optimum extraction pH, which in any case should be accurately determined [30], but the extraction of neutral analytes is independent of the pH value. For more hydrophilic compounds in contrast, low partition coefficients are observed even for their undissociated forms. Nevertheless, it may be proven sometimes extremely difficult to extract highly polar analytes at any pH value. In that case, salting-out or ion-pair extraction may be required to ensure a high-extraction efficiency, or alternatively, liquid-liquid partitioning may be accomplished with very high organic phase/aqueous phase ratios.

However, the analyte may occasionally be chemically unstable in the pH range that is necessary for an efficient liquid-liquid partitioning. To overcome this problem, the analyte can be derivatised with an appropriate reagent to form a suitable derivative that can be subsequently quantitatively extracted. On the other hand, a derivatisation step is frequently introduced to enhance the selectivity and capacity of the extraction phase for analytes such as polar or ionic species, which are difficult to extract. The objective of derivatisation is not only to convert the native analytes into stable or less polar derivatives that are extracted more efficiently, but also to label them for better detection or chromatography [28,31]. The most interesting implementation of this approach is the simultaneous extraction/derivatisation in one step since the derivatisation reagent is present in the extraction phase during the extraction process.

In addition to high extraction efficiency, successful liquid-liquid partitioning relies on a preconcentration step to obtain high analyte concentration in the final solution (high analyte enrichment). Thus, it is preferable to isolate the analytes of interest in a volatile organic solvent, because the analytes can be generally concentrated by evaporation of the solvent. Normally, the extract (organic solvent) is evaporated to dryness and the residue is reconstituted in a small volume to ensure high analyte enrichment. For practical reasons, the volume of reconstitution is normally in the range of 100 to 1000 µl, giving the maximum theoretical analyte enrichment factor in the range of 10-100 (with 100% extraction efficiency), if an initial food sample of 10 g (or 10 ml) is used. From a practical point of view, the evaporation step of liquid-liquid partitioning is cumbersome and loss of analyte may occur following partial evaporation or adsorption to the equipment. Moreover, care should be exercised during evaporation, as traces of acids or alkalis present in the extract may degrade acid- or base-labile compounds when solvent volume approaches dryness [32,33] unless a small quantity of a "keeper" solvent, such as ethylene glycol, has been introduced.

For difficult separations, multiple extractions are frequently carried out, although in many cases the background is also co-extracted. Using multiple extractions, polar interferences may sometimes be transferred from the aqueous into organic solvents that can dissolve minute amounts of water. This problem cannot be eliminated by simple presaturation of the extraction solvent but by washing the extract with small amounts of water [30]. Another relevant issue to be considered in trace residue analysis concerns the purity of the organic solvents, as they can introduce solvent impurities into the sample extract. Therefore, the need for high solvent purification should not be overlooked in some applications.

3.2. Ion-pair partitioning

The efficiency of a liquid-liquid partitioning process can be greatly improved by applying ion-pairing techniques. In these techniques, specific ion-pairing reagents are used in order to enable ionic analytes of opposite charge to be transferred from the aqueous solutions into the organic solvents as neutral well-extractable complexes. Typical ion-pairing reagents contain a non-polar portion, such as a long chain aliphatic hydrocarbon, and a polar portion, such as an acid or a base. The polar portion of the ion-pairing reagent interacts with the charged group on the analyte forming an ion-pair, which is quantitatively extracted into an organic solvent (mainly dichloromethane or chloroform). Ion-pairing techniques are recommended for the extraction of highly polar compounds [34].

The extent of extraction is predominantly controlled by the nature and concentration of the ion-pairing reagent. The polarity of the solvent also influences the ion-pair extraction ability and, in many instances, the hydrogen-accepting or hydrogen-donating properties are considered important. Examples of effective ion-pairing clean-up procedures have been described in the analysis of tetracycline [35] and penicillin [33] residues in milk using tetrabutylammonium, and xylazine [36] and albendazole [37] residues in plasma and animal

tissues, respectively, using octanesulphonate as ion-pairing reagents. The resulting ion pairs proved to be fairly lipophilic and readily extractable with organic solvents (chloroform or dichloromethane).

The commonly used ion-pairing reagents, which are available for both acidic and basic analytes, are listed in Table 1.

Table 1. Commonly used ion-pairing reagents

Positive charged reagents	Negative charged reagents
Tetramethylammonium hydrogen sulphate*	1-Pentanesulphonic acid sodium salt
Tetraethylammonium hydrogen sulphate*	1-Hexanesulphonic acid sodium salt
Tetrapropylammonium hydrogen sulphate*	1-Heptanesulphonic acid sodium salt
Tetrabutylammonium hydrogen sulphate*	1-Octanesulphonic acid sodium salt
Tetrapentylammonium bromide	1-Decanesulphonic acid sodium salt
Tetrahexylammonium hydrogen sulphate*	1-Dodecanesulphonic acid sodium salt
Triethylamine	Dodecylsulphate sodium salt
	Trifluoroacetic acid
	Pentafluoropropionic acid
	Heptafluorobutyric acid

* Also used as bromide or hydroxide

3.3. Other liquid-liquid partitioning approaches

A specific form of liquid-liquid partitioning is the use of the time- and solvent-saving diatomaceous earth columns or cartridges [27,38]. Although they contain solid diatomaceous earth, these columns are not considered to constitute solid-phase extraction systems, because the transfer of the analytes from the aqueous to the organic phase obeys all the laws of the conventional liquid-liquid partitioning. The sorbent in the column is a special grade of flux-calcined, high-purity diatomaceous earth [27]. An aqueous extract is applied to the sorbent and is partitioned over the porous matrix, which acts as a stationary phase. The analytes are eluted with a water-immiscible organic solvent. The high surface area of the packing aids efficient, emulsion-free interactions between the aqueous extract, which is absorbed by the diatomaceous earth, and the organic extraction solvent. This technique is especially applicable to the isolation of lipophilic compounds from liquid aqueous samples or extracts, because the material can hold up to four times its weight of water. In comparison with the conventional liquid-liquid partitioning, the application of prefabricated or manually packed diatomaceous earth columns offers distinct advantages including the elimination of mechanical stirring, the substantial reduction of emulsification during the extraction process and a high recovery increase [27,38,39]. A major disadvantage is that relatively large volumes of the organic solvent are needed.

A second interesting approach closely related to liquid-liquid partitioning is the use of supported liquid membranes for sample clean-up and analyte enrichment, prior to analysis [40-42]. The supported liquid membrane technique involves extraction of the analytes from a stream of aqueous sample (donor) into an organic solvent immobilised by capillary forces in a porous membrane and subsequently back-extraction into a stagnant aqueous phase on the other side of the membrane (acceptor). Supported liquid membrane extraction, which is applicable to analytes of high or moderate polarity, offers a high degree of selectivity and enrichment, uses small amounts of organic solvents and provides convenient possibilities for direct and automated interfacing with chromatography systems and other analytical instruments [43].

Clean-up methods based on partitioning are simple, rapid, flexible and easy to perform with standard laboratory equipment and often still form a part of official methods. These methods are readily applicable to all types of matrices and a wide range of analytes ranging from fairly polar to nonpolar compounds [44]. Despite the fact that they are simple and rapid, clean-up methods based on partitioning may result in highly selective isolation. However, these methods lack specificity, use large volumes of toxic and inflammable organic solvents (sometimes chlorinated), favours formation of emulsions, may cause sample losses by occlusion or adsorption onto glass surfaces, is often laborious and costly and difficult to fully automate for unattended operations [45]. During the volume reduction step of most extraction procedures, the solvents are frequently disposed of into the atmosphere, which causes pollution and contributes to unwanted atmospheric effects, such as smog and ozone holes. To address this issue the Montreal Protocol treaty, signed over a decade ago, stipulated reduction of solvent use [28,45]. The analytical community responded to this challenge by increasing research on chromatography sorbents (such as C_{18}, ion-exchange and size-exclusion phases), immunosorbents and molecularly imprinted polymers as other, less-solvent consuming, alternatives to liquid-liquid partitioning.

4. Methods based on chromatography

Chromatography is one of the oldest separation techniques and is still widely used nowadays. One of its major applications today is for the clean-up of complex matrices before their qualitative and quantitative determination, usually employing an instrumental technique such as liquid or gas chromatography. In most cases these clean-up methods are very simple, both conceptually and in practice, relying on the separation of liquid chromatography, where the solubility and functional group interactions of sample, solvent and sorbent are optimised to affect the retention and elution. Moderately polar to polar analytes are extracted from non-polar solutions onto polar sorbents, whereas non-polar-to-moderately polar analytes are extracted from polar solutions onto non-polar sorbents. Clean-up methods based on chromatography are carried out in an open tube (column) similar to a standard burette fitted

with a stopcock at one end to control the flow rate of the mobile phase percolating through the system, however, the syringe-barrel and/or cartridge types are the most popular formats.

Most analytical procedures include at least one clean-up method using adsorption, partition, ion-exchange or size-exclusion chromatography, or methods based on molecular recognition using immunosorbents or molecularly imprinted polymers.

4.1. Adsorption chromatography clean-up

Adsorption chromatography or liquid-solid chromatography, first discovered by Tswett in 1903, is probably the oldest mode of chromatography [46]. This chromatographic mode is used to separate analytes of a relatively narrow polarity range from interfering compounds of different polarity. Separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid. In addition to removing interferences, adsorption chromatography can be used to fractionate complex mixtures of analytes.

The separation is carried out in a preparative-sized (*e.g.* 30 cm x 2 cm) glass column filled with particles of the chromatographic sorbent; however, the syringe-barrel and/or cartridge types are the most popular formats. Various types of sorbents have been used, including silica (commonly referred to as silica gel), alumina (*e.g.* aluminium oxide), charcoal, Florisil (*e.g.* magnesium silicates), polyamides, celite and diatomaceous earth. The most popular sorbents in adsorption-based clean-up are silica and alumina. These materials adsorb polar substances via Lewis acid-type interactions with the –OH and =O moieties present at their surfaces. The number and topographical arrangement of these moieties determine the activity of the sorbent, thus the greater the number of hydroxyl groups the greater the retention power of the sorbent [46]. In general, in adsorption chromatography the non-polar analytes will not be retained and, as a consequence, elute earlier than polar analytes.

Adsorption chromatography columns are usually made by the user to suit the nature of the extract, the analytes of interest and the amount of lipids present in the extract. The columns can be dry or slurry packed. Prior to use, the materials are normally heated at appropriate temperatures to remove water. After cooling, a specific amount of water, base or acid is added. This amount can be varied to slightly change the elution characteristics of the column; *e.g.* the addition of more water results in earlier elution of polar analytes, whereas when no water is added to the adsorbent all analytes, including the non-polar ones, are retained on the column. The degree of deactivation is critical and difficult to keep under control. Elution profiles need to be established regularly, and certainly from batch to batch since differences may strongly influence the elution pattern.

Many analytical procedures include a clean-up step using adsorption columns packed with silica gel, alumina and Florisil sorbents. Most adsorption columns provide good clean-up only when they are eluted with solvent mixtures of low polarity, eluting less polar analytes and leaving more polar co-extractives in the column. The more the eluting solvent polarity is

increased, the greater will be the portion of interfering compounds eluted and the less effective the clean-up will be [47]. Table 2 lists some separation characteristics of adsorption chromatography.

Alumina and silica gel have gained the greatest attention of all the sorbents used in adsorption-based clean-up of food extracts. Several reports presented the application of alumina [48-61] and silica [62-67] adsorption columns in veterinary drug residues analysis for the clean-up of extracts from food of animal origin, whereas the application of Florisil columns is limited [68-70]. However, Florisil columns have been used by many authors [71-77] for the clean-up of plant extracts. Moreover, adsorption chromatographic separations based on silica, alumina and Florisil sorbents, have long been regarded as important for the analysis of chlorinated dibenzo-p-dioxins [78,79], pyrethrin and pyrethroid [80], and halogenated contaminants and polycyclic aromatic hydrocarbons [81,82] in biological matrices.

Table 2. Separation characteristics of different chromatographic modes

Separation characteristics	Adsorption chromatography	Partition chromatography		Ion-exchange chromatography
		Normal phase	Reversed phase	
Sorbent polarity	High	High	Low	High
Solvent polarity	Low to medium	Low to medium	High to medium	High
Sample loading solvent	Hexane, toluene, CH_2Cl_2	Hexane, toluene, CH_2Cl_2	H_2O, buffers	H_2O, buffers
Eluting solvent	Acetone, CH_3CN, ethyl acetate	Acetone, CH_3CN, ethyl acetate	H_2O mixtures with CH_3CN CH_3OH	Buffers with high ionic strength
Polarity/ionic strength of eluting solvent	Increased polarity	Increased polarity	Decreased polarity	Increased ionic strength
Sample components elution order	Least polar components first	Least polar components first	Most polar components first	Most weakly ionised components first

In some applications, the clean-up process included passing the sample extract through mixed sorbents to combine different adsorption properties. Increased attention is devoted by some authors [83-86] to silica-Florisil columns for the clean-up of food extracts.

The majority, however, of the published articles deals with the application of solid-phase extraction (SPE) cartridges (filled mainly with silica or Florisil sorbents) for the clean-up of food extracts. This is attributed to the great advantages offering the cartridges over the preparative-sized glass columns. An enormous number of applications of solid-phase extraction cartridges in food toxicants analysis can be found in relevant books [87-89].

4.2. Partition chromatography clean-up

Partition or liquid-liquid chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase whose composition is different from that of the moving liquid phase. Chromatographic separation is governed by distribution equilibria of sample molecules between the mobile and liquid stationary phases. Silica is currently the most popular support material. However, a wide variety of porous polymeric resins have also been used [90].

The most widely used sorbents in partition chromatography clean-up methods are those with surface-reacted (chemically bonded) organic stationary phases. By now, bonded-phase silicas are the dominant sorbents used due to their widespread availability. The desired major functional group is introduced into porous silica particles by reaction of silanol groups on the silica surface with a three-reactive functionality organosilane (chloro- or methoxyorganosilane). This type of reaction generates cross-linking between more OH-active groups resulting in reaction of two adjacent silanols and better endcapping of the free silanols [38,91]. Using various radicals R in the organosilane, the attached group to the silica backbone can be alkyl (C_2 to C_{18}), phenyl, cyclohexyl, cyanopropyl, 3-propyloxypropane-1,2-diol, etc. Reagents with shorter alkyl groups are typically used for endcapping. Proper preparation of the silica-based sorbents with complete consumption of the organosilane reagent during derivatisation is important for the quality of the bonded phase [92,93].

Table 3. Polarity of the packing materials used in chromatography-based clean-up procedures

Packing material	Polarity	Separation mode
Octadecyl (endcapped)	Strongly apolar	Reversed-phase
Octadecyl	Strongly apolar	Reversed-phase
Octyl	Moderately apolar	Reversed-phase
Ethyl	Slightly apolar	Reversed-phase
Cyclohexyl	Slightly polar	Normal- or reversed-phase
Phenyl	Slightly polar	Normal- or reversed-phase
Cyanopropyl	Moderately polar	Adsorption, normal- or reversed-phase
Diol	Moderately polar	Normal- or reversed-phase
Alumina	Polar	Adsorption or normal phase
Florisil	Polar	Adsorption or normal phase
Silica gel	Polar	Adsorption or normal phase
Carboxymethyl	Weak cation exchanger	Ion exchange
Aminopropyl	Weak anion exchanger	Ion exchange, normal- or reversed-phase
Propylbenzene sulfonic acid	Strong cation exchanger	Ion exchange
Trimethylaminopropyl	Strong anion exchanger	Ion exchange

Bonded-phase silicas are chemically stable in the presence of most organic solvents. The average pore size of approximately 60 Å makes it possible to separate organic compounds of up to ~ 15,000 molecular weight. Sample solutions much above pH 8 cause some hydrolysis of bonded-phase silica particles [91]. Several supply houses offer a full line of silica-based sorbents for solid-phase clean-up. The major types of them are listed in Table 3 in their approximate order of increasing polarity.

Polymeric materials are also commonly used in partition-based clean-up. Many of them are based on a poly(styrene-co-divinylbenzene) using either *p*-divinylbenzene or *m*-divinylbenzene for the polymerisation. This type of polymer can be made with various functionalities such as acetyl, alkyl, benzoyl, etc. Copolymers with methyl methacrylate, acrylonitrile, vinylpyrrolidone, vinylpyridine, etc. are commercially available [90,93,94]. Unlike bonded-phase silicas, polymeric materials can be used at virtually any pH, and they contain no troublesome silanol groups. The surface area is generally higher than that of silica sorbents, and uptake of organic analytes thus tends to be more complete. In most cases adsorbed analytes are easily and completely eluted from polymeric sorbents by a small volume of an organic solvent.

The type of sorbent to be selected is determined by both the nature of the analyte and that of the solution from which the analyte has to be extracted (Table 2). Two types of bonded-phase sorbents are available for partition-based clean-up, normal-phase and reversed-phase. Normal phase normally involves a polar sorbent used in conjunction with less polar solvents (Table 2). On the contrary, reversed phase involves a relatively nonpolar sorbent used in conjunction with very polar solvents [91]. It is a matter of fact that, in a suitable method, the analyte is easily retained, by the sorbent bed, from the sample solution and is easily eluted as well with a small volume of eluent.

The analyte is isolated from the sample extract on the basis of the principles of liquid chromatography. The retention of the analyte may be based on more than one type of interaction [95-98]. As the sample solution passes over the activated sorbent bed, analytes are retained according to the degree to which each component is partitioned and/or adsorbed by the sorbent. The sorptive properties of bonded-phase silicas, for example, vary with the percentage of carbon in the bonded phase and whether the sorbent is end-capped. The retention on a bonded-phase silica is usually caused by the functional groups bonded to the silica, but may also be due to the interaction between the analyte and the unbound silanol groups remaining on the silica support (secondary interaction). Occasionally this secondary interaction may give rise to such a strong retention of the analyte that complete elution becomes a problem, as was observed in solid-phase clean-up of tetracyclines on a reversed-phase column. The trend is to minimize the number of unbound silanol groups by endcapping them by reaction with a highly-reactive trimethylsilane [96,99,100].

Nowadays, a plethora of polar and nonpolar sorbents, originally designed for normal-phase and reversed-phase liquid chromatography, respectively, is marketed for solid-phase clean-up

applications [38]. Similarly to liquid chromatography, the sorbent is chosen in such a way that the interactions between the stationary phase and the analyte will be stronger than the interactions between the analyte and the mobile phase; for selective isolation of the analytes solvents must have the weakest solvent strength possible in combination with the chosen sorbent. Hydrophobic sorbents including octadecyl, octyl, ethyl, diphenyl, cyclohexyl, phenyl, butyl, ethyl, methyl, and copolymers of styrene and divinylbenzene can be applied for retaining nonpolar to medium polar analytes. In this list, the styrene-divinylbenzene copolymers such as PRP-1 and Amberlite XAD-2 are the most nonpolar sorbents, while the methyl phase is the least nonpolar one [90].

For the isolation of polar to medium polar analytes, polar sorbents (Table 3) including adsorbent packings (silica, alumina, Florisil, etc.) or bonded-phase packings (diol, aminopropyl, cyanopropyl, etc.) are used. Silica, alumina and Florisil are high polarity sorbents that can be used in both the normal-phase and the adsorption chromatography. The cyanopropyl bonded sorbent is of medium polarity and can be applied in the adsorption, the normal and the reversed-phase mode. The aminopropyl phase can be used in both the normal and the reversed-phase mode, and as a weak anion exchanger, but it should not be used in combination with aldehydes and ketones. Because of its mixed retention mechanism, isolations that are not possible with the usual hydrophobic or ion-exchange sorbents can be executed on this material [46,90,92].

Recently, a copolymer of divinylbenzene and N-vinylpyrrolidone (Oasis HLB from Waters) is available in cartridges and a 96-well plate format. The presence of both hydrophilic and lipophilic moieties supposedly make this a balanced sorbent. It is capable of extracting acidic, basic and neutral compounds of varying polarities [95].

The choice of the washing and eluting solvents for a particular sorbent is most critical when solid-phase clean-up procedures are applied. Since these procedures aim in isolation of analytes within a narrow polarity range while keeping all other sample components totally unretained or completely retained, a number of physicochemical and other parameters should always be taken into consideration [90,91,101,102]. The physicochemical properties of the analyte including its molecular weight, solubility, polarity and acidity, the composition and sorption behaviour of the sorbent including its polarity and stability, the nature of the matrix, and the forces of the different interactions between the analyte and the sorbent, the analyte and the matrix, and the sorbent and the matrix are all parameters that can greatly help in optimising a solid-phase clean-up procedure.

The analytes can bind to the solid phase by hydrogen bonding, dipole-dipole interactions, hydrophobic dispersion (van der Waals) forces, and electrostatic interactions. Any or all of these forces can be involved during a solid-phase clean-up, and it is the mastery of these forces that will determine the specificity of the procedure [46,90]. The energies involved in the various bonding forces vary considerably. Hydrophobic bonding energies from dipole-dipole, dipole-induced dipole, and dispersive interactions range from 1-10 kj/mol. Hydrogen

bonding between suitable polar groups has an energy of 5-10 kj/mol, whereas electrostatic or ionic interactions between oppositively charged species involve energies of up to 50 kj/mol.

The pK of a silanol group is not easy to determine because the surrounding environment influences it; however, at a pH of 2, the silanol is uncharged. Above this pH value it becomes increasingly dissociated and able to influence an extraction by virtue of its negative charge. Therefore, if a mixed retention mechanism is present, the influence of residual silanols should be either reduced or enhanced, depending on the extraction mechanism desired. To reduce the influence of silanol groups during an isolation, the residual silanols must be masked by using a competing base such as triethylamine or ammonium acetate. Alternatively, ion suppression can be employed by choosing a pH value at which either the silanol or the ionisable groups on the analyte molecule is uncharged. If silanol activity cannot be reduced using the above suggestions, increasing the ionic strength of the conditioning solvent, sample, and washing solvent may be tried as a means to compete with the silanols and prevent analyte bonding. To enhance the influence of silanol groups during an isolation, a conditioning buffer with a pH value higher than 4 should be used to ensure that the residual silanol groups are ionised. A buffer is recommended as the second conditioning solvent because water can have a variable pH value and has little buffering capacity.

Partition chromatography clean-up with silica-based or polymeric sorbents is generally carried out in pre-prepared solid-phase extraction (SPE) cartridges, or in pre-packed SPE tubes of varying dimensions. The size of the sorbent particles ranges from 10 to 60 μm, but typically averages around 40 or 50 μm in diameter [95,103,104]. Many automated SPE devices and robotics adaptations are also available in the market.

Many books [91,105] and review articles [93,95,106] provide in-depth coverage of normal- and reversed-phase solid phase clean-up and readers are referred to these reference texts for detailed information. Many food matrices have been cleaned up by these clean-up procedures, for the determination of such a large number of analytes that it is not possible to conduct a comprehensive survey of the literature. Usually books [87-89] and review articles [97,104,107-112] deal with the isolation of specific toxicants from foods.

High-performance liquid chromatography (HPLC) can also be successfully used in food toxicants analysis as clean-up procedure (fractionation) for a further chromatographic analysis. The sample is typically separated in specific fractions, and the fraction(s) of interest are submitted for the core chromatographic analysis [113-129]. HPLC as sample preparation technique benefited considerably from the progress made in the field of analytical chromatography. However, a discussion regarding the progress in analytical chromatography is beyond the purpose of this chapter.

4.3. Ion-exchange chromatography clean-up

Ion-exchange mechanisms are employed to separate ionic or easily ionisable compounds on the basis of differences in net charge. The prerequisite for these mechanisms is that the

column sorbent should carry an opposite charge to that on compounds of interest if retention is to take place. In pure ion exchange the interaction results entirely from the coulombic attraction between these opposite charges. There are two types of ion-exchange chromatography, depending on the net charge of the compounds being retained; anion exchange where the retained compounds are negatively charged (anionic compounds), so the ion-exchange sorbent will carry a positive charge, and cation exchange where the relative charges are reversed. In ion-exchange separations, analyte ions and ions of like charge in the sample matrix compete for sites (X) on the column sorbent:

$$A^- + X^+B^- \rightleftharpoons B^- + X^+A^- \qquad \text{(anion exchange)}$$
$$C^+ + X^-B^+ \rightleftharpoons B^+ + X^-C^+ \qquad \text{(cation exchange)}$$

The extent to which these ions compete with B for the charged sites (X) will determine their retention. In general, ion-exchange clean-up may be used to isolate ionic compounds, such as organic acids or bases, which can be ionised under certain pH conditions. Besides the reaction with ionic sites on the sorbent, retention may also be affected by the partitioning of solutes between the sample solvent and the column sorbent, as in reversed-phase chromatography. Thus, ion-exchange sorbents may retain analytes by adsorption as well as by ion exchange (ionic) interactions. A suitable organic solvent elutes analytes that are retained solely by adsorption, while those retained by an ion-exchange mechanism are best desorbed by an acidic or basic eluent that converts the analyte ions back to their molecular form [46,91].

Commonly used sorbents in ion-exchange clean-up procedures include (Table 3) silica particles with attached propylbenzene sulphonic acid groups (strong cation exchanger, SCX) or with trimethylaminopropyl groups (strong anion exchanger, SAX). Weak cation exchangers with carboxymethyl groups and weak anion exchangers with aminopropyl groups are also available. Polymeric-based ion exchangers with similar ion-exchange groups are also available or can be prepared by a simple organic reaction such as sulphonation [90,94,95]. A severe drawback of the use of ion exchange sorbents is that, because of the limited capacity of the resin-based materials, a precipitation step should be introduced in the procedure when the matrix under investigation contains a relatively high concentration of ionic components [130,131].

In ion-exchange clean-up, anionic compounds can be isolated on SAX silica-based or polymeric sorbents. The primary retention mechanism of the compounds is based on the electrostatic attraction of the charged functional group of the analyte to the charged group on the sorbent surface of the column. The pH of the sample matrix must be set to a value, at which the compound of interest and the functional group on the sorbent are charged. Other matrix components of the same charge may interfere with the adsorption and reduce the selectivity. For the elution of the analyte, the functional group that binds to the sorbent must

be neutralised. The electrostatic force that binds the two together is then disrupted and the compound is eluted. Alternatively, a solution with high ionic strength, or an ionic species that replaces the adsorbed analyte, is used for elution.

Usually SAX sorbent is made of an aliphatic quaternary amine group that is bonded to the silica surface. It is a strong base and exists as a positively-charged cation that exchanges or attracts anionic species. Its pK_a is greater than 14, which makes the bonded functional group charged at all pH levels in an aqueous solution. As a result, SAX columns are used to isolate strong anionic (very low pK_a, <1) or weak anionic (low pK_a, >2) compounds (mostly strong or weak acids). A simultaneous concentration and group separation of acidic and neutral organic compounds from aqueous extracts can be obtained using a macroporous anion-exchange resin of fairly low exchange capacity. The resin column is treated with dilute sodium hydroxide to convert it to the OH^- form. The aqueous extract is also made basic to convert the acidic organic solutes to the anionic form. Neutral analytes are retained by simple adsorption, while acidic organic compounds are retained by an ion-exchange mechanism. After passing an aqueous sample extract through the resin column, neutral solutes are eluted with dichloromethane, whereas the acidic compounds are eluted with 1 M HCl in acetonitrile or methanol [95].

Basic compounds can be isolated on cation exchangers with a high concentration of sulphonic acid groups but tend to retain neutral organics weakly, if at all, from aqueous solutions. However, retention of both basic and neutral compounds can be performed using cation exchangers containing a lower concentration of sulphonic acid groups [132]. Neutral analytes are retained by simple adsorption, while protonated bases are retained by an ion-exchange mechanism. Simultaneous concentration of organic compounds thus becomes possible from aqueous extracts, followed by selective elution of neutral and basic compounds [95].

Ion-exchange columns that are used with non-polar organic solvents should be conditioned with the sample solvent. It is important to note that the packing should not dry up between conditioning and sample addition. After regeneration columns can be used again several times.

Many clean-up procedures in food toxicants analysis employ fractionation of food extracts on the basis of differences in net charge. Open column ion-exchange chromatography, either its anion (strong or weak) or its cation (strong or weak) exchange form, has been successfully applied for the purification of food samples. Examples of these applications deal with the determination of sulphamethazine [133], hygromycin B [134], organophosphate pesticide [135,136] and herbicide [137-142] residues using anion-exchangers, and herbicide residues [141-147] using cation-exchangers.

The majority, however, of the reported applications deals with the use of ion-exchange solid-phase extraction cartridges for the clean-up of food extracts. There is a large body of literature concerning the application of anion- and cation-exchange SPE cartridges in food

toxicants analysis and the readers are referred to relevant books [87-89] for detailed information. Some representative applications include the use of cation-exchange cartridges for the purification of antibacterials [148,149], anthelminthics [150], β-agonists [151-153], urea pesticides [154], carbamate pesticides [155], fungicides [156], and herbicides [157] residues, and anion-exchange cartridges for the purification of antibacterials [158], urea pesticides [159,160], carbamate pesticides [135,161], fungicides [135], and organophosphate pesticides [135] residues in various food matrices.

4.4. Size-exclusion chromatography clean-up

Size exclusion chromatography is a technique that separates compounds from one another on the basis of differences in molecular size. The terms "gel-filtration" and "gel-permeation chromatography" were used earlier to describe this process when the stationary phase is a swollen gel. The packing material is porous and is characterised by the range or uniformity (exclusion range) of that pore size. In the choice of packing material, the exclusion range must be larger than the molecular size of the molecules to be separated.

The retention mechanisms in size-exclusion chromatography involve adsorption, partition and size exclusion [162]. Which of these processes dominates in the final separation depends on factors such as the sample solution composition, the type of gel, and the pore size of the packing material used. Size exclusion and adsorption mechanisms predominate when large pore sizes (1000-2000 molecular weight exclusion) are used with poorly-solvating solvents (*e.g.* cyclohexane, toluene-ethyl acetate). Highly polar solvents (*e.g.* tetrahydrofuran) and smaller pore sizes (M_r 400) will result in size exclusion as the sole process governing separation. The most common sorbents used in size-exclusion chromatography are Sephadex and Bio-Beads, but other types such as Lipidex have been shown to be successful as well. Size exclusion is of minor importance in gels such as the Lipidex gel, where the distribution of substances is mainly based on the polarity of compounds and solvents. This mechanism has been referred to as liquid gel chromatography [163].

The separation takes place in a preparative-sized (*e.g.* 30 cm x 2 cm) glass column filled with beads of a rigid porous material (also called a gel). The pores in this packing material are of the same range as the dimensions of the analytes. This material retains molecules that are small enough to enter the pores. The larger molecules, such as proteins, cannot enter the pores, thus they are not retained and are therefore eluted from the column first, whereas, slightly smaller molecules can enter some pores, and so take longer to elute. In general, size-exclusion clean-up is recommended for the elimination from the sample of lipids, proteins, polymers, steroids, cellular components, and dispersed high-molecular weight compounds. This technique is appropriate for both polar and non-polar analytes, therefore, it can be effectively used to clean-up extracts containing a broad spectrum of analytes.

Since the introduction by Stalling *et al.* [164] of size-exclusion chromatography as a versatile and easy to use clean-up procedure in the area of analysis of organic contaminants,

the technique has not been as popular as other clean-up procedures, such as solid-phase extraction. However, size-exclusion chromatography is the most universal clean-up procedure for semivolatile organic compounds and pesticides. Separation is generally performed by using divinylbenzene-linked polystyrene gels, mostly Bio-Beads SX-3 (200-400 mesh, Bio-Rad, USA), in a range of column sizes and solvents [77,133,165-172].

A lot of articles have been published dealing with the applicability of size-exclusion chromatography in the area of trace analysis of organic contaminants. It was found to be suitable for sample clean-up in the case of trenbolone and 19-nortestosterone [169], pyrethroid [173], organochloride and organophosphate pesticide residues [168,174-176], urea pesticides [133,171,172], carbamate pesticides [177,178], chlorinated dibenzo-p-dioxins [170,179], and polycyclic aromatic hydrocarbons and polychlorobiphenyls [165,180-182] from various food matrices. Separation takes place primarily between lipid material of molecular mass > 500 Da, which is the first to elute from the column (usually Bio-Beads SX-3, 200-400 mesh), followed by the small molecules of the organic contaminants (molecular mass between 200 and 400).

Application to drug residues analysis was studied by Petz and Meetschen [183]. Using Sephadex LH-20, they observed a good separation of a number of drugs from co-extractives from liver, kidney, muscle, egg and milk. However, the time needed for separation and the large eluate volume (which has to be evaporated prior to analysis) greatly restrict the application of size-exclusion chromatography to the cleaning-up step in drug residue analysis. The authors state that a clean-up of similar efficiency could be obtained with solid-phase extraction (SPE) silica cartridges. This column (Sephadex LH-20) was also used for the purification of anticoccidial drugs [49] and polyether antibiotics [48] from co-extractives from chicken and bovine liver, respectively.

Size-exclusion clean-up can be fully automated and, in contrast with other chromatographic techniques (such as adsorption chromatography), it is also more applicable to the isolation of unknown contaminants, for which there is little information on the polarity or chemical functionality of the molecule. Two relatively fast and practically fully automated methods were developed by Mengelers et al. [184,185] for the analysis of sulphonamides, trimethoprim and their metabolites in pig tissues using on-line size-exclusion chromatography clean-up.

The essential features of size-exclusion chromatography clean-up include the high efficiency of the process, the variety of compounds of different chemical types that can be isolated in the same fraction and the life time of the column. In general, it could be used for several months without any effect on the clean-up capacity [77,78,186]. However, the large solvent consumption and the relatively large volume of the fraction containing the organic contaminants, causing a high degree of dilution of the sample, are important drawbacks of this technique. Evaporation of the solvent is possible but it is time-consuming and may cause losses of volatile compounds when heating and/or vacuum are used [162]. Moreover, the

removal of interfering substances is not complete in many instances, and therefore these samples need to be analysed twice in order to eliminate the matrix interferences.

4.5. Clean-up based on molecular recognition

Conventional chromatography sorbents, such as C_{18}, ion-exchange and size-exclusion phases, are routinely used for the clean-up and fractionation of target analytes from complex matrices. Despite their attractive features, these sorbents retain analytes by non-selective hydrophobic interactions that lead to a partial co-extraction of interfering substances. Furthermore, mass spectrometry signal suppression or enhancement effects (i.e., matrix effects) occur when matrix components are co-eluted with an analyte [1-4,187,188]. These effects are especially problematic in the case of ultra-trace assays of target analytes. To enhance extraction selectivity new materials based on molecular recognition were recently developed, namely immunosorbents and molecularly imprinted polymers.

4.5.1. Immuno-based clean-up

Immuno-based clean-up is based on the selective and reversible interaction between the analyte molecule and an antibody raised against it. Generally, the antibody, immobilized on a support to form an immunosorbent, is transferred to a small column. The sample solution is drawn through the column by gravity flow or by means of a peristaltic pump. The use of a pump is a preferred procedure because ensures a constant flow through the column during loading and offers the possibility to process more columns simultaneously.

As a result of the immunochemical interaction, the immobilized antibodies retain the analyte molecules, whilst matrix components fail to interact and pass through the column. A washing step using a few millilitres of water (either pure or containing a small percentage of organic solvent) can be used to eliminate non-specifically retained compounds. Following washing, adsorbed analytes are desorbed from the column by the percolation of a few millilitres of an appropriate eluent, which in most of the reported off-line procedures is an organic solvent (methanol, ethanol or acetonitrile), mixed with water, sometimes at low pH [189,190].

The first requirement for preparing immunosorbents is to develop antibodies with the ability to recognize either one or a group of analytes. Antibodies are proteins produced by mammalian immune systems in response to the presence of a foreign substance (antigen). Although compounds of high molecular mass, such as proteins, glycoproteins and carbohydrates, can readily elicit a potent antibody formation, compounds of low molecular mass (<1000 da) are unable to evoke an immune response. The later compounds, widely known as haptens, must first be modified by binding to a larger carrier molecule, usually a protein such as bovine serum albumin (see also chapter 5) [191,192].

The reactions of chemicals with proteins lead to the formation of bonds of different strengths. The formed chemical bonds are characterised by their energies, which are a direct

reflection of their stabilities. Weak interactions, such as hydrophobic, dipolar (including hydrogen bonds) and ionic bonds, involve energies of up to 50 kJ/mol, whereas strong interactions, such as covalent and co-ordination bonds, involve energies ranging from 200 to 420 kJ/mol. The evidence to date indicates that the interaction between the hapten and protein must result in the formation of a strong bond (covalent bond) so that a non-self antigen is produced [193,194].

Functional groups on protein carriers that are used to form a suitable bond include the N-terminal amino group, the ε-amino group of lysine, the sulfydryl group of cysteine, the imidazole group of histidine, the indole group of tryptophan, the free carboxyl of aspartate or glutamate, and the guanidine group of arginine. Albumin provides approximately 60 free amino groups for linkage while thyroglobulin has about 400 sites available for binding [193,195,196].

Functional groups on haptens that are mostly used for linkage are the amino and carboxyl groups. As a result, most common linkages are peptide bonds between a carboxyl and an amino group. For hapten-protein binding to occur, a hapten must be electron deficient (electrophilic). A hapten may not be initially electrophilic but can be converted to a protein-reactive species by air oxidation or derivatisation in order to create a site for attachment to the carrier protein [193]. The chemistry and orientation used in attaching the hapten to the carrier molecule have a great impact on the specificity of the antibodies that are subsequently elicited. Specificity is lost to the region of the hapten molecule involved in the protein linkage because this is sterically shielded from interaction with the immune system. Therefore, the structure of an analyte should be carefully considered in order to design an appropriate antigen based upon the reactivity spectrum desired in the final assay [197].

As many soluble antigens are rapidly metabolised and/or excreted and, therefore, can be cleared from the circulation by routes that usually bypass the lymph nodes and spleen, production of antibodies is rarely stimulated by these antigens, unless adjuvants are used. Adjuvants are substances that provide a long-lasting antigen reservoir, which releases antigen slowly into the body. In this way, macrophages become activated by the antigen and in turn stimulate production of lymphocytes. The most popular adjuvants are the incomplete and the complete forms of Freund. The incomplete adjuvant is a water-in-oil emulsion by which the antigen is slowly released providing a long period of contact between antigen and immune system. The complete adjuvant contains heat-killed *Mycobacteria,* which cause a local lesion and granuloma formation, thus providing a non-specific stimulus [196,198-200].

The target is to immunise host animals with a specific antigen carrying a number of antigenic determinants and to obtain antibodies that have the specificity and sensitivity required for the intended application. Thus, after immunisation either polyclonal or monoclonal antibodies can be obtained. Serum from immunised animals contains a large number of antibodies of varying isotype, affinity and specificity, called polyclonal because they are products of different cell types. Different clones respond to different antigenic sites

on the hapten to create a mixture of antibodies. Some of these antibodies will have a strong affinity for and bind readily to the selected antigen. Monoclonal antibodies, on the other hand, are derived from a single clone and produce a homogenous population of antibodies. The procedure for obtaining monoclonal antibodies involves removing mammalian splenic antibody-producing cells, usually from a mouse. Each one of these cells is fused with an immortal line of myeloma cells in culture. The resulting hybrid cells are screened in order to select one cell that will produce a desired antibody indefinitely. As the resulting antibodies are homogenous, they are referred to as monoclonal [201,202].

Although both polyclonal and monoclonal antibodies have been effectively used in immuno-based clean-up applications, only the latter can provide reproducible immunosorbents and the high specificity required in some applications. The principal advantage of monoclonal antibodies is that they are highly standard reagents and can be produced in virtually unlimited quantities [203]. Antibody specificity, on the other hand, is both a major advantage and disadvantage for immunochemical methods. It enables highly selective detection of analytes but at the same time may complicate the development of multiresidue methods. Moreover, production of monoclonal antibodies requires special expertise and it is much more expensive compared to polyclonal antibodies [191,201]. Thus, in cases where a range of analytes similar in molecular structure is required to be determined, a polyclonal may be more suitable than a monoclonal antibody.

The choice of the host animal into which the antigen is introduced, is based on the amount of antisera and the type of antibodies required, species response to the introduced antigen, and the genetic makeup of the animal. Polyclonal antibodies can be raised in many different animals; however rabbits, guinea pigs, sheep and goats are most frequently used. Use of sheep and goats enables larger quantities of sera to be rapidly collected with a minimum amount of pooling and are often chosen for producing commercial quantities of polyclonal antibodies. If monoclonal antibodies are required, host animals most commonly employed are mice [198,201,202,204].

The route of antigen administration depends on the nature of the antigen itself, the animal species, the use of an adjuvant, and the immunological response. When producing antisera from rabbits and goats, subcutaneous and intradermal are the most popular routes of administration, whereas mice are injected intraperitoneally. Intramuscular injections can be used in the presence of Freund adjuvants because this route provides rapid access to the lymphatic system. The intravenous route is used with particulate antigens because injection can produce a response, which though rapid, is not sustained [198,202,204].

To elicit an antibody response, host animals are administered the antigen periodically according to an immunisation schedule (see also chapter 5). The first dose primes the animal, as the developing immunological response is short-lived, with the resulting antibodies produced for only a few weeks. During this period, memory cells are produced which, when stimulated by booster injections of antigen, produce antibody more rapidly for a longer period

of time and at higher titres. The antisera should be collected 1 to 2 weeks after the last booster injection. During the course of the immunisation schedule, test bleeds from the animal must be screened against a panel of target and non-target analytes to identify the presence and quantity of the antibodies specific to the target analyte [196,204,205].

Prior to immobilisation of antibodies on solid-supports, isolation of pure antibodies from crude antisera is required. Various degrees of antibody purification can be performed. A classical preliminary step in antibody purification is precipitation with ammonium sulphate. With the use of this reagent, most immunoglobulins are precipitated at 35-40% saturation. Concentrations greater than this level seldom increase the yield of immunoglobulins but, instead, result in further increase of antibody contamination by other proteins. Following immunoglobulin precipitation, ammonium sulphate is eliminated commonly with dialysis [190,206]. Further antibody purification can be performed by applying ion-exchange chromatography based on diethylaminoethylcellulose resin or gel-permeation chromatography [198,206,207]. If higher purification of the antibodies is required, the highly efficient preparative-scale generic immunoaffinity chromatography method can be applied [206].

Following their purification, the antibodies have to be bound to a solid support in such a manner that they do not lose appreciable activity. It is important to choose a support, which in addition to basic properties such as chemical and biochemical inertness, good mechanical stability and uniformity in particle size, should be easily activated to allow antibody attachment, have large pore size and should be hydrophilic in order to avoid any non-specific interactions [191]. The most common approach involves covalent bonding of the antibodies onto supports such as silica, agarose, trisacryl, polyacrylamide, dextran, and cellulose. Prior to coupling, the supports must be activated using reagents such as cyanogen bromide, carbonyldiimidazole, or N-hydroxysuccinimide. Activated supports such as cyanogen bromide-activated Sepharose, and carbonyldiimidazole-activated trisacryl are available in the market. The cyanogen bromide coupling procedure has some disadvantages; the isourea link introduced is quite unstable, and charged isourea groups are formed, which are responsible for undesirable non-specific binding due to ion-exchange effects [191,208-211].

Several studies have attempted to optimise the antibody immobilisation through oriented bonding procedures. The coupling of antibodies to activated supports is considered random immobilisation because it involves covalent coupling via lysine ε-amino groups, which are encountered throughout antibody molecules, allowing thus several different antibody orientations. Generally, such immunoaffinity sorbents exhibit lower analyte capacity than theoretically calculated due mainly to partial inactivation of the antibodies during immobilisation and to shielding of the analyte-binding sites resulting from random immobilisation of the immunoglobulin molecules on the support [191,209,212]. In contrast, some hydrazide activated supports involves covalent bonding via carbohydrate moieties and are thought to provide greater column binding capacity because immobilisation occurs away from the antigen bonding sites and results in a more oriented reaction [210,213]. Another way

to contribute to a better orientation and to increase the binding surface area of immobilised antibodies is to employ antibody fragments, because these fragments will increase the number of integral binding sites without causing steric hindrance [214].

It is also possible to immobilise antibodies by physicochemical interactions. Group-specific adsorbents including immobilised protein-A, which is a cell wall component produced by several strains of *Staphylococcus aureus*, immobilised protein-G, which is a recombinant form of a bacterial cell wall protein isolated from group G *Streptococcus*, and immobilised (strept)avidin immunosorbent, can be used for this purpose. Protein-A and protein-G are able to bind specifically to the crystallisable fragment (Fc) of immunoglobulin G [215], whereas (strept)avidin has an extraordinary high affinity to biotin. The binding of antibodies via the Fc portions to protein-A leaves the analyte-combining site in the correct orientation for the binding of the analyte [191,216].

Recently, another method, the sol-gel method, has been used successfully for entrapping antibodies in the pores of a hydrophilic glass matrix. Activation and bonding are no longer necessary and the coupling is carried out in milder conditions, so that one can expect the antibodies to retain their affinity and specificity. The entrapment is a simple and rapid two-step procedure in which hydrolysis is followed by polymerisation of tetramethoxysilane after addition of antibody solution. Immunosorbents against polycyclic aromatic hydrocarbons [217], nitroaromatic compounds [218], bisphenol A [219] and *s*-triazines [220] have been prepared by this method.

Following its preparation, the immunosorbent can be packed either into a disposable cartridge, for off-line immunoextraction procedure, or into a precolumn for use in on-line immunoextraction systems. The capacity of these immunoaffinity columns corresponds to the total number of accessible specific immobilised antibodies. The capacity determines the maximum amount of the target analyte that can be extracted by a given amount of immunosorbent. However, it cannot be calculated directly because with polyclonal or monoclonal antibodies, random orientation and steric hindrance might prevent the access of the analyte to the specific retention sites of the antibodies. Moreover, the binding efficiency of an immunoaffinity column may vary with the number of the antibody molecules on the sorbent, the flow rate, the concentration of the analyte, and the nature of the sample solution. Generally, the amount of analyte practically retained is always smaller than that theoretically calculated. It may approximate the theoretically calculated amount only when low flow rates combined with low analyte concentrations pass through the column, and the medium of the sample solution is close to the physiological conditions. The former condition favours a strong binding reaction, because the number of analyte molecules passing through the column per time unit greatly influences this retention. As a result, retention in immunoaffinity column can be improved with sample dilution but the time of analysis inevitably also increases [191,201].

Today, the capacity of an immunosorbent is measured experimentally using the target antigen [221,222]. However, the most important feature is that capacities are always in the range of the hundreds of ng to some μg for 1 g of sorbent. Consequently, much care should be given to this value and one has to keep in mind that immunosorbents are only devoted to trace analysis. Moreover, it is very important to not overload the capacity in quantitative analysis because the linear range of the capacity curve corresponds to the linear part of the quantification range.

In order to elute the analytes from an immunoaffinity column, specific conditions within the column have to be applied. Effective elution solutions should ideally disrupt the analyte-antibody interactions, which are generally of weak physical nature, without adversely affecting the immobilised antibodies. As the types of physical bonds, which are involved in immunochemical interactions, vary considerably among the different antibody-analyte systems, each antibody-mediated clean-up procedure has its own optimal conditions for elution [223]. In general, elution can be made by either specific or non-specific desorption. Specific desorption can be achieved, for example, by ligand competition and co-substrate elution. Non-specific desorption can be accomplished by a variety of different procedures, all aiming at the dissociation of the antibody-analyte complex. However, the elution conditions must be chosen in such a way that the reduction of the affinity constant be a reversible process, because otherwise the antibody will be damaged and the repeated use of the immunoaffinity column will be strongly restricted. The most common strategies are reducing the pH to 2 or 3, using water-miscible organic solvents, or chaotropic agents, or a combination of these conditions [208]. The choice first depends on the affinity between antibodies and analytes. It also depends on the nature of the analyte, because of the ratio between electronic and hydrophobic interactions involved in the antigen-antibody interactions. One inconvenience of chaotropic agents and pH variation is that large volumes are required for complete desorption of small molecules. So, in most of the reported off-line procedures desorption was achieved with a high percentage of an organic solvent mixed with water, sometimes at low pH [189,211,224-235]. In this instance, a small volume of eluent is required and provides a high enrichment factor.

Antibodies generated against small molecules may have some affinity for compounds with a structure similar to the analyte that induced the immune response (cross-reactivity). Although cross-reactivity is usually considered as a negative feature for immunoassays, it is exploited in extraction, because immunoaffinity extraction is followed by a separation procedure allowing individual quantification of each trapped analyte. In food analysis, it is often interesting to determine a group of related toxicants and their metabolites. To develop a group-specific immunosorbent one must obtain antibodies that exhibit a wide cross-reactivity. A key parameter in this process is hapten design. Nevertheless, antibodies often do not have the same affinity towards different members of the group. An easy experimental estimation of the affinity order may consist in recovery measurements. The stronger is the affinity of the

antibodies for an analyte, the higher is the extraction recovery for a given sample volume. So, the measure of the extraction recoveries gives information about the specificity of the immobilised antibodies [191,201]. On the other hand, to achieve immunoextraction with high recoveries it is important to ensure that the immunosorbent is not overloaded and to take note of the breakthrough volume of each analyte. It has been demonstrated that increasing the amount of the immunosorbent used increases both capacity and breakthrough volume. Therefore, it is possible to control extraction by altering the amount of immunosorbent used [201].

The conditions that can improve the elution of the analyte are not always compatible with the immunoaffinity column matrix. It has long been established that changing the polarity of the aqueous eluent by adding methanol or ethanol, or using denaturing agents, antibodies may be inactivated to some extent. Also, chaotropic ions have the tendency to reduce hydrophobic interactions and can cause some antibody denaturation. In addition, as the column temperature rises, antibodies have a tendency to slowly denature; the lower the temperature the longer the life of the column and the better the peak resolution [236]. Therefore, the less vigorous elution solvent has to be chosen in practice. It is common experience that immunosorbents are generally very stable as long as the elution conditions are not too extreme.

The extent to which the immunoaffinity column can be re-used depends mainly on the nature and the volume of the analysed samples, and the stability of the antibody and the support. The most important step is to remove any of the material physically adsorbed to the antibody so that the column may be re-used with reproducibility. All that is required for a column to be re-equilibrated is the passage of several volumes of the starting buffer and stored in PBS at 4^{o}C when not in use. An antimicrobial agent, such as sodium azide is often added. Under these conditions immunoaffinity columns can be stored for at least 3 months [191,231]. However, with time and a number of runs, a decrease in capacity is often observed. Many studies reported the reusability of commercial and laboratory-made agarose or sepharose-based supports [231,234,237-239]. Nevertheless, when they are used for large sample volumes, their reusability can be reduced because of their low mechanical resistance.

Multi-immunoaffinity clean-up (MIAC), in which different polyclonal antibodies were combined in one column, has also been described [239-243]. A mixture of two different polyclonal antibodies was coupled to one support for the determination of nortestosterone and methyltestosterone in bovine muscle tissue by GC-MS [240], whereas seven individual immunosorbents were combined in a single column for the analysis of seven anabolics including nortestosterone, methyltestosterone, trenbolone, zeranol, diethylstilbestrol, testosterone and estradiol, in muscle tissue by LC [239]. Methods based on the same analytical strategy have also been developed for the determination of clenbuterol in urine by LC [241], and for the analysis of both 17-β-nortestosterone and 17-α-nortestosterone in urine [242] and muscle tissue [243] of calves.

Table 4. Selected applications of immuno-based clean-up for the analysis of organic contaminants in biological matrices

Analytes	Matrix	Method of analysis	Sensitivity (μg/kg)	Ref.
Aflatoxins B_1 & M_1	Pig liver	Direct Fluorometric	1.0	[230]
Aflatoxin M_1	Milk, milk powder	LC-MS/MS	0.0006-0.0085	[232]
Aflatoxin M_1	Milk	LC-FLD	0.05	[249]
Aflatoxin M_1	Milk, milk powder, yogurt	LC-FLD	0.02-0.002	[250]
Aflatoxin B_1	Animal liver	LC-FLD	0.002	[233]
Ochratoxin A	Muscle	LC-FLD	0.04	[251]
Clenbuterol	Beef muscle & liver	LC-UV	0.3	[234]
β-Agonists	Bovine urine	LC-MS/MS	0.01-0.05	[246]
β-Agonists	Calf & human urine	LC-EC	4	[252]
Dexamethasone, flumethasone	Equine urine	LC-MS	3-4	[253]
Corticosteroids	Bovine milk, liver, urine	GC-MS	0.5	[254]
Okadaic acid, dinophysistoxins	Shellfish	LC-FLD	3000	[255]
Okadaic acid, dinophysistoxins	Shellfish	LC-FLD or LC-MS	1000	[227]
Chloramphenicol	Pig tissues & urine	GC-ECD	0.2-2.0	[256]
Sulphonamides	Pig muscle	LC-UV	1-2	[257]
Tetracycline	Milk	Direct Fluorometric	19	[258]
Zeranol, stilbene estrogens	Bovine urine	GC-MS	0.15-0.84	[259]
Zearalenone, deoxynivalenol	Egg	LC-MS/MS	0.1-0.01	[260]
Diethylstilbestrol, dienestrol, hexestrol	Plasma, urine	GC-MS	0.01	[261]
Nortestosterone, methyltestosterone	Bovine muscle	GC-MS	1	[262]
Halofuginone	Sturgeon muscle	LC-UV	10.0	[211]
Halofuginone	Egg	LC-MS/MS	1.2	[263]
Avermectins	Pig liver	LC-MS	5	[264]
Avermectins	Bovine liver	LC-FLD	2	[265]
Ivermectin	Pig liver	LC-UV	5	[266]

A large number of papers have been published illustrating the various features of immuno-based clean-up using relevant applications. Selected applications that refer to food toxicants analysis are presented in Table 4. Many of these take advantage of the cross-reactivity of antibodies to develop multiresidue analysis targeting a parent compound and its metabolites or a class of structurally related analytes. Most applications deal with the extraction and clean-up of mycotoxins in food matrices, whereas limited number of studies refer to other toxicants.

So far, the most selective phases used for sample clean-up are based on immunoaffinity. These phases strongly contribute to the selectivity of trace analytical methods, especially for identifying pollutants and drug residues [201]. However, to become a truly popular clean-up procedure, more is required for immuno-based clean-up. It is not without reason that immunosorbents are used less widely for many different compounds because a selective antibody must be developed for each analyte. The availability of immunosorbents is the major factor, which will determine its success or not. With rare exceptions, most analytical laboratories are not equipped to produce antibodies. Developing a specific antibody is a long and arduous process that can take as long as 12 months. Furthermore, because of the costs of producing immunoaffinity columns, long-term use of those columns is necessary. Operational conditions that cause denaturation or mobilisation of the antibodies bound to the column should not be used [244-248]. The above mentioned and other drawbacks noted in Table 5, have contributed to the development of an alternative technology using molecularly imprinted polymers.

4.5.2. Molecular imprinting clean-up

The technique of molecular imprinting introduced in 1972 by Wulff and Sarhan [267], and much expanded by the work of the group of Arshady and Mosbach in the 1980s [268], has been shown to be capable of producing materials with “antibody-like” selectivity [269]. Molecularly imprinted polymers (MIPs), artificial antibodies or plastic antibodies, are highly stable synthetic polymeric materials that possess recognition sites within the polymer matrix (specific cavities) that are adapted to the three-dimensional (3-D) shape and functionalities of an analyte of interest [270,271]. The molecular imprinting step is critical for obtaining optimal selectivity. Specific cavities must be designed for the print molecule (the so-called template molecule). For this reason, monomers are chosen to develop strong interactions with the target analyte (or a structural analogue) acting as the template in a porogen solvent. In the presence of a large amount of cross-linking agent, polymerisation takes place around the template molecules. At the end of the polymerisation process, the templates are removed to produce a polymer with binding sites complementary to the template in size and shape [248,272-275].

Table 5. Advantages and disadvantages of immunoaffinity columns for analytical applications

Advantages	Disadvantages
Excellent selectivity	Custom-made products must be developed for new analytes
Simple to use	Chemically unstable (organic solvents, strong acids and bases)
Proven technology for analysis of complex matrices	Reduced thermal and storage stability
Use of aqueous solutions	Non-selective interactions with analytes and matrix components
	Analytical applications should permit development of an antibody with the desired selectivity profile
	Developing antibodies may take as long as 12 months
	Different immunisations will yield different antibodies
	Animals are needed

The challenge of designing and synthesising a MIP can be a daunting prospect to the uninitiated practitioner, not least because of the sheer number of experimental variables involved, *e.g.* the nature and levels of template, functional monomer(s), cross-linker(s), porogen solvent(s) and initiator, the method of initiation and the duration of polymerisation. Attention has to be drawn to a number of factors pertaining to the template molecule and the selection of suitable functional monomers, cross-linkers, porogen solvents, initiators and general polymerisation procedures.

Table 6. Selected functional monomers used for molecular imprinting

Acidic	Basic	Neutral
Methacrylic acid (MAA)	4-Vinylpyridine (4-VP)	Acrylamide
p-Vinylbenzoic acid	2-Vinylpyridine (2-VP)	Methacrylamide
Acrylic acid (AA)	4-(5)-Vinylimidazole	Acrylonitrile (AN)
Itaconic acid	1-Vinylimidazole	Methyl methacrylate (MMA)
2-(Trifluoromethyl)-acrylic acid (TFMAA)	Allylamine	2-Hydroxyethyl methacrylate (2-HEMA)
Acrylamido-(2-methyl)-propane sulfonic acid (AMPSA)	N,N'-diethyl aminoethyl methacrylamide (DEAEM)	Trans-3-(3-pyridyl)-acrylic acid
	N,N'-diethyl-4-styrylamidine	Styrene
	N-Vinylpyrrolidone (NVP)	Ethylstyrene

In all molecular imprinting processes the template is of central importance in that it directs the organisation of the functional groups pendent to the functional monomers (Table 6), which are responsible for the binding interactions in the imprinted binding sites. As regards cross-linkers (Table 7), they fulfil three major functions. First of all they are important in controlling the morphology of the polymer matrix, secondly, they serve to stabilise the imprinted binding site, and finally, they impart mechanical stability to the polymer matrix. The solvent (porogen), on the other hand, serves to bring all the components in the polymerisation, *i.e.* template, functional monomer(s), cross-linker(s) and initiator into one phase. However, it serves a second important function in that it is also responsible for creating the pores in macroporous polymers. Finally, many chemical initiators (Table 7) with different chemical properties can be used as the radical source in polymerisation. Normally they are used at low levels compared to the monomer. The rate and mode of decomposition of an initiator to radicals can be triggered and controlled in a number of ways, including heat, light and by chemical/electrochemical means, depending upon its chemical nature. However, there may well be drivers for selecting one over another arising from the system under study [275].

MIPs can be produced in a covalent or non-covalent manner. In covalent imprinting, the template molecule is chemically coupled with one of the building blocks of the polymer, and, after the polymerisation, the resulting bond must be cleaved to obtain free selective binding sites. The most common approach, however, is non-covalent imprinting, which relies upon self-assembly between the template and carefully selected functional monomers. After polymerisation in the presence of a cross-linking monomer, the resulting highly reticulated polymer will contain a binding site equipped with functional groups in a defined 3-D arrangement [247]. It is good to emphasise that both the morphology and the number of functional groups of the template and analyte play important roles in selectivity recognition [276,277]. An increasing number of interacting functional groups in a molecule will increase the selectivity, whereas the morphology is important for site accessibility and the mass transfer properties of the sorbent. With the present systems, the upper limit in the molecular weight of the template-analyte is approximately 1000. On one hand these molecules must be able to diffuse into and out of the polymer, on the other hand, an increased number of interactions will slow dissociation from the binding sites. MIPs can recognise one or a group of analytes depending upon the template choice [247].

Removing the template molecule from the polymer is necessary not only to make the binding sites available for binding of the analyte but also to limit possible interference of the template with the analytes quantification. Some small amount of template molecule always remains behind in the polymer despite the best attempts to extract it. Residual template presence in the MIP and subsequent leaching in practical usage is considered to be a drawback of the present state of MIP technology [278].

Table 7. Selected cross-linkers and initiators used for molecular imprinting

Cross-linkers	Initiators
p-Divinylbenzene (DVB)	Azobisisobutyronitrile (AIBN)
Ethylene glycol dimethacrylate (EGDMA)	Azobisdimethylvaleronitrile (ABDV)
1,3-Diisopropenyl benzene (DIP)	Dimethylacetal of benzil
Tetramethylene dimethacrylate (TDMA)	Benzoylperoxide (BPO)
N,N'-Methylene bisacrylamide (MDAA)	4,4'-Azo(4-cyanovaleric acid)
N,N'-1,3-Phenylenebis(2-methyl-2-propenamide) (PDBMP)	
2,6-Bisacryloylamidopyridine	
1,4-Diacryloyl piperazine (DAP)	
1,4-Phenylene diacrylamide	
N,O-Bisacryloyl-L-phenylalaninol	

MIPs are very stable and can withstand extremes of heat and pH and treatment with organic solvents with only small losses in selectivity [279,280]. Chemical stability studies [281] demonstrated that the polymers kept >95% of their affinity for the imprinted molecule of theophylline even after 24 h of exposure to autoclaving treatment, triethylamine, 10 M HCl and 25% NH_3. Heat treatment revealed that the MIPs retain their chemical affinity since they can withstand temperatures of up to 150 ^{o}C. At higher temperatures de-carboxylation is induced causing loss of MIP specificity. It is interesting to note the enhanced storage stability since polymers synthesized 8 years prior to this study were found to have largely retained their affinity for the template.

From an analytical separation point of view, a MIP may be best characterised as being a material, which in addition to the imprinted affinity sites contains both polar and lipophilic surface functionality. Thus, retention in MIPs systems is due to a mixed-mode mechanism involving both selective affinity binding with imprints and non-specific physicochemical adsorption on polymer surface. Accordingly, the optimisation of MIP-based separation requires a fundamental understanding of the strength and nature of imprint-analyte and polymer surface-analyte interactions, respectively, and how these vary with the type of solvent or buffer employed [274]. Consequently, using MIPs the required selectivity can be obtained in two ways; non-selective and selective adsorption. If the analyte can be trapped on the MIP by ionic or hydrophobic interactions, users can load an aqueous sample directly and retain it by using non-selective interactions. Following, the washing step with organic solvent will remove non-selectively bound matrix components, while the retaining of the analyte of interest on the MIP will change from non-selective to selective binding. Washing solvents can be dichloromethane or acetonitrile, the latter solvent with or without small amounts of basic

or acidic modifiers. In an alternative approach, users can transfer the analyte into an organic solvent first, thus providing selective binding when loaded onto the MIP [247].

The analyte of interest can be eluted from the MIP with a solvent or mixtures of solvents that break or disrupt the strong and multiple interactions of the analyte with the polymer. This solvent can be methanol that contains a larger amount of a modifier, such as acetic acid or triethylamine, or mixtures of water with either methanol or acetonitrile. The elution volume can be less than 1 ml, but volumes as much as 5 ml, depending upon the elution and binding strengths have been used [282,283].

The excellent clean-up obtained with MIPs can be explained by the fact that little discrimination will occur between individual components of the same polarity when using conventional sorbents, whereas matrix components with the same polarity will be washed away with the MIP phase because they lack the same spatial arrangement of functional groups present in the analyte that causes selective recognition [247]. In principle, MIPs can be made with selectivity for essentially any of a diverse range of analyte species, such as drugs, pesticides, hormones, toxins, short peptides and nucleic acids. These polymers can then be employed for separation of the analyte in matrices ranging from pure organic solvents to biological samples.

For practical use MIPs can be packed in disposable cartridges or in columns for off-line or on-line solid-phase extraction. Experimental parameters such as particle-size distribution, packing uniformity and flow characteristics affect recovery and reproducibility in the same manner as they do with conventional solid-phase extraction materials [284].

The operational use of solid-phase extraction consumables containing MIPs is very similar to the use of other solid-phase extraction sorbents for preconditioning, sample loading, washing and elution. Using the MIP with the same range of solvents used in solid-phase extraction, the polymer will behave like an ordinary reversed-phase or ion-exchange material. It has been largely demonstrated that MIPs offer the highest selectivity when samples are dissolved in the organic solvent that was used in the polymerisation process [191,247,277].

The past decade was characterised by the increasing transition of molecular imprinting techniques from a predominantly fundamental experimental and theoretical research area to practically useful analytical applications. In recent years, the potential of MIPs for these applications has been thoroughly investigated culminating in the commercial availability of MIP-based SPE (MISPE) cartridges by at least four companies, namely Aspira Biosystems, Inc. (San Francisco, California, USA) [285], Ellipsa AG (Berlin, Germany) [286], and MIP Technologies AB (Lund, Sweden) [287].

Trace and ultra-trace analysis of complex biological samples often rely on selective sample clean-up prior to a chromatographic separation. Affinity separation materials, such as MIPs, potentially offer a high degree of sample clean-up efficiency and recent years have seen increasing research activity into the use of SPE that incorporates MIP technology for extracting drugs and pollutants from different matrices. Nowadays, it is well known that

MISPE procedures constitute a clear alternative to classic methodologies for the extraction and clean-up of organic contaminants in complex biological matrices, and successful applications (Table 8) have been described in the literature. Biofluids, such as plasma, serum and especially urine have been the most analysed biomatrices [283,288-296] and only a few studies have been performed concerning the extraction of compounds from food of animal origin [297-301].

Table 8. Selected applications of molecularly imprinted polymers for the clean-up of organic contaminants in biological matrices

Analytes	Template	Matrices	Monomers	Porogenic solvent	Ref.
Naproxen	S-Naproxen	Urine	4-Vinylpyridine	Toluene	[288]
Clenbuterol	Brombuterol	Calf urine	Methacrylic acid	Acetonitrile	[291]
Clenbuterol	Brombuterol	Calf urine	NR	NR	[292]
β-Agonists	NR	Calf urine	NR	NR	[293]
Clenbuterol	Clenbuterol	Calf urine & liver, milk	Methacrylic acid	Acetonitrile	[294]
Triazines	Simazine	Urine	Methacrylic acid	Dichloromethane	[283]
Scopolamine	Hyoscyamine	Urine, serum	Methacrylic acid	Toluene	[296]
Triazines	Atrazine	Bovine liver	Methacrylic acid	Acetonitrile	[297]
Clenbuterol	Bromoclenbuterol	Bovine liver	Methacrylic acid	Acetonitrile	[298]
Tetracyclines	Oxytetracycline	Pig kidney	Methacrylic acid	Dimethylsulfoxide	[299]
Sulfamethazine	Sulfamethazine	Milk	Methacrylic acid	Acetonitrile	[300]
Sulfamethazine	Sulfamethazine	Milk	Methacrylic acid	Acetonitrile	[301]

NR : Not reported (in these studies the MIP4SPE® columns from MIP Technologies were used)

MIPs can provide a strong contribution to the selectivity and sensitivity of analytical methods and compared with immunosorbents and conventional solid-phase extraction sorbents provide many potential advantages (Table 9). Theoretically, they can solve a given selectivity problem, reduce method development time, reduce analysis time, and, possibly, allow the use of more conventional detectors. From a practical point of view, developing a MIP sorbent is much faster than developing biological antibodies (i.e., weeks versus one year). In a controlled environment, MIPs can be produced in a reproducible fashion. The batch-to-batch variation of parameters such as avidity, binding-site density and selectivity are small and can be considered negligible [247]. Still another advantage of MIPs is their high capacity, which averages 30-fold that of immunosorbents [191].

Table 9. Advantages and disadvantages of molecularly imprinted polymers for analytical applications

Advantages	Disadvantages
Cost-effective alternative to immunosorbents	Custom-made products must be developed for new analytes
Ease of preparation	Reliable methods for imprinting large molecules are still not available
Enhanced thermal stability (up to 150°C)	Difficult to remove template from polymer
Enhanced chemical stability (organic solvents, strong acids and bases)	Binding site heterogeneity providing a distribution of binding site affinities
Enhanced storage stability (for years)	Analytical applications should permit development of a MIP with the desired selectivity profile
High capacity (30-fold that of immunosorbents)	Lower catalytic capabilities than immunosorbents
Production period of weeks	Possible leaching during use
No animals are needed	Grinding and sieving of bulk polymer for MISPE applications is labor-intensive and inefficient in material yield

Current research demonstrates the progression of MIP chemistry and the breadth of its appeal contribution to solving a wide variety of analytical problems. However, it is also apparent that continuous progress in MIP technology relies on better analytical and rational understanding of MIP synthesis and technology enabling rational design, development and optimisation along with generic synthesis strategies for effective MIPs adaptable to any given analyte. This prerequisites detailed understanding on the nature of interactions between the essential building blocks of the MIP matrix and the template analyte for advanced tailoring of the polymer properties. Hence, the next critical step or advancement in MIP research targets fundamental understanding and subsequent rational tenability of analyte-polymer interactions aiming at the development of methods for modelling and prediction of MIP selectivity and polymer behaviour at the molecular level [302].

5. Co-distillation/forced volatilisation methods

Distillation is a widely used method for separating the components of liquid mixtures based on the distribution of mixture constituents between liquid and vapour phase. The main purpose of distillation is the separation of volatile components in a mixture from either the semi-volatile components or each other. The variation in the saturation vapour pressure of a material versus its temperature is the basis of a distillation separation. Overall, if two

components of a liquid mixture have different vapour pressures, analysts can enrich the more volatile component in the vapour phase. Two phases (the liquid and the vapour phase) can be recovered separately with the more volatile compounds enriched in the vapour phase and the less volatile compounds concentrated in the liquid phase.

Analysts have many distillation techniques for use in the analytical laboratory. Among these techniques, steam distillation and sweep co-distillation can be applied successfully for sample clean-up before chromatographic analysis.

Steam distillation is an alternative approach for the purification of heat-sensitive samples. It is also useful for liquids with such poor heat-transfer characteristics that excessive local overheating would cause direct heating. Steam distillation is performed as a batch process in which steam flows continuously through the sample mixture in the still pot. The steam carries the more volatile materials in the vapour phase and the volatile materials' concentrations are related to their vapour pressures in the steam mixture. Steam distillation is rather a gentle technique in that the distilled materials are never heated to temperatures greater than that of the steam. At the end of the process, the steam and the separated materials are condensed. Normally, they are immiscible and the two phases can then be separated [303].

Steam distillation clean-up has been used for many years as a technique for the isolation of petroleum hydrocarbons from fish muscle tissue [304,305] and mussels [306,307], and organochlorine compounds from milk products [308].

Sweep co-distillation, however, is the distillation technique of choice in the majority of applications. Sweep co-distillation is based upon the low vapour tension of analysed compounds in a stream of inert gas (e.g., nitrogen). Evaporated extracts of a sample in organic solvent is distilled in a glass tube filled with silanized glass wool or glass beads. The filling material traps less volatile components [77,309]. After freezing, the distillate is evaporated again and analysed by gas chromatography. It is an efficient purification technique for organochlorine [310-313] and organophosphate pesticide residues [314,315]. Moreover, the sweep co-distillation clean-up technique was efficiently used for the determination of environmental contaminants [316], phthalate esters [317], and drugs (neutral poisons) [318] from food and biological matrices, but its use is now being replaced by the more universal size-exclusion chromatography. Sweep co-distillation gained great popularity in Australia for the analysis of meats and dairy products [310,314,319,320]. Results of this clean-up procedure are equivalent to those of size-exclusion chromatography and adsorption chromatography on Florisil column clean-up [320].

6. Precipitation clean-up methods

The most frequently used precipitation methods for the clean-up of food sample extracts involve protein precipitation and low-temperature fat precipitation.

For trace analysis in samples with high protein content, precipitation of the proteins becomes necessary in order to prevent the formation of emulsions during extraction with organic solvents. This procedure is most critical when there is a hint of binding of the analyte to proteins. Widely used techniques to destroy analyte-protein binding are based on either protein denaturation or enzymatic/chemical hydrolysis of the analyte-protein complexes [63,119,321,322].

Protein precipitation in a food sample can be affected by a variety of means including addition of various protein-precipitating reagents, sample boiling, or use of microwave irradiation. Precipitation of sample proteins can be performed with a great number of reagents including hydrochloric acid [234,323,324], perchloric acid [325,326], tungstic acid [50], trichloroacetic acid [327-329], acetonitrile [37,330-332], ethanol [51], acetone [333-335], methanol [336,337] ammonium sulphate [338], or ammonium chloride [339].

With the use of the inorganic sulphates or chlorides, protein precipitation does occur but the process is reversible. This does not occur when acids or organic solvents are used for protein precipitation as the biological activity of the proteins in this case is irreversibly destroyed. On the other hand, use of mineral or organic acids, although valuable for deproteinisation purposes may be, has raised problems with regard to precision and accuracy of the analysis due to occasional adsorption and/or occlusion of the analytes in the precipitate. This effect can be minimised by not adding the deproteinising agent to the homogenised sample, but by slowly adding the latter to a surplus of deproteinising agent. Unlike acids, use of water-miscible organic solvents, has consistently offered, with proper pH adjustment, almost quantitative recoveries for a large number of food contaminants in a simple and rapid way.

Methanol and acetonitrile are the most frequently used water-miscible organic solvents for protein precipitation. The main advantage of using methanol for protein precipitation is that a clear supernatant is obtained and a flocculent precipitate is formed. Acetonitrile, on the other hand, gives a hazy supernatant with a fine precipitate. The compatibility of these solvents with the reversed-phase liquid chromatographic eluents commonly used for separation purposes is an added advantage.

In the case of acetonitrile the denaturating procedure requires mixing of one volume of sample homogenate with 1-4 volumes of the solvent, the ratio being depended on the composition of the sample itself. Residues are uniformly distributed in the melange formed by the addition of the organic solvent and, therefore, lengthy multiple extractions of the precipitate are not required. However, this procedure may lead to co-extraction of water-insoluble compounds and considerable dilution of the sample. One way to overcome this problem is to add dichloromethane that removes acetonitrile and water-insoluble interferences, leading to an increased concentration of the analyte in the aqueous supernatant.

The use of heat for precipitation of proteins [340-343] should be avoided on account of possible deterioration of the analyte while microwave irradiation is a particularly attractive

technique because it can denature both quaternary and tertiary structures of proteins within 1 s [344].

The various methods used to precipitate proteins, although fast and simple, remove proteins with limited efficiency, and still results in fast column deterioration. Precipitation with organic solvents makes it difficult to match the solvent strength with mobile phase, and the use of strong acids is incompatible with pH labile analytes. Subsequent removal of precipitated proteins by high-speed centrifugation or filtration is difficult to automate. In addition, precipitation gives relatively high background of impurities in the chromatogram, which presents a serious problem in trace analysis [345].

In the case of lipid-containing sample extracts, further treatment by applying low temperature proved to be useful for the separation of pesticides and other lipophilic chemical contaminants (*e.g.* polychlorinated biphenyls, chlorinated dibenzodioxins and dibenzofurans) of toxicological concern through fat precipitation.

In a very early application [346], low-temperature fat precipitation was used in combination with column chromatography to clean-up chlorinated hydrocarbon pesticides from plant extracts. The procedure involves the removal of waxes by precipitation from an acetone solution of the extract at -70^{o}C. This methodology was further evaluated for clean-up of several polar and apolar pesticides from sample lipid, waxes and water [347]. Samples were extracted by refluxing with acetone-benzene (19:1) and extracts were cleaned-up by freezing out the lipids, waxes and water of solution, leaving the pesticides in the supernatant acetone-benzene extraction solvent. The precipitated material is merely filtered out, leaving the solution sufficiently clean for gas chromatographic analysis.

However, a study performed with meat and fatty matrices has shown that gravimetric fat removal through ice cooling the extract in ethyl acetate-methanol (3:7, v/v) gives sufficient clean-up for organophosphate pesticide determination when combined with solid-phase extraction [348]. Organophosphate insecticides along with triazine herbicides were recently evaluated using a low-temperature clean-up method developed for their determination in olive oil [349]. Acetonitrile extracts were stored in a freezer at -20^{o}C and were allowed to stand overnight for lipid precipitation and separation.

Although apparently simple, this technique has not been widely used. The loss of analytes by occlusion in the precipitated matrix and by adsorption in the filtration process, proved to be deterrents to its widespread practicability and general acceptability [350].

7. Chemical clean-up methods

Derivatisation, saponification and oxidative dehydration by sulphuric acid are the most frequently used chemical methods in the cleaning-up of food sample extracts [28,45,110,186,351].

To enhance selectivity and capacity of the extraction phase for analytes such as polar or ionic compounds, which are difficult to extract, a derivatisation step is frequently introduced [31,45]. The objective of derivatisation is not only to convert the native analytes into less polar derivatives that are extracted more efficiently but also to label them for better detection or chromatography. The most interesting implementation of this approach is simultaneous extraction/derivatisation. In this technique, the derivatisation reagent is present in the extraction phase during the extraction. The main advantage of this approach is that two steps are combined. The major limitation, which describes this combination, refers to the usually slow reaction rate when compared with transport of analytes to the extraction phase.

A very large number of derivatisation reactions have been reported but in reality only a few have been used in routine analyses. Even though many textbooks and monographs have reported compilations of derivatisation techniques as part of sample preparation [352,353], this is an approach that most analytical chemists will avoid for a number of reasons. The problem is that derivatisation adds an additional step to the sample preparation procedure. As well as the extra costs involved, care must be taken to ensure that the reaction is working by introducing derivatisable standards. The additional manual or reagent addition stages introduce additional uncertainty into quantification [45].

In the case of lipid-containing sample extracts, further treatment by chemical reaction (such as alkaline treatment and oxidative dehydration by sulphuric acid) can result in better separation of the target analytes from interfering lipid co-extractives. Alkaline treatment resembles the saponification used in extraction, but is applied to the solvent extract instead of the food matrix. Lipids can be saponified by heating the sample extract in a small volume of solvent with 20% ethanolic potassium hydroxide at ca. 70^{o}C for 30 min. However, because the method is exhaustive, some loss in recoveries may be obtained, as was reported for polychlorinated biphenyls [354-356] and chlorinated dibenzo-p-dioxins [179].

Lipids can also be destroyed by applying concentrated sulphuric acid. The latter is mixed with the lipid extract (which is usually dissolved in pentane or hexane) and vigorously stirred for a few minutes; this clean-up procedure is repeated 4-6 times. Following separation in a separatory funnel the organic layer is evaporated to dryness under a gentle stream of nitrogen (20^{o}C). Finally, the cleaned extract is reconstituted in an organic solvent for analysis. Sulphuric acid clean-up is widely used for the determination of polychlorinated biphenyls [357,358] and organochlorine pesticides [359-361] in various food matrices.

8. Final remarks

In many cases, the clean-up procedure constitutes the weakest link in the whole analytical methodology chain. Owing to the wide range of matrices encountered in food of animal and plant origin and, the wide range of different food toxicants, a typical universal clean-up scheme cannot be established. Instead, the extent of the clean-up procedure strongly depends

on the concentration of the analyte, the composition of the matrix, and the detection system chosen.

If analysis is to be attempted with a detection system of only moderate selectivity, a substantial clean-up procedure may be required in order to enhance the concentration of the extracted trace residue while decreasing the concentration of possible interfering substances in the sample extract. This is the case with most of the relatively nonspecific physicochemical detection systems used in food analysis. Occasionally a sample may be suitable for direct physicochemical analysis after an extraction and concentration step. However, the majority of food samples need extensive clean-up to isolate the compounds of interest from lipids and other naturally occurring organic substances prior to detection. For such detection systems, there has been a general rule dictating that the cleaner the sample the better the result obtained.

When a more selective detection system is used instead, a rigorous sample clean-up may not be necessary since the interfering compounds are simply not detected. In this context, single and multiple ion monitoring in GC-MS are quite helpful and LC-MS/MS methods can greatly increase specificity. Effectively these methods filter the ions produced by the MS process to separate the characteristic ions for the analyte of interest and to ignore those from the matrix or from other components, hence reducing the background signal. However, some sample preparation may still be needed, otherwise interferences and signal suppression for LC-MS can occur, even when there is no obvious sample signal.

In this chapter the author attempted to provide an overview of methods that can be used in the cleaning-up of samples for food analysis. Food analysis is a complex area that has led to the development of a plethora of approaches due to the complexity of the sample matrices. Emphasis has been placed here on methods based on molecular recognition using immunosorbents or molecularly imprinted polymers since they are rapid, selective and minimise the generation of chemical waste (*e.g.* organic solvents). The author hope that the 361 references that have been cited may aid the analyst in further selecting the clean-up method that is most appropriate for solving the problem being investigated.

References

[1] J. Hajslova and J. Zrostlikova, J. Chromatogr. A 1000 (2003) 181.
[2] F. Klink, LC-GC Eur. 13 (2000) 396.
[3] M. Fiori, C. Civitareale, S. Mirante, E. Magaro and G. Brambilla, Anal. Chim. Acta 529 (2005) 207.
[4] B.K. Matuszewski, M.L. Constanzer and C.M. Chanez-Eng, Anal. Chem. 75 (2003) 3019.
[5] R. King, R. Bonifiglio, C. Fernandez-Metzler, C. Miller-Stein and T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[6] R. Dams, M.A. Huestis, W.E. Lambert and C.M. Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.
[7] C. Chin, Z.P. Zhang and H.T. Karnes, J. Pharm. Biomed. Anal. 35 (2004) 1149.
[8] F. Pommier and R. Frigola, J. Chromatogr. B 784 (2003) 301.

[9] S. Zhou, Q. Song, Y. Tang and W. Naidong, Current Pharmaceutical Analysis 1 (2005) 3.
[10] M. Becker, E. Zittlau and M. Petz, Anal. Chim. Acta 520 (2004) 19.
[11] B.K. Matuszewski, M.L. Constanzer and C.M. Chanez-Eng, Anal. Chem. 70 (1998) 882.
[12] J. Smeraglia, S.F. Baldrey and D. Watson, Chromatographia 55 (2002) S95.
[13] J. Qu, Y. Wang and G. Luo, J. Chromatogr. A 919 (2001) 437.
[14] B.K. Choi, D.M. Hercules and A.I. Gusev, J. Chromatogr. A 907 (2001) 337.
[15] P. Zollner, A. Leitner, D. Berner, M. Kleinova, J. Jodlbauer, B.X. Mayer and W. Lindner, LC-GC Eur. 16 (2003) 163.
[16] P. Zollner, A. Leitner, J. Jodlbauer, B.X. Mayer and W. Lindner, LC-GC Eur. 16 (2003) 354.
[17] C. Sanchez-Brunete, B. Albero, G. Martin and J.L. Tadeo, Anal. Sci. 21 (2005) 1291.
[18] M. Anastassiades, K. Mastovska and S.J. Lehotay, J. Chromatogr. A 1015 (2003) 163.
[19] J. Hajslova, K. Holadova, V. Kocourek, J. Poustka, M. Godula, P. Cuhra and M. Kempny, J. Chromatogr. A 800 (1998) 283.
[20] D.R. Erney, T.M. Pawlowski and C.F. Poole, J. High Resolut. Chromatogr. 20 (1997) 375.
[21] K. Mastovska, S.J. Lehotay and M. Anastassiades, Anal. Chem. 77 (2005) 8129.
[22] P.L. Wylie and K. Uchiyama, J. AOAC Int. 79 (1996) 571.
[23] M. Godula, J. Hajslova, K. Mastovska and J. Krivankova, J. Sep. Sci. 24 (2001) 355.
[24] F.J. Egea Gonzales, M.E. Hernande Torres, E. Almansa Lopez, L. Cuadros-Rodriguez and J.L. Martinez-Vidal, J. Chromatogr. A 966 (2002) 155.
[25] L. Cuadros-Rodriguez, A.M. Garcia-Campana, E. Almansa Lopez, F.J. Egea Gonzales, M.L.C. Cano, A.G. Frenich and J.L. Martinez Vidal, Anal. Chim. Acta 478 (2002) 281.
[26] F.J. Schenck and S.J. Lehotay, J. Chromatogr. A 868 (2000) 51.
[27] R.E. Majors, LC-GC Eur. 10 (1997) 93.
[28] J. Pawliszyn, Anal. Chem. 75 (2003) 2543.
[29] A. Berthod and S. Carda-Broch, J. Chromatogr. A 1037 (2004) 3.
[30] M. Uihlein, Practice of High Performance Liquid Chromatography, Ed. H. Engelhardt, Springer-Verlag, Berlin, Germany, 1986.
[31] J. Rosenfeld, Sampling and Sample Preparation for Field and Laboratory, Ed. J. Pawliszyn, Elsevier, Amsterdam, 2002.
[32] N.A. Botsoglou, J. Agric. Food Chem. 36 (1988) 1224.
[33] D.J. Fletouris, I.E. Psomas and A.I. Mantis, J. Agric. Food Chem. 40 (1992) 617.
[34] M.C. Carson, J. Chromatogr. A 885 (2000) 343.
[35] D.J. Fletouris, I.E. Psomas and N.A. Botsoglou, J. Agric. Food Chem. 38 (1990) 1913.
[36] I.E. Psomas and D.J. Fletouris, J. Liq. Chromatogr. 15 (1992) 1543.
[37] D.J. Fletouris, E.P. Papapanagiotou, D.S. Nakos and I.E. Psomas , J. Agric. Food Chem. 53 (2005) 893.
[38] S.C. Moldoveanu, J. Chromatogr. Sci. 42 (2004) 1.
[39] S.J. Dickson, W.T. Cleary, A.W. Missen, E.A. Queree and S.M. Shaw, J. Anal. Toxicol. 4 (1980) 74.
[40] S. Palmarsdottir, B. Lindegard, P. Deininger, L.E. Edholm, L. Mathiasson and J.A. Jonsson, J. Cap. Electrophoresis 2 (1995) 185.
[41] S. Palmarsdottir, L. Mathiasson, J.A. Jonsson and L.E. Edholm, J. Chromatogr. B 688 (1997) 127.
[42] S. Pedersen-Bjergaard, K.E. Rasmussen and T.G. Halvorsen, J. Chromatogr. A 902 (2000) 91.
[43] J.A. Jonsson and L. Mathiasson, LC-GC Eur. 16 (2003) 683.
[44] R.D. McDowall, J.C. Pearce and G.S. Murkitt, J. Pharm. Biomed. Anal. 4 (1986) 3.

[45] R.M. Smith, J. Chromatogr. A 1000 (2003) 3.
[46] S. Ahuja, Ed., Selectivity and Detectability Optimizations in HPLC, Wiley Interscience, New York, 1989.
[47] A. Ambrus and H.P. Their, Pure Appl. Chem. 58 (1986) 1035.
[48] E.E. Mertinez and W. Shimoda, J. Assoc. Off. Anal. Chem. 69 (1986) 637.
[49] K.D. Gallicano, H. Park, J. Yee, L.M. Young and P.W. Saschenbrecker, J. Assoc. Off. Anal. Chem. 71 (1988) 48.
[50] H. Terada, M. Asanoma and Y. Sakabe, J. Chromatogr. 318 (1985) 299.
[51] A.I. MacIntosh and G.A. Neville, J. Assoc. Off. Anal. Chem. 67 (1984) 958.
[52] J. Unruh, E. Piotrowski, D.P. Schwartz and R.A. Barford, J. Chromatogr. 519 (1990) 179.
[53] J. Unruh, D.P. Schwartz and R.A. Barford, J. AOAC Int. 76 (1993) 335.
[54] A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, J. Assoc. Off. Anal. Chem. 74 (1991) 292.
[55] D.P. Schwartz and A.R. Lightfield, J. AOAC Int. 78 (1995) 967.
[56] Y.S. Endoh, Y. Takahashi and M. Nishikawa, J. Liq. Chromatogr. 15 (1992) 2091.
[57] T. Nagata and M. Saeki, J. Assoc. Off. Anal. Chem. 70 (1987) 706.
[58] E.E. Mertinez and W. Shimoda, J. Assoc. Off. Anal. Chem. 68 (1985) 1149.
[59] Y. Takahashi, T. Sekiya, M. Nishikawa and Y.S. Endoh, J. Liq. Chromatogr. 17 (1994) 4489.
[60] M.B. Medina and D.P. Schwartz, J. Chromatogr. B 581 (1992) 119.
[61] J.E. Roybal, R.K. Munns, J.A. Hurlbut and W. Shimoda, J. Assoc. Off. Anal. Chem. 73 (1990) 940.
[62] K. Takatsuki, S. Suzuki, N. Sato, I. Ushizawa and T. Shoji, J. Assoc. Off. Anal. Chem. 70 (1987) 708.
[63] M.G. Lauridsen, C. Lund and M. Jacobsen, J. Assoc. Off. Anal. Chem. 71 (1988) 921.
[64] A.E. Mooser and H. Koch, J. AOAC Int. 76 (1993) 976.
[65] G.P. Dimenna, B.E. Walker, L.B. Turnbull and G.J. Wright, J. Agric. Food Chem. 34 (1986) 472.
[66] K. Takatsuki, S. Suzuki and I. Ushizawa, J. Assoc. Off. Anal. Chem. 69 (1986) 443.
[67] G. Formica and C. Giannone, J. Assoc. Off. Anal. Chem. 69 (1986) 763.
[68] J.R. Markus and J. Sherma, J. AOAC Int. 76 (1993) 451.
[69] J.R. Markus and J. Sherma, J. AOAC Int. 76 (1993) 459.
[70] T. Nagata, M. Saeki, H. Nakazawa, M. Fujita and E. Takabatake, J. Assoc. Off. Anal. Chem. 68 (1985) 27.
[71] F.J. Schenck and R. Wagner, Food Addit. Contam. 12 (1995) 535.
[72] J.H. Petersen and K.G. Jensen, Z. Lebensm. Unters. Forsch. 182 (1986) 489.
[73] A.J. Cessna and D.L. Benoit, Pestic. Sci. 35 (1992) 355.
[74] A.J. Cessna, J. Agric. Food Chem. 40 (1992) 1154.
[75] H. Wan, J. Chromatogr. 516 (1990) 446.
[76] Y. Tsumura, I. Wada, Y. Fujiwara, Y. Nakamura, Y. Tonogai and Y. Ito, J. Agric. Food Chem. 42 (1994) 2922.
[77] J. Tekel and S. Hatrik, J. Chromatogr. A 754 (1996) 397.
[78] J.-F. Focant, C. Pirard and E. de Pauw, Talanta 63 (2004) 1101.
[79] R. Loos, S. Vollmuth and R. Niessner, Fresenius J. Anal. Chem. 357 (1997) 1081.
[80] Z.-M. Chen and Y.-H. Wang, J. Chromatogr. A 754 (1996) 367.
[81] J. de Boer and R.J. Law, J. Chromatogr. A 1000 (2003) 223.
[82] S. Moret and L.S. Conte, J. Chromatogr. A 882 (2000) 245.

[83] J. Okada and S. Kondo, J. Assoc. Off. Anal. Chem. 70 (1987) 818.
[84] M.M.L. Aerts, W.M.J. Beek, H.J. Keukens and U.A.Th. Brinkman, J. Chromatogr. 456 (1988) 105.
[85] G.M. Binnendijk, M.M.L. Aerts, H.J. Keukens and U.A.Th. Brinkman, J. Chromatogr. 541 (1991) 401.
[86] H.J. Keukens, C.A. Kann, M.J.H. Tomassen, Proc. EuroResidue III Conference, Eds. N. Haagsma and A. Ruiter, Veldhoven, University of Utrecht, The Netherlands 1996, p 611.
[87] N.A. Botsoglou and D.J. Fletouris, Eds., Drug Residues in Foods. Pharmacology, Food Safety, and Analysis, Marcel Dekker, New York, 2001.
[88] L.M.L. Nollet, Ed., Handbook of Food Analysis, 2nd Edition, Vol. 2: Residues and Other Food Component Analysis, Marcel Dekker, New York, 2004.
[89] H. Oka, H. Nakazawa, K.-I. Harada and J.D. MacNeil, Eds., Chemical Analysis for Antibiotics Used in Agriculture, AOAC INTERNATIONAL, Arlington, VA, USA, 2001.
[90] S.C. Moldoveanu and V. David, Eds., Sample Preparation in Chromatography, Elsevier, Amsterdam, The Netherlands, 2002.
[91] J.S. Fritz, Ed., Analytical Solid-Phase Extraction, Wiley-VCH, New York, 1999.
[92] R. Chaney, M.J. Telepchak and T.F. August, Eds., Forensic and Clinical Applications of Solid Phase Extraction, Humana Press, Totowa, NJ, 2003.
[93] M.-C. Hennion, J. Chromatogr. A 856 (1999) 3.
[94] T. Vaananen, P. Kuronen and E. Pehu, J. Chromatogr. A 869 (2000) 301.
[95] J.S. Fritz and M. Macka, J. Chromatogr. A 902 (2000) 137.
[96] N. Haagsma, Proc. EuroResidue II Conference, Eds. N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg, Veldhoven, University of Utrecht, The Netherlands 1993, p 40.
[97] Y. Pico, G. Font, J.C. Molto and J. Manes, J. Chromatogr. A 885 (2000) 251.
[98] A. Vailaya and C. Horvath, J. Chromatogr. A 829 (1998) 1.
[99] M.C. Hennion, C. Cau-Dit-Coumes and V. Pichon, J. Chromatogr. A 823 (1998) 147.
[100] B. Buszewski, R. Gadzala-Kociuchn, R. Kaliszan, M. Markuszewski, M.T. Matyska and J.J. Pesek, Chromatographia 48 (1998) 615.
[101] V. Pichon, J. Chromatogr. A 885 (2000) 195.
[102] L.G. Rushing, S.F. Webb and H.C. Thompson Jr., J. Chromatogr. B 674 (1995) 125.
[103] R.E. Majors, LC-GC Int. 6 (1993) 336.
[104] P.L. Buldini, L. Ricci and J.L. Sharma, J. Chromatogr. A 975 (2002) 47.
[105] E.M. Thurman and M.S. Mills, Eds., Solid-Phase Extraction: Principles and Practice, Wiley Interscience, New York, 1998.
[106] M.E. Leon-Gonzales and L.V. Perez-Arribas, J. Chromatogr. A 902 (2000) 3.
[107] R.M. Marce and F. Borrull, J. Chromatogr. A 885 (2000) 273.
[108] V. Ruiz-Gutierrez and M.C. Perez-Camino, J. Chromatogr. A 885 (2000) 321.
[109] J.P. Franke and R.A. de Zeeuw, J. Chromatogr. B 713 (1998) 51.
[110] I. Mukherjee and M. Gopal, J. Chromatogr. A 754 (1996) 33.
[111] F.J. dos Ramos, J. Chromatogr. A 880 (2000) 69.
[112] A.A.M. Stolker and U.A.Th. Brinkman, J. Chromatogr. A 1067 (2005) 15.
[113] W.A. Moats, J. AOAC Int. 76 (1993) 535.
[114] W.A. Moats, J. AOAC Int. 77 (1994) 41.
[115] J.A. Tarbin, W.H.H. Farrington and G. Shearer, Anal. Chim. Acta 318 (1995) 95.
[116] G. Carignan and K. Carrier, J. Assoc. Off. Anal. Chem. 74 (1991) 479.
[117] C. Hummert, B. Luckas and H. Siebenlist, J. Chromatogr. 668 (1995) 53.

[118] G. Knupp, G. Bugl-Kreickmann and C. Commichau, Z. Lebensm. Unters Forsch. 184 (1987) 390.
[119] L.A. van Ginkel, P.L.W.J. Schwillens, M. Jaquemijns and G. Zomer, Proc. EuroResidue Conference, Eds. N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg, Noordwijkerhout, University of Utrecht, The Netherlands 1990, p 189.
[120] W.A. Moats, Proc. EuroResidue Conference, Eds. N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg, Noordwijkerhout, University of Utrecht, The Netherlands 1990, p 280.
[121] W.A. Moats and R.D. Romanowski, J. Agric. Food Chem. 46 (1998) 1410.
[122] W.A. Moats, J. Chromatogr. 507 (1990) 177.
[123] W.A. Moats and R. Malisch, J. AOAC Int. 75 (1992) 257.
[124] W.A. Moats and R. Harik-Kham, J. AOAC Int. 78 (1995) 49.
[125] R. Harik-Kham and W.A. Moats, J. AOAC Int. 78 (1995) 978.
[126] W.A. Moats and R.D. Romanowski, J. Chromatogr. 812 (1998) 237.
[127] A.D. Cooper, G.W.F. Stubbings, M. Kelly, J.A. Tarbin, W.H.H. Farrington and G. Shearer, J. Chromatogr. A 812 (1998) 321.
[128] W.A. Moats, J. Agric. Food Chem. 39 (1991) 1812.
[129] L.A. van Ginkel, H.J. van Rossum, P.W. Zoontjes, H. van Blitterswijk, G. Ellen, E. van de Heeft and A.P.J.M. de Jong and G. Zomer, Anal. Chim. Acta 237 (1990) 61.
[130] M.W.F. Nielen, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr. 317 (1984) 557.
[131] M. Agudo, A. Rios and M. Valcarcel, Anal. Chim. Acta 281 (1993) 103.
[132] J.S. Fritz, P.J. Dumont and L.W. Schmidt, J.Chromatogr. A 691 (1995) 133.
[133] A. Balinova, J. Chromatogr. A 823 (1998) 11.
[134] M.B. Medina and J.J. Unruh, J. Chromatogr. B 663 (1995) 127.
[135] Y. Yamazaki and T. Ninomiya, J. AOAC Int. 82 (1999) 1474.
[136] D. Bennett, A.C. Chung and S.M. Lee, J. AOAC Int. 80 (1997) 1065.
[137] J.E. Cowell, J.L. Kunstman, P.J. Nord, J.R. Steinmetz and G.R. Wilson, J. Agric. Food Chem. 34 (1986) 955.
[138] A.J. Cessna and N.P. Cain, Can. J. Plant Sci. 72 (1992) 1359.
[139] N.P. Sen and P.A. Badoo, Int. J. Environ. Anal. Chem. 63 (1996) 107.
[140] J.N. Seiber, M.M. McChesney, R. Kon and R.A. Leavitt, J. Agric. Food Chem. 32 (1984) 681.
[141] Y.Y. Wigfield and M. Lanouette, Pestic. Sci. 33 (1991) 491.
[142] Y.Y. Wigfield and M. Lanouette, J. Assoc. Off. Anal. Chem. 74 (1991) 842.
[143] P.L. Alfernes and Y. Iwata, J. Agric. Food Chem. 42 (1994) 2751.
[144] R.A. Guinivan, N.P. Thompson and W.B. Wheeler, J. Assoc. Off. Anal. Chem. 65 (1982) 35.
[145] D.N. Roy, S.K. Konar, S. Banerjee and D.A. Charles, Can. J. For. Res. 19 (1989) 842.
[146] H.A. Moye, C.J. Miles and S.J. Scherer, J. Agric. Food Chem. 31 (1983) 69.
[147] T. Nagayama, T. Maki, K. Kan, M. Iida and T. Nishima, J. Assoc. Off. Anal. Chem. 70 (1987) 1008.
[148] M. Horie, K. Saito, R. Ishii, T. Yoshida, Y. Haramaki and H. Nakazawa, J. Chromatogr. A 812 (1998) 295.
[149] S. Horii, J. Liq. Chromatogr. 17 (1994) 213.
[150] P.S. Chu, R.Y. Wang, T.A. Brandt and C.A. Weerasinghe, J. Chromatogr. 620 (1993) 129.
[151] G. van Vyncht, S. Preece, P. Gaspar, G. Maghuin-Rogister and E. de Pauw, J. Chromatogr. A 750 (1996) 43.
[152] L.X. Whaites and E.J. Murby, J. Chromatogr. B 728 (1999) 67.
[153] P.A. Guy, M.C. Savoy and R.H. Stadler, J. Chromatogr. B 736 (1999) 209.

[154] A.J. Krynitsky and D.M. Swineford, J. AOAC Int. 78 (1995) 1091.
[155] M. Okihashi, H. Obana and S. Hori, Analyst 123 (1998) 711.
[156] A. di-Muccio, S. Girolimetti, D.A. Barbini, P. Pelosi, T. Generalli, L. Vergori, G. de Merulis, A. Leonelli and P. Stefanelli, J. Chromatogr. A 833 (1999) 61.
[157] J.R. Pardue, J. AOAC Int. 78 (1995) 856.
[158] M.G. Beconi-Barker, R.D. Roof, L. Millerioux, F.M. Kausche, T.J. Vidmar, E.B. Smith, J.K. Callaham, V.L. Hubbard, G.A. Smith and T.J. Gilbertson, J. Chromatogr. 729 (1996) 229.
[159] M. Gamon, A. Saez, R. Pelegri, I. Peris, J.G. de la Cuadra and R. Coscolla, J. AOAC Int. 81 (1998) 1037.
[160] M. Martinez-Galera, T. Lopez-Lopez, M.D. Gil-Garcia, J.L. Martinez-Vidal and P. Parrilla-Vazquez, J. Chromatogr. A 918 (2001) 79.
[161] Y. Tsumura, K. Ujita, Y. Nakamura, Y. Tonogai and Y. Ito, J. Food Protect. 58 (1995) 217.
[162] P. Fernandez, C. Porte, D. Barcelo, J.M. Bayona and J. Albaiges, J. Chromatogr. 456 (1988) 155.
[163] J. Ellingboe, E. Nystrom and J. Sjovall, J. Lipid Res. 11 (1970) 266.
[164] D.L. Stalling, R.C. Tindle and J.L. Johnson, J. Assoc. Off. Anal. Chem. 55 (1972) 32.
[165] F.E. Ahmed, Trends Anal. Chem. 22 (2003) 170.
[166] D. Puig and D. Barcelo, J. Chromatogr. A 673 (1994) 55.
[167] A.H. Roos, A.J. van Munsteren, F.M. Nab and L.G.M.Th. Tuinstra, Anal. Chim. Acta 196 (1987) 95.
[168] J.A. van Rhijn and L.G.M.Th. Tuinstra, J. Chromatogr. 552 (1991) 517.
[169] G.W. Stubbings and M.J. Shepherd, J. Liq. Chromatogr. 16 (1993) 241.
[170] P.T. Holland, Pure Appl. Chem. 75 (2003) 1843.
[171] T. Tomsej and J. Hajslova, J. Chromatogr. A 704 (1995) 513.
[172] K.A. Barnes, J.R. Startin, S.A. Thorpe, S.L. Reynolds and R.J. Fussell, J. Chromatogr. A 712 (1995) 85.
[173] A. di Muccio, P. Pelosi, D.A. Barbini, T. Generali, A. Ausili and F. Vergori, J. Chromatogr. A 765 (1997) 51.
[174] M.J. Shepherd, International Analyse 2 (1988) 14.
[175] U. Uygun, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 771.
[176] V. Kocourek, J. Hajslova, K. Holadova and J. Poustka, J. Chromatogr. A 800 (1998) 297.
[177] S. Rossi, A.P. Dalpero, S. Ghini, R. Colombo, A.G. Sabatini and S. Girotti, J. Chromatogr. A 905 (2001) 223.
[178] W. Specht, S. Pelz and W. Gilsbach, Fresenius J. Anal. Chem. 353 (1995) 183.
[179] R.J. Norstrom, M. Simon and M.J. Mulvihill, Int. J. Environ. Anal. Chem. 23 (1986) 267.
[180] S.A. Wise, B.A. Benner, G.D. Byrd, S.N. Chesler, R.E. Rebbert and M.M. Schantz, Fresenius J. Anal. Chem. 60 (1988) 887.
[181] M.M. Schantz, B.J. Koster, L.M. Oakley, S.B. Schiller and S.A. Wise, Anal. Chem. 67 (1995) 901.
[182] P. Hess, J. de Boer, W.P. Coffino, P.E.G. Leonards and D.E. Wells, J. Chromatogr. A 703 (1995) 417.
[183] M. Petz and U. Meetschen, Z. Lebensm. Unters. Forsch. 184 (1987) 85.
[184] M.J.B. Mengelers, J.F. Staal, M.M.L. Aerts, H.A. Kuiper and A.S.J.P.A.M. van Miert, Proc. EuroResidue Conference, Eds. N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg, Noordwijkerhout, University of Utrecht, The Netherlands 1990, p 276.

[185] M.J.B. Mengelers, A.M.M. Polman, M.M.L. Aerts, H.A. Kuiper and A.S.J.P.A.M. van Miert, J. Liq. Chromatogr. 16 (1993) 257.
[186] A.K.D. Liem, R.A. Baumann, A.P.J.M. de Jong, E.G. van der Velde and P. van Zoonen, J. Chromatogr. 624 (1992) 317.
[187] T. Reemtsma, Trends Anal. Chem. 20 (2001) 533.
[188] J. Haginaka, Trends Anal. Chem. 24 (2005) 407.
[189] N. Delaunay-Bertoncini, V. Pichon and M.-C. Hennion, J. Chromatogr. B 745 (2000) 15.
[190] W.L. Shelver, Generation and Testing of Immunoaffinity Columns, in Methods in Molecular Biology, vol. 295: Immunochemical protocols, Ed. R. Burns, 3rd Edition, Humana Press, Totowa, Nj, 2005, pp 269-280.
[191] M.-C. Hennion and V. Pichon, J. Chromatogr. A 1000 (2003) 29.
[192] M.A.J. Godfrey, Analyst 123 (1998) 2501.
[193] M. Divkovic, C.K. Pease, G.F. Gerberick and D.A. Basketter, Contact Dermatitis 53 (2005) 189.
[194] J.P. Lepoittevin, Ed., Molecular Aspects of Allergic Contact Dermatitis, 3rd Edition, Springer Verlag, Heidelberg, Berlin, 2001.
[195] S.E. Katz and M.S. Brady, J. Assoc. Off. Anal. Chem. 73 (1990) 557.
[196] E. Watanabe, H. Eun, K. Baba, T. Arao, S. Endo, M. Ueji and Y. Ishii, J. Agric. Food Chem. 53 (2005) 7395.
[197] J.W. Goding, Ed., Monoclonal Antibodies, Principles and Practice, 3rd Edition, Academic Press, New York, 1996.
[198] W.L. Shelver, H-J. Kim and Q.X. Li, J. Agric. Food Chem. 53 (2005) 3273.
[199] P.L. Carpenter, Ed., Immunology and Serology, 3rd Edition, W.B. Saunders Co, Philadelphia, 1975.
[200] S. Sell, Ed., Immunology, Immunopathology and Immunity, 3rd Edition, Harper and Row, Hagerstown, MD, 1980.
[201] N. Delaunay-Bertoncini, V. Pichon and M.-C. Hennion, LC-GC Eur. 14 (2001) 162.
[202] M. Vass, L. Kotkova, I. Diblikova, Z. Nevorankova, K.M. Cooper, D.G. Kennedy and M. Franek, Vet. Med.-Czech 50 (2005) 300.
[203] G.C. Howard and D.R. Bethell, Eds., Basic Methods in Antibody Production and Characterisation, CRC press, LLC, USA, 2000, pp. 63-70.
[204] W.L. Shelver, Y-S. Keum, H-J. Kim, D. Rutherford, H. Hakk, A. Bergman and Q.X. Li, J. Agric. Food Chem. 53 (2005) 3840.
[205] J.G. Tew and T.E. Mandel, Immunology 37 (1979) 69.
[206] H.M.T. Griffith and R.A. Abuknesha, J. Chromatogr. B. 827 (2005) 182.
[207] Affinity Chromatography Handbook, Pharmacia Fine Chemical Co., Piscataway, NJ, 1985.
[208] P.D.G. Dean, W.S. Johnson and F.A. Middle, Eds., Affinity Chromatography, a Practical Approach, IRL Press, Oxford, UK, 1985.
[209] J. Yan, M.P.Zhao and Y.Z. Li, J. Sep. Sci. 28 (2005) 1163.
[210] L. Li, J. Yan and M-P. Zhao, J. Chromatogr. A 1103 (2006) 350.
[211] S.Y. Ding, Y.L. Hou, N.P. Wu and J.Z. Shen, J. AOAC int. 88 (2005) 1644.
[212] V.S. Prisyaznoy, M. Fusek and Y.B. Alakhov, J. Chromatogr. 424 (1988) 243.
[213] W. Clarke, J.D. Beekwith, A. Jackson, B. Reynolds, E.M. Karle and D.S. Hage, J. Chromatogr. A 888 (2000) 13.
[214] N.A. Guzman, J. Chromatogr. B. 749 (2000) 197.
[215] G. Rule and J.D. Henion, J. Chromatogr. 582 (1992) 103.

[216] T.M. Philips, N.S. More, W.D. Queen and A.M. Thompson, J. Chromatogr. 327 (1985) 205.
[217] M. Cichna, P. Markl, P. Knopp and R. Niessner, J. Chromatogr. A 919 (2001) 51.
[218] A. Bronshtein, N. Aharonson, A. Turniansky and M. Altstein, Chem. Mater. 12 (2000) 2050.
[219] R. Braunrath and M. Cichna, J. Chromatogr. A 1062 (2005) 189.
[220] C. Stalikas, D. Knopp and R. Niessner, Environ. Sci. Technol. 36 (2002) 3372.
[221] N. Delaunay-Bertoncini, V. Pichon and M.-C. Hennion, Chromatographia 53 (2001) S224.
[222] E. Watanabe, Y. Yoshimura, Y. Yuasa and H. Nakazawa, Anal. Chim. Acta 433 (2001) 199.
[223] C.J. van Oss, R.J. Good and M.K. Chaudhury, J. Chromatogr. 376 (1986) 111.
[224] A.E. Prince, T.S. Fan, B.A. Skoczenski and R.J. Bushway, Anal. Chim. Acta 444 (2001) 37.
[225] A. Kussak, B. Andersson and K. Andersson, J. Chromatogr. A 708 (1995) 55.
[226] A. Kussak, B. Andersson and K. Andersson, J. Chromatogr. B 672 (1995) 253.
[227] N. Delaunay, V. Pichon, J.-P. Le Caer and M.-C. Hennion, Anal. Chim. Acta 407 (2000) 173.
[228] M. Cichna, P. Knopp and R. Niessner, Anal. Chim. Acta 339 (1997) 241.
[229] J. Li and C. Qian, J. AOAC int. 79 (1996) 1062.
[230] E. Chiavaro, C. Cacchioli, E. Berni and E. Spotti, Food Addit. Contam. 22 (2005) 1154.
[231] W.L. Shelver and D.J. Smith, J. AOAC int. 85 (2002) 1302.
[232] C-Y. Chen, W-J. Li and K-Y. Peng, J. Agric. Food Chem. 53 (2005) 8474.
[233] G. Tavcar-Kalcher, K. Vrtac, U. Pestevsek and A. Vengust, Food Control (2006) in press.
[234] J.F. Lawrence and C. Menard, J. Chromatogr. B 696 (1997) 291.
[235] E. Horne, T. Coyle, M. O'Keeffe, M. Alvinerie, P. Galtier and D.L. Brandon, J. Agric. Food Chem. 51 (2003) 5552.
[236] S.E. Katz and M. Siewierski, J. Chromatogr. 624 (1992) 403.
[237] F. Kondo, Y. Ito, H. Oka, S. Yamada, K. Tsuji, M. Imokawa, Y. Niimi, K. Harada, Y. Ueno and Y. Miyazaki, Toxicon. 40 (2002) 893.
[238] H.H. Maurer, C.J. Schmitt, A.A. Weber and T. Kraemer, J. Chromatogr. B 748 (2000) 125.
[239] L.A. van Ginkel, J. Chromatogr. 564 (1991) 363.
[240] L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, H. van Blitterswijk, P.W. Zoontjes, R.C.M. Hooijschuur and J. Zuydendorp, J. Chromatogr. 489 (1989) 95.
[241] W. Haasnoot, M.E. Ploum, R.J.A. Paulussen, R. Schilt and F. Huf, J. Chromatogr. 519 (1990) 323.
[242] A. Farjam, G.L. de Jong, R.W. Frei, U.A.Th. Brinkman, W. Haasnoot, A.R.M. Hamers, R. Schilt and F.A. Huf, J. Chromatogr. 452 (1988) 419.
[243] W. Haasnoot, R. Schilt, A.R.M. Hamers, F.A. Huf, A. Farjam, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr. 489 (1989) 157.
[244] J.G. Rollag, M. Beck-Westermeyer and D.S. Hage, Anal. Chem. 68 (1996) 3631.
[245] S. Ouyang, Y. Xu and Y.H. Chen, Anal. Chem. 70 (1998) 931.
[246] J. Cai and J. Henion, J. Chromatogr. B 691 (1997) 357.
[247] K. Ensing, C. Berggren and R.E. Majors, LC-GC Eur. 15 (2002) 16.
[248] R.J. Ansell, J. Chromatogr. B 804 (2004) 151.
[249] S. Dragacci, F. Grosso and J. Gilbert, J. AOAC int. 84 (2001) 437.
[250] L.C. Lin, F.M. Liu, Y.M. Fu and D.Y.C. Shih, J. Food Drug Anal. 12 (2004) 154.
[251] E.M. Guillamont, C.M. Lino, M.L. Baeta, A.S. Pena, M.I.N. Silveira and J.M. Vinuesa, Anal. Bioanal. Chem. 383 (2005) 570.
[252] A. Koole, J. Bosman, J.P. Franke and R.A. de Zeeuw, J. Chromatogr. B 726 (1999) 149.
[253] C.S. Creaser, S.J. Feely, E. Houghton and M. Seymour, J. Chromatogr. A 794 (1998) 37.

[254] Ph. Delahaut, P. Jacquemin, Y. Colemonts, M. Dubois, J. de Graeve and H. Deluyker, J. Chromatogr. A 696 (1997) 203.
[255] L. Puech, S. Dragacci, E. Gleizes and J.M. Fremy, Food Addit. Contam. 16 (1999) 239.
[256] Th. Gude, A. Preiss and K. Rubach, J. Chromatogr. B 673 (1995) 197.
[257] J.S. Li, X.W. Li, J.X. Yuan and X. Wang, J. AOAC int. 83 (2000) 830.
[258] A.L. Savage, S.H. Sarijo and J. Baird, Anal. Chim. Acta 375 (1998) 1.
[259] L.C. Dickson, J.D. MacNeil, J. Reid and A.C.E. Fesser, J. AOAC int. 86 (2003) 631.
[260] Z. Sypecka, M. Kelly and P. Brereton, J. Agric. Food Chem. 52 (2004) 5463.
[261] R. Bagnati, M.G. Castelli, L. Airoldi, M.P. Oriundi, A. Ubaldi and R. Fanelli, J. Chromatogr. 527 (1990) 267.
[262] L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, H.M. Steinbuch, G. Zomer, E. van de Heeft and A.P.J.M. de Jong, J. Chromatogr. 489 (1989) 111.
[263] L. Mortier, E. Daeseleire, A.C. Huet, P. Delahaut and C. van Peteghem, Rapid Commun. Mass Spectrom. 18 (2004) 1817.
[264] Z.G. Wu, J.S. Li, L. Zhu, H.P. Luo and X.J. Xu, J. Chromatogr. B 755 (2001) 361.
[265] J.H. He, X.L. Hou, H.Y. Jiang and J.Z. Shen, J. AOAC int. 88 (2005) 1099.
[266] J.S. Li, X.W. Li and H.B. Hu, J. Chromatogr. A 696 (1997) 166.
[267] G. Wulff and A. Sarhan, Angew. Chem. 84 (1972) 364.
[268] R. Arshady and K. Mosbach, Makromol. Chem. 182 (1982) 687.
[269] L.I. Andersson, R. Muller, G. Vlatakis and K. Mosbach, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4788.
[270] K. Ensing and T. de Boer, Trends Anal. Chem. 18 (1999) 138.
[271] B. Sellergren, Ed., Molecularly Imprinted Polymers – Man-Made Mimics of Antibodies and Their Application in Analytical Chemistry, Elsevier, Amsterdam, The Netherlands, 2001.
[272] F. Chapuis, V. Pichon and M.-C. Hennion, LC-GC Eur. 17 (2004) 408.
[273] J. Haginaka, Anal. Bioanal. Chem. 379 (2004) 332.
[274] L.I. Andersson and I.A. Nicholls, J. Chromatogr. B 804 (2004) 1.
[275] P.A.G. Cormack and A. Zurutuza Elorza, J. Chromatogr. B 804 (2004) 173.
[276] B. Sellergren, M. Lepisto and K. Mosbach, J. Am. Chem. Soc. 110 (1988) 5853.
[277] K. Yoshizako, K. Hosoya, Y. Iwakoshi, K. Kimata and N. Tanaka, Anal. Chem. 70 (1998) 386.
[278] A. Ellwanger, L. Karlsson, P.K. Owens, C. Berggren, C. Crecenzi, K. Ensing, S. Bayoudh, P. Cormack, D. Sherrington and B. Sellergren, Analyst 126 (2001) 784.
[279] Y. Chen, M. Kele, P. Sajonz, B. Sellergren and G. Guiochon, Anal. Chem. 71 (1999) 928.
[280] D. Kriz and K. Mosbach, Anal. Chim. Acta 300 (1995) 71.
[281] J. Svenson and I.A. Nicholls, Anal. Chim. Acta 435 (2001) 19.
[282] W.M. Mullett and E.P.C. Lai, Anal. Chem. 70 (1998) 3636.
[283] B. Bjarnason, L. Chimulka and O. Ramstrom, Anal. Chem. 71 (1999) 2152.
[284] R.E. Majors, LC-GC Int. (September 1998) S8-S16.
[285] www.aspirabio.com.
[286] www.elipsa.de.
[287] www.miptechnologies.se.
[288] E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington and F. Borrull, J. Chromatogr. B 813 (2004) 137.
[289] P. Martin, G.R. Jones, F. Stringer and I.D. Wilson, Analyst 128 (2003) 345.
[290] B. Dirion, F. Lanza, B. Sellergren, C. Chassaing, R. Venn and C. Berggren, Chromatographia 56 (2002) 237.

[291] C. Berggren, S. Bayoudh, D. Sherrington and D. Ensing, J. Chromatogr. A 889 (2000) 105.
[292] A. Blomgren, C. Berggren, A. Holmberg, F. Larsson, B. Sellergren and K. Ensing, J. Chromatogr. A 975 (2002) 157.
[293] C. Widstrand, F. Larsson, M. Fiori, C. Civitareale, S. Mirante and G. Brambilla, J. Chromatogr. B 804 (2004) 85.
[294] G. Brambilla, M. Fiori, B. Rizzo, V. Crescenzi and G. Masci, J. Chromatogr. B. 759 (2001) 27.
[295] H. Sanbe and J. Haginaka, Analyst 128 (2003) 593.
[296] G. Theodoridis, A. Kantifes, P. Manesiotis, N. Raikos and H. Tsoukali-Papadopoulou, J. Chromatogr. A 987 (2003) 103.
[297] M.T. Muldoon and L.H. Stanker, Anal. Chem. 69 (1997) 803.
[298] C. Crescenzi, S. Bayouth, P.A.G. Cormack, T. Klein and K. Ensing, Anal. Chem. 73 (2001) 2171.
[299] E. Caro, R.M. Marce, P.A.G. Cormack, D.C. Sherrington and F. Borrull, Anal. Chim. Acta 552 (2005) 81.
[300] A. Guzman-Vazquez de Prada, P. Martinez-Ruiz, A.J. Reviejo and J.M. Pingarron, Anal. Chim. Acta 539 (2005) 125.
[301] A. Guzman-Vazquez de Prada, A.J. Reviejo and J.M. Pingarron, J. Pharm. Biomed. Anal. 40 (2005) 281.
[302] J.O. Mahony, K. Nolan, M.R. Smyth and B. Mizaikoff, Anal. Chim. Acta 534 (2005) 31.
[303] R.E. Majors, LC-GC Eur. 12 (1999) 19.
[304] A. Isigigur, H. Heras and R.G. Ackman, Food Chem. 57 (1996) 457.
[305] J.A. Lebo, J.L. Zajicek, T.R. Schwartz, L.M. Smith ans M.P. Beasley, J. Assoc. Off. Anal. Chem. 74 (1991) 538.
[306] T.L. Shchekaturina, A.L. Khesina, O.G. Mironov and L.G. Krivosheeva, Mar. Pollut. Bull. 30 (1995) 38.
[307] K. Granby and N.H. Spliid, Mar. Pollut. Bull. 30 (1995) 74.
[308] G. Filek, M. Bergamini and W. Lindner, J. Chromatogr. A 712 (1995) 355.
[309] R.W. Storherr and R.R. Watts, J. Assoc. Off. Anal. Chem. 48 (1965) 1154.
[310] B.G. Luke, J.C. Richards and E.F. Dawes, J. Assoc. Off. Anal. Chem. 67 (1984) 295.
[311] E. Neidert and P.W. Saschenbrecker, J. Assoc. Off. Anal. Chem. 67 (1984) 773.
[312] M. Lopez-Mesas and M. Crespi, Grasas Y Aceites 51 (2000) 183.
[313] S. Noegrohati and W.E. Hammers, Tox. Envir. Chem. 34 (1992) 219.
[314] B.G. Luke and J.C. Richards, J. Assoc. Off. Anal. Chem. 67 (1984) 902.
[315] R.W. Storherr, E.J. Murray, I. Klein and L.A. Rosenburg, J. Assoc. Off. Anal. Chem. 50 (1967) 605.
[316] J. Mes and D.J. Davies, Int. J. Environ. Anal. Chem. 19 (1985) 203.
[317] B.D. Page and G.M. Lacroix, Food Addit. Contam. 9 (1992) 197.
[318] R.H. Drost and R.A.A. Maes, Forensic Science 3 (1974) 175.
[319] R.L. Brown, C.N. Farmer and R.G. Millar, J. Assoc. Off. Anal. Chem. 70 (1987) 442.
[320] P. Armishaw and R.G. Millar, J. AOAC Int. 76 (1993) 1317.
[321] Z. Deyl, J. Hyanek and M. Horakova, J. Chromatogr. 379 (1986) 177.
[322] M.J. Lynch, F.R. Mosher, R.P. Schneider, H.G. Fouda and J.E. Risk, J. Assoc. Off. Anal. Chem. 74 (1991) 611.
[323] N. Takeda and Y. Akiyama, J. Chromatogr. 607 (1992) 31.
[324] L.A. Lin, J.A. Tomlinson and R.D. Satzger, J. Chromatogr. A 762 (1997) 275.
[325] G.C. Gerhardt, C.D.C. Salisbury and J.D. MacNeil, J. AOAC Int. 77 (1994) 334.

[326] G.C. Gerhardt, C.D.C. Salisbury and J.D. MacNeil, J. AOAC Int. 77 (1994) 765.
[327] V. Hormazabal and M. Yndestad, J. Liq. Chromatogr. 18 (1995) 2695.
[328] V. Hormazabal and M. Yndestad, J. Liq. Chromatogr. 20 (1997) 2259.
[329] F. Sar, P. Leroy, A. Nicolas and P. Archimbault, Anal. Chim. Acta 275 (1993) 285.
[330] K. Takeba, K. Fujinuma, T. Miyazaki and H. Nakazawa, J. Chromatogr. A 812 (1998) 205.
[331] C.C. Hong, C.L. Lin, C.E. Tsai and F. Kondo, Am. J. Vet. Res. 56 (1995) 297.
[332] M. Janecek, M.A. Quilliam, M.R. Bailey and D.H. North, J. Chromatogr. 619 (1993) 63.
[333] V. Hormazabal, I. Steffenak and M. Yndestad, J. Chromatogr. 616 (1993) 161.
[334] A. Sannino, J. AOAC Int. 81 (1998) 1048.
[335] G. Y. Eng, R. J. Maxwell, E. Cohen, E. G. Piotrowski and W. Fiddler, J. Chromatogr. A 799 (1998) 349.
[336] S. Hartmann and H. Steinhart, J. Chromatogr. A 704 (1997) 105.
[337] R.J. Bushway and Z. Fan, Food Agric. Immunol. 10 (1998) 215.
[338] L.C. Nicholls, Ed., Toxicology in the Scientific Investigation of Crime, Butterworth, London, 1956.
[339] H.M. Stevens, J. Forens. Sci. Soc. 7 (1967) 184.
[340] B. Shaikh, E.H. Allen and J.C. Gridley, J. Assoc. Off. Anal. Chem. 68 (1985) 29.
[341] B. Shaikh and J. Jackson, J. AOAC Int. 76 (1993) 543.
[342] V.P. Agarwal, J. Liq. Chromatogr. 12 (1989) 613.
[343] M. J. B. Mengelers, A. M. M. Polman, M. M. L. Aerts, H. A. Kuiper and A. S. J. P. A. M. van Miert, J. Liq. Chromatogr. 16 (1993) 257.
[344] M. Rubenstein, S. Stein, and S. Udenfriend, Eds., Hormonal Proteins and Peptides, Vol. 9, Academic Press, New York, 1985.
[345] M. Gilar, E.S.P. Bouvier and B.J. Compton, J. Chromatogr. A 909 (2001) 111.
[346] C. Anglin and W.P. McKinley, J. Agric. Food Chem. 8 (1960) 186.
[347] H.A. McLeod and P.J. Wales, J. Agric. Food Chem. 20 (1972) 624.
[348] R.K. Juhler, J. Chromatogr. A 786 (1997) 145.
[349] Ch. Lentza-Rizos, E.J. Avramides and F. Cherasco, J. Chromatogr. A 912 (2001) 135.
[350] S.M. Walters, Anal. Chim. Acta 236 (1990) 77.
[351] D.E. Wells, Pure Appl. Chem. 60 (1988) 1437.
[352] G. Lunn and L.C. Hellwig, Eds., Handbook of Derivatisation Reactions for HPLC, Wiley Interscience, New York, 1998.
[353] T. Toyo'oka, Ed., Modern Derivatisation Methods for Separation Science, Wiley Interscience, New York, 2002.
[354] S.B. Singh and G. Kulshrestha, J. Chromatogr. A 774 (1997) 97.
[355] F. van der Valk and Q.T. Dao, Chemosphere, 17 (1988) 1735.
[356] N. Kannan, D. Schultz-Bull and J.C. Duinker, J. Chromatogr. 642 (1993) 425.
[357] L. Berdie and J.O. Grimatt, J. Chromatogr. A 823 (1998) 373.
[358] J.N. Huckins, M.W. Tuberger, J.A.M. Lebo, R.W. Gale and T.R. Schwartz, J. AOAC Int. 73 (1990) 290.
[359] M. Gopal and I. Mukherjee, Pesticide Sci. 37 (1993) 67.
[360] P. Aplada-Sarlis, K.S. Liapis and G.E. Milisdis, Bull. Environ. Contam. Toxicol. 52 (1994) 135.
[361] P. Bottomley and P.G. Baker, Analyst 109 (1984) 85.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 11

Automated clean up techniques

Hans G.J. Mol

RILKILT-Institute of Food Safety, PO Box 230, 6700 AE Wageninge, The Netherlands

1. Introduction

1.1. Scope

The growing concern of consumer, legal authorities and food industry over food safety results in a constant increase in the number of samples to be tested and a demand for shorter turnaround times. Consequently, interest in automated sample pre-treatment is on-going. This chapter is devoted to automation of clean up procedures in analysis methods based on chromatography (capillary electrophoresis will be dealt with in Chapter 16). This means that the main focus is on organic food toxicants. Analysis of trace elements, microbiological assays and analysis based on molecular biological techniques are, although certainly not less relevant with respect to food safety and quality control, considered beyond the scope of the current review. Emphasis is on contributions presented the past five years. Since a clear distinction between extraction and clean up can not always be made, automated extraction procedures are also covered where considered relevant. The principles of the various extraction and clean up techniques are dealt with elsewhere in this book and not presented in detail here. Brief descriptions are, however, given and inevitably there will be some overlap. The literature survey for this chapter was focusing on contributions on food and feed analysis, although some food related matrices are also addressed (e.g. in case of hormones/veterinary drugs). While this review cannot be fully comprehensive, examples have been selected to highlight current practise and new developments. It should be realised, however, that the number of papers on automated clean up in clinical and environmental analysis outnumbers that on food (for reasons given below) and that interesting approaches, that might also be applicable to food analysis, have been described in the literature on those areas. This is especially true for analysis of drinking water, for this the reader is recommended to explore the environmental literature which is not covered in this chapter. Reviews and articles related to automation in sample preparation in general include those by Smith [1] (sample preparation), Buldini et al. [2] (sample preparation techniques in food analysis), Rossi et al. [3] (automating SPE), Venn et al. [4] (96-well SPE), Shepherd [5] (automated clean up trace component analysis), Stolker et al [6] (automated extraction/clean up technologies), Bovanova et al. [7] (automated sample prep in food based on LC), Hennion et al. [8] (immuno-based sample prep, MIP), Goosens et al [9], Hyötyläinen et al. [10] (on-line extraction-chromatography), Ramos et al [11] (miniaturization in sample pre-treatment), O'Reilly et al [12] (automation of SPME), Focant et al. [13] (automated sample prep/fractionation dioxins and related compounds].

1.2. Reasons for clean up in food analysis

Food matrices are often highly complex and raw extracts can contain a wide variety of compounds that may interfere in the analysis. The raw extract that is obtained after the initial extraction of toxicants from a homogenised sample is often unsuited for direct instrumental analysis, especially when this extract has been concentrated in order to bring the mass of analyte in the detectable range of the final measurement system. Clean up of extracts is often needed for two main reasons: 1) compensation for limitations in selectivity of the final determinative separation/detection step by removing compounds that co-elute with the target analytes (in other words, prevention of false positives and false negatives), 2) removing bulk matrix constituents/co-extractants that, besides the previous reason, can have an adverse effect on the quantitative performance of the chromatographic equipment. These instruments can handle only limited amounts of extracted material because otherwise deterioration of quantitative performance can occur, i.e. matrix-dependent response and retention times, loss of response, loss of separation efficiency, peak tailing and, consequently, difficulties in integration. For example, fat containing extracts in GC analysis contaminate the GC inlet and analytical column which results in adsorption (loss of response) and retention time shift and/or peak tailing of analytes, respectively. The presence of co-extracted bulk matrix in GC can result in an enhanced response of especially more polar and thermolabile analytes due to competition of active sites in the chromatographic system [14]. In LC-MS/MS, high levels of co-extracted matrix can affect the ionisation process and results in a matrix dependant analyte response which complicates accurate quantification [14, 15].

With respect to clean up for increasing selectivity of the analytical method it should be realised that selectivity can be introduced at several stages in the analytical method: during sample preparation (initial extraction and subsequent steps thereafter), during the chromatographic separation, during the detection, and finally during data processing. The combined selectivity obtained in all these stages should be such that the method is fit for purpose, i.e. has adequate LOQs to allow testing against legal MRLs and fulfils requirements with respect to accuracy, precision and identity confirmation. Although the main topic in this chapter is automated clean up during the sample preparation stage, developments in this field can not been seen separately from possibilities and developments in the other stages of the analytical method because they are strongly interrelated. In a way, the chromatographic separation, detection and even data processing (e.g. deconvolution) can be seen as a kind of automated clean up since they all contribute to isolating the target analyte from other compounds. The interrelationship between the extend to which clean up is required (in the sample preparation stage) and the chromatographic system used (for analysis of the extract) is presented in Table 1. It has to be noted that in case of two-dimensional chromatography (GC-GC heart-cutting, LC-LC column switching and the currently emerging comprehensive 2D chromatography approaches) the borders between sample preparation and final

chromatographic separation/detection become rather unclear. When still distinguishing between the two main reasons for clean up indicated above, it can be seen that with some separation/detection systems there is a contradictionary effect on the need for clean up. For example, in GC, narrow bore columns improve selectivity which reduces the need for clean up, but on the other hand the system becomes less tolerant for bulk matrix which should be more carefully eliminated. In LC, MS/MS allows very selective detection of the analytes (less clean up required), but is more prone to inaccuracies in quantification due to bulk matrix (more clean up required).

In general, the more complex the matrix (=> more co-extractants), the lower the level of toxicants, and the lower the selectivity of the final separation/detection system, the more clean up will be required. As an example, for the determination of a food toxicant at mg/kg level in a relatively clean matrix, like a pesticide in tomato, using a selective analytical technique such as GC-MS, the initial organic extract typically requires little or no further clean up at all. On the other hand, analysis of the same sample extract with a non selective technique like GC-FID would probably call for an additional clean up during sample preparation, or, alternatively, a pre-separation in the GC (heart cut GC-GC or comprehensive GCxGC) in order to achieve the same result. For determination of ultra low levels of food toxicants in very complex matrices, like pg/kg of dioxins in liver, even the use of very selective instrumental techniques, such as GC with high resolution mass spectrometry, would still require extensive clean up of the extract before analysis.

Over the past decade, the selectivity that is obtained in the instrumental analysis has strongly improved. Mass spectrometric detection is becoming the standard for food toxicant analysis. Especially the strongly increased use of MS/MS as detector for food analysis in LC has substantially reduced the need for extensive clean up procedures during sample preparation. The selectivity is superb and the high-end instruments are so sensitive that adverse effects of bulk matrix on the system's performance (contamination of LC column and ion suppression) can be overcome simply by dilution instead of clean up while still achieving adequate detection limits. This does not mean that clean up in sample preparation has become obsolete.

GC instruments in general remain intolerant to high amounts of involatile matrix injected, and most analyses where LOQs of low µg/kg levels are needed, both in GC and LC, require at least some basic form of clean up (for removal of bulk co-extractants) to ensure robustness and high accuracy. On the other hand, it does mean a reduced need for very dedicated clean up procedures during sample preparation. This further opens the way for determination of multi-class analytes in one single run, which from an efficiency point of view might be very beneficial.

Table 1
Implications of chromatographic system used for final analysis of the extract on clean up requeriments

	Effect on selectivity	Need for clean up	Tolerance to bulk matrix with respect to prolongued quantitative performances	Need for clean up
GC injection				
> On-column	-	-	Low: non-volatile material deposited in analytical	↑↑
> Splitless	-	-	column	↑
> DMI	-	-	Intermediate: non-volatile material is retained in the inlet	↓
			High: no build up/accumulation of non-volatile matrial	
GC separation				
>Wide bore columns	Low resolution	↑	High	↓
> Narrow bore columns	High resolution (better sep. and/or faster	↓	Low: overloading of column, shift in retention times	↑
> GC-GC	analysis)	↓	-	-
> GCxGC	Very high selectivity (small # analytes)	↓	-	-
	Very high selectivity (multi-analyte)			
LC separation				
> Smaller particle	Higher resolution (better sep and/or faster	↓	-	-
diameters (UPLC)	analysis)	-	Faster overloading of analytical column (usually	-
> Smaller ID columns	-	↓	compensated for by reduction in injection volume)	-
(typical in LC-MS)	Very high selectivity (small # analytes)	↓	-	-
> LC-LC	Very high selectivity (multi-analyte)		-	
> LCxLC				

	Effect on selectivity	Need for clean up	Tolerance to bulk matrix with respect to prolongued quantitative performances	Need for clean up
Detector selectivity				
> GC: (hr)MS(/MS) instead of ECD/NPD/FPD	Improves ability to separately detect co-eluting compounds	↓		
> LC: MS/MS instead of UV, Flu	Improves ability to separately detect co-eluting compounds	↓	bulk matrix affects ionisation: suppression or enhancement of response (in case of sufficient sensitivity, e.g. LOQs at mg/kg range, such effects can be minimised by reducing the amount of matrix equivalent introduced in the chromatographic system, see next item)	↑
Detector sensitivity				
> GC: e.g. ECD, SIM (hr)MS(/MS)	-	-	Reduces amount of matrix equivalent that needs to be introduced in chromatographic system	↓
> LC: e.g. Flu, MS/MS				
Data processing				
Deconvolution (full scan DAD and MS data)	Improves spectral quality, allows better (automatic) identification of analytes	↓	-	-

To summarise this section, dedicated clean up during sample preparation continues to be important for ultra-trace analysis, while for other analyses there is, or can be, a shift to more simplified (generic) clean up procedures when laboratories take advantage of the highly sensitive and selective instruments available today.

1.3. Rational of automated clean up

Traditional approaches that still are frequently applied for clean up of sample extracts include liquid-liquid partitioning (LLE), low resolution column chromatography, gel permeation chromatography (GPC) and solid phase extraction (SPE). Often, in one method, more then one clean up step is conducted. A classical method for food toxicant analysis of solid samples, would first extract the food toxicants from a homogenised sample with a water miscible solvent (e.g. acetone) by shaking, blending or otherwise. After separation of the aqueous extract from the solid sample matrix, the analytes are partitioned into an organic solvent (e.g. hexane, dichloromethane) using a separation funnel. The remaining aqueous phase is collected and extracted another time. For basic or acidic analytes, a repeated LLE procedure is often done with appropriate adjustment of pH (e.g. for acidic analytes, first remove basic and neutral co-extractants from the aqueous phase at high pH, then acidify and extract the acidic analytes into the organic phase). The organic extract obtained is concentrated using a rotary evaporator. This extract is then further cleaned by GPC or column chromatography which strongly dilutes the extract and requires, again, an evaporative concentration step. In case of GPC clean up, an additional SPE clean up is often employed, requiring another evaporative concentration step. Then, after reconstitution in a suitable solvent, the cleaned extract is ready for instrumental analysis. In such methods, typically a lot of laboratory glass ware is used that needs to be cleaned before re-use.

It will be clear from the above that the subsequent manual clean up and concentration procedures are tedious and time consuming. It is also clear that there is room for improvement of efficiency and automation should be considered.

1.3.1. Reasons to automate clean up

Automation of clean up steps, or integrating the clean up in the analytical instrument by on-line coupling of the clean up with final chromatographic analysis, can replace many if not all of the manual procedures. This means a reduction in analyst time spend on the sample (=costs). Automated systems can operate more or less continuously and in certain cases can process samples in parallel which can increase sample throughput considerably (= shorter turnaround times). Automation can improve productivity of personnel and analytical instrumentation. In addition, it can improve the quality of the data because a greater precision can be achieved in an automated process where samples will be treated identically. Finally,

since no or less manual handling is required, there is less exposure of personnel to toxic solvent vapours.

1.3.2. Reasons not to automate clean up

Automation involves initial investment in equipment, method development and validation, and training of personnel to use the system. The cost for this can be quite substantial. For accredited routine methods historic data on method performance becomes void when switching to a new concept and need to be newly generated which may require analysis of extra QC samples with each batch of samples. After starting routine use, the equipment needs to be maintained (usually involves a service agreement with the instrument supplier) and dedicated consumables may be needed. Once implemented, the laboratory heavily relies on such instrumentation and robustness. Minimal downtime and quick supplier support are essential. To many analysts automated systems are a black box, a complex bunch of valves and tubing where it is not always obvious to understand how it works exactly. For further development or extension of the system and for trouble shooting, skilled personnel is required which usually involves a higher educational level compared to manual sample pretreatment. In a typical routine environment, such analysts are often rare. Furthermore, automated systems can be less flexible when it comes to dealing with deviating situations, i.e. urgent samples, samples that behave different from the bulk. Especially in food analysis, this is likely to occur since the matrix variability is enormous. When no precautions are made, stability of analytes in liquid samples or extracts may be an issue because vials may stay a long time in an autosampler tray before being analysed.

Automation does not necessarily increase sample throughput. When in the overall method the clean up is not the time limiting step, the gain in throughput by automation of this step is limited. In some cases, manual procedures can be quite efficient. An example is the dispersive clean up step which has been described for pesticide residue analysis [16]. Here the extract is cleaned by addition of loose solid phase material to the extract, rather then performing SPE in the classical way. After centrifugation the cleaned extract is ready for analysis. Compared to that approach, the gain in efficiency by using automated SPE would be limited and, depending on the number of samples, may not justify the investment in a SPE workstation.

1.3.3. To automate or not?

The cost reduction and other benefits need to outweigh the additional cost and possible drawbacks like increased complexity and loss of flexibility mentioned above. The situation will differ for each laboratory and depend on variety and numbers of samples, labor cost, required turnaround times, capabilities of analysts and support from suppliers. The sample number for the method to be automated and the time spend on sample clean up usually will be the most important factors, assuming that labor is the cost determinative factor. In the

literature there is only limited data on time savings and numbers of samples being routinely analysed after implementation. The focus, obviously, is on reporting the development and validation of the automated methods and systems rather than on the retrospective evaluation of cost-benefits. In some papers, e.g. on fully automated (on-line) SPE-LC methods, to demonstrate reliability of the automated method, it was reported that the systems were successfully used for 500-2000 samples over a period ranging from 6 months to 2 years. This might be a rough indication that such numbers of samples were considered sufficient to initiate automation.

Changing to an alternative, more straightforward method (with or even without automation) might be more effective then just automating the existing manual procedures and should be considered. A complicating factor in food analysis is that in many countries official standardised methods exist which are demanded by the laboratories' customers or for legal purposes. This may severely limit the freedom to take advantage of new approaches with respect to automation of sample clean up and improved instrumentation for final separation and detection. Implementation of completely new approaches in such standardised methods often is a (very) slow process. As a consequence, when it comes to automation, one ends up increasing the efficiency of an inefficient method by automation of all the subsequent steps, rather then switching to an entirely new approach that would be less complex and much more effective.

1.3.4. Relative backwardness of automation in food toxicant analysis

Automation in general is easier when dealing with fluid flows and when analyte/matrix combinations that are to be analysed are very similar. Large numbers of similar samples increase attractiveness of automation. For these reasons, automated procedures are widely used in bio/pharmaceutical analysis where very high numbers of plasma or urine sample need to be analysed. Even though there is a range of (candidate) pharmaceuticals to be determined, one basic approach can often be used for the majority of the tests (e.g. SPE using automated 96 well plates). In environmental analysis, water samples are also well suited for (on-line) automated extraction/clean up procedures and many applications can be found in literature as demonstrated in a recent review [11].

In food toxicant analysis, except for beverages and oils, the samples are solid. Apart from requiring a homogenisation step, an initial extraction of the analytes into a fluid medium often (headspace analysis being an exception) needs to be carried out which is less suited for automation. Once a liquid extract is obtained, automation of subsequent clean up steps can be considered. Due to the large variety of food products, and the wide variety of physico-chemical properties of the target analytes there also is a wide variety of analytical methods for food toxicant analysis. Consequently, the number of samples for a certain method is relatively small compared to bioanalysis, and thus, automation less obvious. However, two trends can

be seen that result in increased attractiveness of automation in food analysis: firstly, in recent years there has been a growing concern about food quality and safety. Food quality guidelines require more and more explicit information on presence of an ever wider range of food toxicants in our food which results in a higher number of samples that need to be analysed, usually without extension of personnel capacity. Secondly, it is believed that a further improvement in efficiency in food toxicant control and monitoring is possible through the development of more generic methods which would lead to a decrease in the number of different methods and, consequently, an increase the number of samples per method remaining.

2. Approaches for automated clean up: off-line vs on-line

In this and the subsequent sections, approaches for automation of clean up techniques (in some cases combined extraction/clean up) will be presented. First off-line or semi automated clean up will be discussed. Next, fully automated clean up combined or integrated with chromatographic analysis will be dealt with. In this case, approaches for GC and LC will be described separately. Examples of applications will be briefly described in the technique sections and are summarized in the application overview at the back of this chapter (Table 4) They are categorized by type of residue or contaminant since up to now methods usually deal with one type of food toxicant (e.g. pesticides, veterinary drugs, mycotoxins, PAH, etc.).

The difference between automated off-line and on-line are not always clear, and in the literature sometimes additional terms like at-line and in-line have been used to indicate the degree of automation. Here, off-line means semi-automated. There is no direct connection, through tubing or an autosampler, between the clean up system and the chromatographic system. The cleaned extract needs to be manually transferred to the GC or LC equipment. With on-line systems a sample or raw extract is put into an autosampler tray and further processing and final analysis is done automatic and fully unattended.

Advantages and disadvantages of off-line and on-line clean up have been discussed before [e.g. 9,10]. In Table 2 an overview is given of the main characteristics of automated off-line, on-line and, for sake of completeness, also manual off-line clean up.

Going from manual off-line to automated off-line and to on-line clean up, the degree of automation in the overall analysis increases, the precision improves, and the risk of loosing analytes (during handling of extracts, by exposure to light) or contaminating the extract (handling of extracts, glass ware) reduces. On the other hand, there is less flexibility in the method, i.e. there can be limitations in the use of aggressive chemicals and/or extreme conditions, there are less options for derivatisation, and solvent switching for matching the solvent(strength) with the subsequent chromatography is more complicated. All steps connected need to be compatible with each other and one may end up making compromises.

Table 2. Description and characteristics of manual, automated off-line and on-line clean up

Manual clean up
Sample clean up steps are performed manually and separated from each other and from the chromatographic system

Advantages	*Disadvantages*
- samples can be processed in parallel	- labour intensive
- better optimisation of individual steps	- time consuming
- simplicity of equipment	- risk of analyte losses
- operational flexibility	- risk of contamination of extract
	- exposure of personnel to solvent (vapours)

Automated off-line clean up (semi-automated clean up)
Sample clean up steps are performed automated, in certain cases combined with other sample preparation procedures, but separately from the chromatographic system.

Advantages	*Disadvantages*
- continuous and unattended operation	- intermediate operational flexibility
- samples can be processed in parallel (depending on set-up)	- more complicated equipment
- optimisation of individual steps still possible	- risk of analyte losses
- improved precision	- risk of contamination of extract
- reduced exposure of personnel to solvent (vapours)	- dependence on instrument supplier in case of mall functioning

Automated on-line clean up (fully automated clean up)
Sample clean up (and in certain cases other steps) are connected with the chromatographic system, via tubing and valves or through a dedicated autosampler/robot

Advantages	*Disadvantages*
- continuous and unattended operation	- often sequential process
- suited for miniaturisation, reduced consumption of chemicals	- low operational flexibility, optimisation of individual steps is more complicated or impossible, clean up conditions need to be compatible with final separation/detection technique
- reduced risk of analyte losses	- complicated equipment
- contamination of extract from external sources is minimized	- dependence on instrument supplier in case of mall functioning
- improved precision	
- reduced or no exposure of personnel to solvent (vapours)	

Other issues that need to be addressed in automated (on-line) systems are carry-over (valves, tubing, cartridges) and stability of analytes in raw sample extracts which in case of sequential processing may spend a long time in the autosampler tray before being analysed. In on-line systems the time required for clean up should not exceed that of the chromatographic separation because this would result in inefficient use of the chromatographic equipment. Furthermore, the complexity of method development and equipment increases. The trade-off

to be made is that the down-stream separation and detection need to be adapted to the on-line extraction in terms of timing and solvent selection etc. and could be sub-optimal.

3. Automated off-line clean up approaches

Here, clean up is not connected directly to the chromatographic system for final separation and detection. Automation often is a quite literally translation of what is manually being done, for example for automated SPE and GPC. This is less so for approaches which in fact combine extraction and clean up. Examples are SFE and the much more popular PLE.

3.1. SFE and PLE

Supercritical fluid extraction (SFE) and pressurised liquid extraction (PLE, also called pressurised solvent extraction (PSE) or accelerated solvent extraction, ASE, which is a Dionex trade mark) are basically extraction methods where the homogenised sample is placed into an extraction cell and the analytes are extracted at elevated temperature and pressure. Details on these techniques can be found elsewhere in this book and have been reviewed earlier (SFE: [17,18], PLE: [19]). Commercial systems equipped with carousels for 12-24 samples are available allowing sequential automatic extraction of a batch of samples. Together with extraction of the analytes, high molecular weight matrix components, like lipids and pigments, and other potential interferences are frequently present in the extract and must be removed before proceeding with the analysis. Most commonly this is done in a post-extraction clean up procedure, but an in-situ clean up is an attractive alternative. By including an adsorbent in the extraction cell (or by mixing the sample with a sorbent before putting it in the extraction cell) a combined automated extraction and clean up can be achieved. Examples are discussed below for both SFE and PLE.

SFE emerged in the 1990s as an attractive alternative to Soxhlet extraction because of its fast extraction kinetics, “tunable” solvation power (i.e. selectivity) via pressure and temperature, easy of solvent (usually carbondioxide) removal, minimal usage of organic solvent. However, initial poor robustness, lack of a universal method for a wide range of analytes/matrices, difficulties in extracting polar analytes, and, especially, complex optimisation procedures have prevented a wide acceptance and implementation of the technique [18]. Nevertheless, (a few) interesting applications of efficient combined extraction/clean up based on SFE can be found in the literature and are worth including here.

Matabudul et al. [20] packed the extraction cell with aluminium oxide and on top of that 1 g of egg, meat of poultry feed. In case of egg and meat the sample was bracketed between layers of anhydrous sodium sulfate which was mixed (in the extraction cell) with a spatula to form a paste. Ethanol was added as modifier and CO_2 used as supercritical fluid. Expansion took place in a flask filled with methanol which only needed to be filled to the mark before LC-UV analysis. Hopper [21] coupled SPE extraction to a column packed with C1 material.

Pesticides (organochlorine, organophosphorus, pyrethroids and others) were extracted from oil- and fat-containing products mixed with hydromatrix and packed in an extraction cell. The SFE extract was then directed through the C1 column for clean up after which the cleaned restrictor effluent was trapped in hexane. Using CO_2 with 3% acetonitrile as modifier in combination with the on-line clean up, the efficiency of the fat removal was comparable to GPC. Polar pesticides (e.g. dimethoate, atrazin) were not recovered from the C1 column. Extracts could be analysed directly using GC-ELCD (halogen mode) and GC-FPD (phosphorus mode). For GC-ECD an additional Florisil clean up was required for certain samples.

PLE appeared in the mid-1990s. Like SFE it has favourable extraction kinetics. The main purpose of the elevated pressure in PLE is to maintain the solvent in the liquid state at a temperature that exceed its boiling point. Compared to SFE, the number of parameters that need to be optimised is less resulting in faster method development. In fact, often Soxhlet methods are more or less directly translated into a PLE method as far as solvents are concerned. For these reasons, PLE has become quite popular.

Several applications of combined automated extraction/clean up using PLE have been presented for food toxicant analysis. Björklund et al [22] described the determination of PCBs in fat containing matrices using GC-MS. The sample (2 g of fish meal or 0.5 g of fish oil) was grinded with sand-sodium sulfate (1:1, w/w) and then packed into a 33 ml extraction cell on top of a layer of silica and a layer of “fat retainer”. For the latter, several materials were tested: basic-, neutral- and acidic alumina, Florisil, and sulfuric acid impregnated silica. All materials were capable of removing the fat for 99% when the fat/fat retainer ratio was 1:40 (w/w). Florisil and sulfuric acid/silica provided the cleanest extracts. Due to the low density of Florisil and limitations in the volume of the extraction cell, the sulfuric acid/silica was considered more practical. However, others reported that this rather aggressive SPE material resulted in accelerated aging of PLE extraction cells and frits, which became more difficult to clean between runs. Also in subsequent clean up of the extract, acid resistant parts had to be used to avoid problems with leaking [13].

In a later contribution [23] the sulfuric acid/silica clean up was further investigated using an extraction cell of different geometry (wider ID, shorter, 34 ml) with a simplified packing procedure. In addition, the effect of solvent (pentane, hexane, heptane) and temperature on fat elimination was investigated. The solvent had no significant effect while a higher temperature (100°C vs 50°C) improved recoveries of PCBs but also resulted in increased amounts of fat in the final extract. In order to avoid suppression of the PCB response and eliminate interferences in the GC-ECD analysis, the remaining fat content in the final extract had to be less than 0.5 mg/ml. This could be achieved by using a ratio between fat and sulfuric acid/silica of 1:40 which in practice translated into 0.25 g of pork fat or fish oil with 10 g of sulfuric acid/silica and 2.5 g of fish meal (280 mg fat) with 11.3 g of sulfuric acid/silica. With

the system used (Dionex ASE300) the carousel could accommodate 12 samples for automatic extraction/clean up. The solvent volume ending up in the collection vials was 30-40 ml which then was concentrated by a rotary evaporator to 1 ml.

A similar application, PCBs in biota, was described by Gómez-Ariza et al [24]. Samples (2 g) were ground with Florisil (4 g) into a homogeneous mixture that was packed into the extraction cell on top of a layer of Florisil (illustrated in Figure 1a). For quantitative recoveries of the PCBs at least 15% of dichloromethane had to be added as modifier to pentane using an eluate volume of 55 ml. Both the efficiency of extraction of the PCBs and removal of the fat by the Florisil depended on the temperature. This was studied and the results are shown in Figure 1b. Higher temperatures resulted in more efficient PCB extraction but also in less clean extracts. At 40°C, fat removal was sufficient (0.8% co-extracted) for the GC-MS analysis. The method was validated using a mussel reference material and the results corresponded well with the certified values. Oysters, mussel, fish and clams (3-10% fat) were analysed and method detection limits ranged from 0.001-0.12 µg/kg using GC-MS/MS andGC-ECD.

The application of the sulfuric acid/silica clean up during PLE extraction was extended to the determination of dioxins in food and feed. The use of this approach by Bernsmann et al [25] reduced the time required for extraction and primary clean up (elimination of bulk fat) to 1 hour and also drastically reduced the amount of solvent required. The extract obtained was further cleaned using the usual AlOx and carbon column purifications. Sporring et al [26] explored the possibilities of extended clean up in the PLE extraction cell.

In the field of veterinary drug analysis several interesting applications of PLE with in-situ clean up were described. Draisci et al. [27] selectively removed fat from the bovine liver/hydromatrix mixture in the extraction cell using hexane. The corticosteroids were retained and subsequently extracted with a mixture of hexane/ethyl acetate (1:1, v/v). This way a cleaned extract was automatically obtained within 35 min. After a solvent-switch the extract was ready for LC-MS/MS analysis. A similar approach was followed by Hooijerink et al. [28] for determination of anabolic steroids in kidney fat. Here the extraction cell was filled with, from bottom to top, 5 g of aluminum oxide, sodium sulfate and then 2 g of the melted fat. An initial extraction with hexane resulted in elution of fat from the cell while the analytes were retained on the aluminum oxide layer. A second extraction cycle with acetonitrile eluted the steroids. Fat remaining in the acetonitrile extract were further removed by freezing out. After a solvent switch an additional clean up by SPE was performed before LC-MS/MS analysis.

The application described by Gentili et al. [29] in fact is an automated way of matrix solid phase dispersion (MSPD) where the analytes (sulfonamides) were automatically extracted with superheated water using a PLE system (Dionex ASE200). 1 g of homogenised meat or infant food was blended with 2 g of pre-cleaned C18 material using a pestle. This mixture was

packed into an extraction cell. C18 was selected in favour of diatomaceous earth because of better dispersing properties and for (partially) retaining the lipids from the matrix. Different temperatures were investigated for the extraction, for good recoveries 130°C or higher temperatures were required. Under these conditions solvation properties of water can change dramatically (polarity decreases) [30] which probably accounts for the improved extraction efficiency and also for the co-extraction of (small amounts) of fat. These were effectively removed by freezing out after which the extract was analysed by LC-MS/MS. No matrix effects were observed for infant foods (meat based) while extracts of fresh beef and porcine showed 30% ion suppression (10 mg equivalent of sample injected onto the column). Detection limits were around 1 µg/kg.

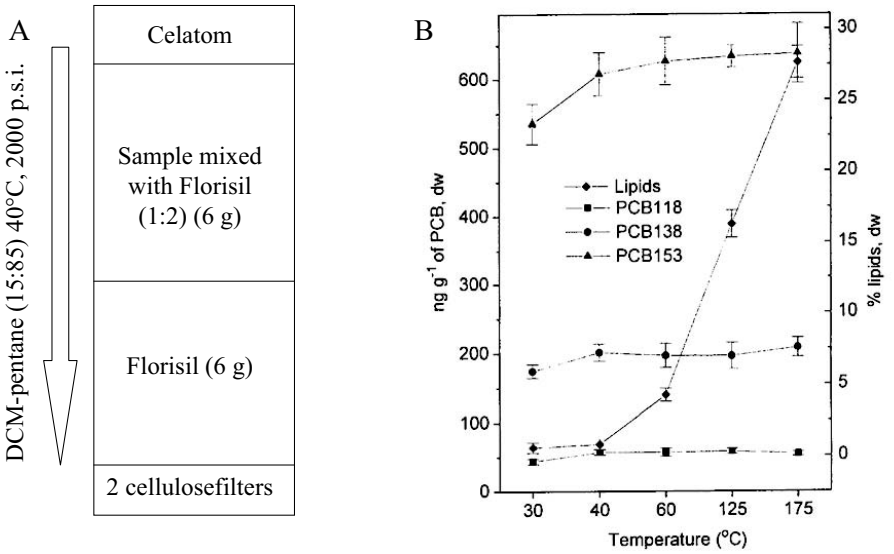


Figure 1. Combined extraction and clean up for determination of PCBs in fat containing biota using PLE. A (left): packing of extraction cell. B (right): effect of temperature on extraction efficiency of PCBs and co-extracted fat, 3x15 min static extraction, 150% flush volume (Reproduced (modified) from [24] with permission from Elsevier© 2003).

3.2. GPC

Gel permeation chromatography (GPC) can be considered as a very valuable universal clean up technique that separates small molecules (food toxicants) from large molecular weight compounds (lipids = waxes, triglycerides, phospholipids etc; pigments = chlorophylls,

cartenoids, etc.; plant resins). GPC is widely used in pesticide residue analysis, especially for removal of fat. For wide-scope multi-residue analysis, as is common in this field, other techniques for fat removal like sulfuric acid treatment or aluminum oxide column chromatography (as used in PCB and PAH analysis) are less suited because many pesticides would not be recovered from such procedure. The major drawback of GPC clean up is that the process is rather time consuming. In order to have a high capacity for fat removal, relatively large column dimension are (still) being used very often (typically 50-60 cm x 27 mm ID, S-X3 Bio-Beads) resulting in processing times that can be more than one hour per sample. The cleaned extract obtained is strongly diluted. Before analysis of the cleaned extract or before a subsequent additional clean up step a manual concentration step by rotary evaporation is usually necessary.

Automated systems for off-line GPC usually consist of an autosampler, a pump, a GPC column, a fraction collector and a controller, and are being used since the early 1970s [31]. Automated GPC is a sequential process, the same column can be used for a very long time. Basically, automated off-line GPC clean up has hardly changed since the early years. Smaller ID columns have been used which result in smaller size fractions that can be concentrated more efficiently (i.e. parallel evaporation of eluates in tubes in a heating block under N_2 instead of one-by-one using a rotary evaporator). Obviously, the sample capacity of the GPC column is lower in this case. To compensate for this, the extract needs to be concentrated more, or as a better alternative, a larger volume of the extract needs to be injected into the chromatographic system.

Systems have been developed where the evaporation of the eluate and/or a subsequent SPE clean up has been automated. Behre at al. [32] described a system (TACS) supplied by LCTech where the 65-100 ml GPC fraction is eluted into a cylindrical flask positioned in a vacuum chamber. There the solvent is automatically evaporated down to 5 ml. Two laser beams monitor the progress and prevent the solvent from complete evaporation which would result in losses of volatile compounds. The concentrated fraction is transferred into glass vials and the flask rinsed automatically to prevent carry-over. Depending on the set up and the number of rinse cycles, up to 20 samples a day can be processed (up to 36 with second vacuum chamber). Rimkus et al. [33] also described the use of GPC with automated evaporation and extended the automation with an HPLC clean up. After evaporating the GPC eluent to dryness, toluene was added for reconstitution and the extract transferred into a sealed vial. From there an aliquot was injected onto a 250 x 4 mm id SiOH column. Using a ternary solvent (hexane/toluene/acetone, 88/10/2), organochlorine pesticides, PCBs and nitro musks were eluted within 7 ml fractions. These were off-line concentrated and analysed by GC-ECD and GC-MS.

3.3. SPE

Solid phase extraction (SPE) is widely used for clean up in food analysis, and has largely replaced classical gravity column chromatography. Disposable cartridges with a range of stationary phases are available. For aqueous samples, i.e. beverages, and aqueous extracts, non-polar phases are frequently used for extraction/clean up. For organic extracts of food samples a range of normal phase materials is available (SiOH, CN, NH_2, Florisil, Al_2O_3). In addition, there are phases for isolating a specific target analyte (immuno-affinity (IA) phases, and molecular imprinted polymers (MIP)) and for removal of large molecules like proteins (RAM, restricted access materials). Details on SPE techniques can be found elsewhere in this book.

There are a number of commercially available systems that can automatically condition the cartridge, load the sample, rinse and dry the cartridge, and then elute the analytes of interest into a tube or autosampler vial. In contrast to manual SPE where a vacuum is usually applied to achieve a flow through the SPE cartridge, automated SPE use syringes or piston pumps to apply either a positive gas pressure or an eluent to transport the liquid flows through the system which results in more uniform flows and therefore better reproducibility.

Three approaches can be distinguished in automated SPE: discrete column workstations, 96-well workstations and on-line coupling of SPE with chromatography (which will be dealt with in section 4). These approaches were reviewed earlier by Rossi et al. [3].

Figure 2. TACS (totally automated clean up system) for automated GPC and evaporative concentration (courtesy LCTech, Germany).

In food toxicant analysis, workstations using discrete SPE cartridges are currently the most widely used manner of automating SPE clean up. Examples of systems frequently mentioned

in literature include ASPEC (Gilson), RapidTrace (Caliper, previously Zymark), Prospekt (currently called Symbiosis) (Spark) and Power-Prep (FMS), photographs for illustration are shown in Figure 3.

The first two systems can accommodate any commercially available 1, 3 and 6 ml SPE cartridges, while the Prospekt and Power-Prep use proprietary pre-packed disposable columns. With the ASPEC system samples are processed sequentially but there are configurations with 4 or 8 probes which allow parallel processing of the same number. The RapidTrace is a modular system. Each module contains 10 SPE cartridges which enables automated sequential clean up of 10 samples. For parallel processing more modules can be attached. With both systems it is possible to process hundreds of samples a day. The Power-Prep system is also a modular system. Each module can process one sample. This system is especially dedicated to automation of multiple clean up steps, i.e. GPC and SPE, or several SPE procedures, as is done in, for example, dioxine analysis. The Prospekt is an automatic cartridge exchanger that processes one sample at a time. It is mostly used in on-line configurations, but use in combination with a fraction collector has also been reported [34].

96-well SPE was developed in the 1990s especially to increase sample throughput in bioanalysis since with the use of LC-MS/MS (with very short run times optimized for the one or few target compounds of interest), sample preparation (mainly SPE) by far was the time limiting factor [4]. The high throughput results from the fact that 96 samples can be processed in parallel with very little operator intervention. While in bioanalysis 96-well SPE is the standard, the current number of sample(extracts) in food toxicant analysis is usually (far) too low, the complexity of the clean up sometimes too high, and the chromatographic analysis not as fast to be beneficial here. Only one application was found in literature (pesticides in tobacco [35]).

Applications of automated off-line SPE clean up in food toxicant analysis from the past five years are included in the application table (Table 4). The ones included are just examples to illustrate the wide applicability and the list is by no means complete. Many interesting earlier applications have been described. For these the reader is referred to earlier reviews, e.g. by Shepherd [5] and Stolker et al. [6].

In most applications of automated SPE the clean up is a fairly straightforward condition/load/wash/elute procedure, sometimes not even much more than just filtering the extract through the cartridge. There are, however, also examples of more complicated multi-step clean up procedures such as those used in dioxine analysis. Focant et al. presented several papers on this using the Power-prep workstation. The system is composed of valve drive modules connected to a pump module. Programming of solvent volumes, types, flow-rates and directions is controlled by a computer linked to the system. The set up of the system, involving three disposable columns: silica (multi-layer acidic, base, neutral), aluminum oxide and carbon, is schematically shown in Figure 4.

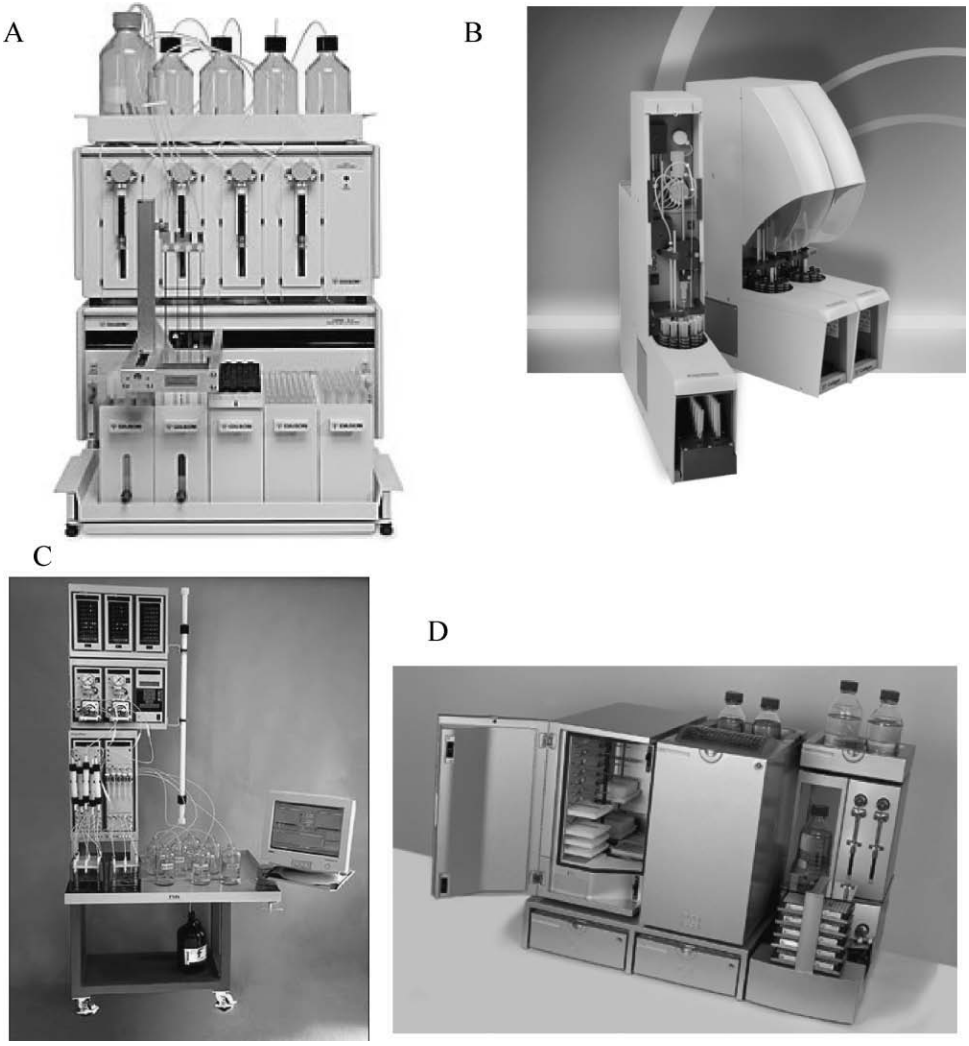


Figure 3. Four commercially available systems for automated SPE clean up. (a) ASPEC (courtesy Gilson, Netherlands), (b) RapidTrace (courtesy Caliper), (c) Prospect (courtesy Spark Netherlands), (d) PowerPrep (courtesy FMS, Germany).

For samples with high fat content initially a separate GPC clean up was required before the automated column clean up which could later be avoided by adding a high capacity disposable silica columns (HCDS, total 50 g of stationary phase!) in front of the standard (7.5 g) column [36]. This system could handle up to 4 g of fat. Fat was dissolved into 50 ml of hexane, loaded onto the HCDS column, eluted to the next column with 200 ml of hexane. 60 ml of hexane/dichloromethane (98/2) were dispensed to the alumina column to eliminate low polarity interferences (certain PCBs). Then dioxins and co-planar PCBs were eluted from the alumina with 120 ml of hexane-dichloromethane (1/1) onto the carbon column. The carbon column was back flushed with 60 ml of toluene to elute the dioxins and co-planar PCBs which was then manually concentrated by rotary evaporation. At the end of the clean up process, a special solvent program was run to clean the system and prevent carry-over. A modification of the system was used for determination of dioxins in milk [37 and in a later contribution the application was extended and included also poly brominated diphenyl ethers (PBDEs) and PCBs [38]. Besides a substantial increase in sample throughput another advantage of the automated clean up system was reduction of the blank levels, and, thus, reduction of the method LOOs.

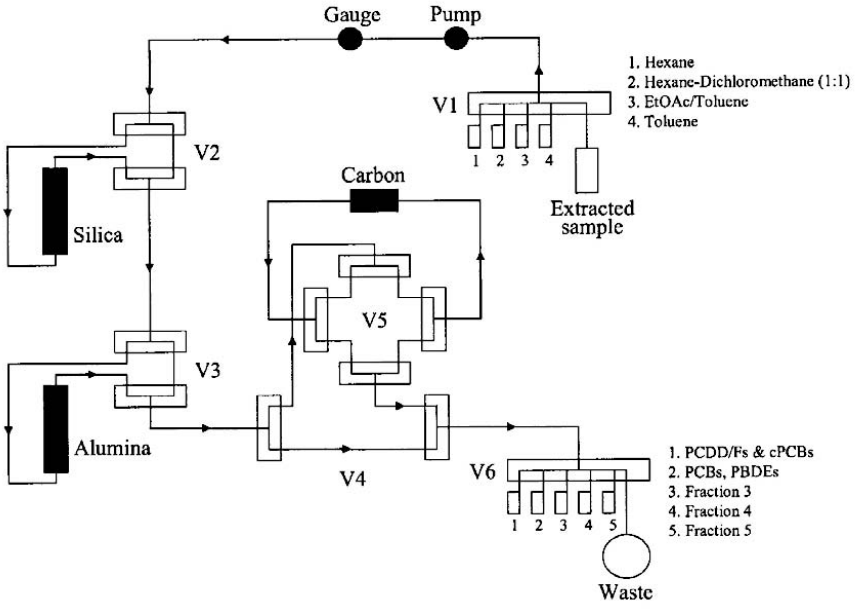


Figure 4. Schematic representation of the Power-Prep automated clean-up system used in dioxine analysis. (Reproduced from [13] with permission from Elsevier© 2004).

Haib et al. [35] nicely illustrated how moving to alternative approaches in both clean up and final chromatographic separation/detection can greatly improve sample throughput in pesticide multi-residue analysis in tobacco. Tobacco extracts in toluene were cleaned by two SPE steps in series. To cover all analytes, three different two-step SPE clean ups were used, one for low polarity compounds, one for intermediate/high polarity compounds, and one for N-methyl carbamates. This resulted in a total of six SPE steps per sample. A 96 well SPE plate set up was used. From the crude extract 0.1 ml was loaded onto the first SPE cartridge. After rinsing, the pesticides were eluted with a small volume 0.5-0.8 ml into the well of the sample collection rack. The content of the well was automatically transferred onto the second SPE cartridge for further clean up. Cleaned extracts were analysed with NCI and EI-GC-MS/MS, and HPLC post column derivatisation fluorescence detection. LODs ranged from 1-60 ug/kg. Six 96-well plates could be loaded on the instrument. All six SPE steps could be performed totally unattended. For each individual SPE step, eight samples were processed in parallel. This resulted in an overall clean-up time of 3.5 hours for 24 samples (144 SPE clean up procedures).

3.4. HPLC

SPE cartridges, due to column dimensions and particle size, are low resolution chromatographic systems which offer a rough clean up of the crude extract. In cases where more efficient clean up is required, HPLC can be an attractive alternative. With respect to automation, there are two options: automated fraction collection and on-line coupling with the final chromatographic separation/detection, which will be discussed later. One reason for choosing for off-line HPLC fractionation is when the analysis involves a derivatisation which is incompatible with the chromatographic set up. Another reason is when the analysis requires several fractions of interest to be collected which makes on-line coupling to another chromatographic system more complicated (although in this area advances are made, see LCxGC and LCxLC).

As far as food (related) analysis is concerned, off-line HPLC fractionation has mainly been used for the determination of anabolic steroids in urine and meat [39,40]. After enzymatic digestion, LLE or SPE, a HPLC fractionation is performed. The relevant fractions are subject to derivatisation and then analysed by GC-MS. This method now is becoming more and more replaced by methods based on LC-MS/MS which eliminates the need for derivatisation and allows replacement of the NPLC by (faster) SPE clean up steps [41].

Another application of HPLC clean up is separation of organochlorine pesticides (OCP) from triglycerides [42]. Using a 50 x 4.6 mm ID LC column, an OCP containing fraction of 12 ml was obtained which was analysed by GC-ECD after evaporation to 1 ml.

Table 3
Overview of clean up techniques used in fully automated systems that combine clean up with final chromatographic separation/detection (overview is restricted to applications for food toxicant analysis).

		Automation through dedicated autosamples/xyz		On-line connection (closed systems)	
Technique	Description	GC	LC	GC	LC
Headspace	Gas phase above sample in autosampler vial at elevated temperature is pressurized and transferred to GC	P[46-48] PP[49-51]			
Pervaporation	Analytes from gas phase above sample in lower donor chamber of pervaporation unit diffuses through membrane into acceptor chamber. Analytes in acceptor gas phase are transferred by switching it into the carrier gas stream of the GC	N[57]			
HS-SPME	Coated fiber attached to autosampler syringe is exposed to headspace of sample in autosampler vial. Analytes are extracted into coating and desorbed thermally (GC) or by liquid (LC)	N[55] PP[54,56]			
SPME, fiber	Coated fiber attached to autosampler syringe is immersed in aqueous sample in autosampler vial, analytes are extracted into coating and desorbed thermally (GC) or by liquid (LC)				
SPME, in-tube (hollow fiber)	Capillary coated on inside extracts analytes from aqueous sample (extract) which is flushed through the capillary				P[114] V[113] PP[115,161]
SDME, single drop micro extraction	Micro drop of water-immiscible extraction solvent at tip of syringe is immersed in aqueous sample. Analytes are extracted into the drop which is injected into GC				
MMLLE, microporous membrane liquid-liquid extraction	Sample (aqueous) and extraction solvent are separated by a microporous membrane (also filled with the extraction solvent). Several configurations exist, flat-sheet membrane, hollow fiber, membrane bag.	P[69]		P[70,71]	P[121] V[119]

Technique	Description	Automation through dedicated autosamples/xyz		On-line connection (closed systems)	
		GC	LC	GC	LC
Similar: MASE, membrane assisted solvent extraction					
Clean up by/in GC inlet	DMI, difficult matrix introduction: liner contains microcup with sample extract. Each sample has its own liner, liners are automatically exchanged which prevents build up of “dirt” in the injector. Laminar cup liners: fat is retained in the liner while analytes are transferred to the analytical column.		P[73, 75-77]		
SLM, supported liquid membrane extraction Or LLLME, liquid-liquid-liquid micro extraction	Analytes are extracted from a donor phase containing the sample (aqueous), through a porous membrane impregnated with a water immiscible organic solvent, and back extracted into an acceptor aqueous phase of different pH then the donor phase		P[120]		
SPE, solid phase extraction	Analytes and matrix interferences are separated on a SPE cartridge (low resolution column) by proper choice of solvents. Is used with organic extracts (normal phase cartridges) as well as aqueous samples/extracts (reversed phase cartridges)		N[122-124]		P[134,145, 1, 25] P[129] V[135,131] V[132,128] N[126-127] N[130,133]
GPC, gel permeation chromatography (also called SEC, size exclusion chromatography	Large molecules (lipids, pigments) are separated from small molecules (food toxicants) on a GPC column. Extracts are strongly diluted.	P[87]		P[86,89]	
Dialysis	Analytes selectively diffuse through porous membrane to aqueous				V[116-118]

Technique	Description	Automation through dedicated autosamples/xyz		On-line connection (closed systems)	
		GC	LC	GC	LC
	acceptor phase while high molecular weight compounds (protein, lipids) remain in aqueous donor phase.				
HPLC, Column-switching	High resolution pre-separation were fraction(s) of interest are transferred to second column for final separation detection.	P[87]		LC-GC P[94], E[92] PP[81,90, 91]	LC-LC P[137-139] E[93]
GC Heart-cutting	High resolution pre-separation were fraction(s) of interest are transferred to second column for final separation detection.			Heart-cutting E[99]	
2D comprehensive chromatography	As two items above but the entire eluate from the pre-separation column is, in distinct segments/fractions, transferred for final separation and detection			GCxGC E[103-108]	

Capitals preceding the references indicate area of application: P = pesticide V = veterinary drugs, N = natural contaminant, E = environmental contaminant, PP = processing/packaging contaminant

Off-line HPLC clean up was applied quite frequently in the 1990s but in the recent literature, only few papers have appeared. One example is the clean up of fish extracts for the determination of organochlorine pesticides and PCBs with GC-MS/MS [43].

4. On-line clean up approaches

As mentioned before, this section will include any approach for fully automated clean up combined with the final chromatographic separation and detection (i.e. on-line, in-line, at-line, robotized). Some of the approaches described here could be considered more as automated extraction combined with chromatographic analysis, rather then automated clean up. However, since a clear distinction between the two processes cannot always be made (with respect to selectivity the two are interrelated anyway), automated extraction coupled to chromatographic analysis is also addressed. Another reason for doing so is that such approaches can be effective alternatives to step-by-step automation of existing manual procedures. Below the most relevant and/or recent techniques described for on-line automated clean up coupled with GC or LC are described. Some approaches are used with both GC and LC while others are more explicitly used with one of both. An overview of the techniques is presented at the end of this section in Table 3.

4.1. Clean up coupled to GC

4.1.1. Headspace techniques

Headspace analysis (vapour phase extraction) provides clean "extracts" in itself because it is expected that the vapour phase mixture contains fewer compounds than the liquid or solid food matrix. In addition, headspace techniques are easily automated. Consequently, it is a very attractive approach for determination of volatile food toxicants in food matrices. Headspace analysis in GC in general has been reviewed by Snow and Slack [44].

Automated headspace GC (static, dynamic, purge and trap) are all well established techniques which are mature and have been reported since the 1980s for fumigant analysis (EDB in cereals [45] and fruits [46], methylbromide in various products [47]).

More recent papers report on the determination of dithiocarbamates (as CS_2) in plant matrices [48], residual solvents in natural food additives [49], 1,3-DCP in soya sauce [50] and off-flavors from irradiated meat [51].

Over the past decade, several alternative approaches have appeared for headspace analysis involving a third phase (besides the sample itself and the headspace) which is either a sorbent or a liquid introduced into the headspace. Some interesting examples are described below.

Headspace solid phase micro extraction (HS-SPME) is based on the distribution of analytes between the sample, the headspace above the sample and a coated fused silica fiber. Analytes

are absorbed by the coating of the fiber until the concentrations in the phases are in equilibrium. The basics of SPME are discussed in more detail in Chapter 10. In the instrumental set up, a fused silica fiber coated with a thermostable polymeric phase is attached to the lower end of the plunger of a modified GC injection syringe. By moving the plunger the fragile coated fiber can be positioned inside the needle of the syringe (during piercing of septa of headspace vial or GC injector) and outside the needle during sorption (exposure to headspace) and desorption in a standard GC split/splitless injector. Compared to direct immersion of the fiber into solution (see later, section 4.1.2), contamination of the fiber is avoided in headspace SPME

In food analysis, HS-SPME is especially used for analysis of flavours and other natural volatile compounds, but several interesting applications in the field of food contaminants have been reported. Examples include the determination of pesticides in herbal infusions [52] and the determination of petroleum contamination in shellfish [53]. In these two examples, however, SPME and injection into the GC was done manually while one of the most attractive features of (HS)SPME is its ease of automation.

In principle, any autosampler can be transferred into an automated SPME system [12]. Varian autosamplers are an example of commercially available systems adapted for this purpose. An application using this set up was presented by Fernando et al [56] who used HS-SPME for the determination of hexanal in a study on oxidation of pork. Raw ground pork was put in the headspace vial, water was added and using a manual procedure the sample was heated for 30 min in a water bath. After cooling to room temperature, the vial was placed in the autosampler adapted to perform HS-SPME. Hexanal was extracted from the headspace for 20 min and after that the fiber was desorbed in the splitless injector and analysed using GC-FID.

In other automated applications xyz-robotic autosamplers like the CTC combi-Pal were used. Goldmann et al [54] developed a rapid method based on HS-SPME-GC-MS for the determination of furan in a wide variety of heated foodstuffs (baby food, coffee, canned food, fruit juices etc.). Good precision and accuracy were obtained using deuterated furan as internal standard. The detection limit was 17 pg as absolute amount of furan in a 10 ml headspace vial.

Another example was given by Lachenmeier et al. [55] for the determination of cannabinoids in hemp food products. The only manual handling was transferring the sample (tea, oil, seed, nibbles, softdrinks, chocolate) and reagents (NaOH, Na_2CO_3, internal standard) in the headspace vial. The sample was hydrolysed for 5 min at 90°C in the agitator of the autosampler. Next, the fiber was exposed to the headspace in the vial for 25 min. Then the fiber was exposed for 8 min at 90°C in a second vial containing MSTFA for derivatisation. Finally, the SPME fiber with the absorbed and silylated cannabinoids was introduced into the injector of the GC-MS for 5 min for quantitative desorption. HS-SPME resulted in much

cleaner chromatograms compared to LLE, was faster while achieving comparable LODs. However, the matrix strongly affected the distribution of the analytes over the phases and thus the response. The hydrolysis step introduced to saponify the fat containing samples improved extraction efficiency (which in absolute terms was low). The use of a deuterated internal standard was essential for obtaining adequate method performance.

Another headspace technique is pervaporation. In this technique there are two distinct chambers separated by a gas permeable membrane. The lower chamber contains the sample, in automated systems a liquid or slurry, with a thin air gap layer above the sample. The upper chamber contains the acceptor phase. Priego-López et al. [57] coupled pervaporation to GC-FID for the determination of acetaldehyde and acetone in yoghurt and pulp containing fruit juices (no filtration was necessary). The sample was pumped through the donor chamber while the acceptor chamber was filled with helium. After 10 min the helium, containing the volatile analytes, was switched into the carrier gas flow towards the GC system.

4.1.2. Automated combined extraction/clean up with GC analysis

The past 10 years a variety of miniaturised extraction/clean up techniques have emerged which can be automated through dedicated autosamplers (xyz robots) of the GC. They have in common that the applications deal with aqueous samples or aqueous sample extracts. Hence, their application is much more abundant in the environmental (water) and clinical (urine, plasma) field than in food analysis. Major advantages include low cost, virtually or completely solvent free, fast and readily automated. For details on the techniques the reader is referred to a number of excellent reviews covering the various approaches [58, 11, 10, 59].

The majority of the applications with respect to food toxicant analysis is on beverages. Here beverages means any drink except (mineral)water for which the reader should consult the extensive literature available on environmental applications. In case of solid samples an initial extraction with a suitable solvent is required followed by a dilution or reconstitution with water after which the micro extraction techniques can be used for clean up.

Solid phase micro-extraction (SPME)

A technique that has become quite popular is SPME. It was also described above in the headspace configuration. Here, the fiber is immersed in the (usually) aqueous solution. Since the extraction is diffusion controlled, some form of agitation or stirring is often performed to speed up the extraction process. New coatings and development of superelastic metal fiber (rather then fused silica) assemblies have improved lifetime of the fiber and the applicability to more polar compounds and low detection limits can be obtained. However, extraction times can be rather long (e.g. 60 min to reach equilibrium), carry-over may occur and the matrix can affect the extraction efficiency considerably (e.g. [60] and references therein). Given the wide variety of food matrices, this might reduce the attractiveness for application to quantitative

analysis, unless stable isotope internal standards are available. The main advantage of the technique is that it is easily automated.

There are a number of papers using SPME for food toxicant analysis. Examples include organophosphorus pesticides in honey and fruit juices and aqueous vegetable extracts [60], triazine herbicides in milk [61], organochlorine pesticides in Chinese teas [62] and in fish tissue [63], organotin and mercury compounds in wine [64], other alcoholic beverages [65] and marine biota [66]. In all these papers, however, a manual device was used for the SPME procedure followed by manual injection into the GC, probably because the focus was on optimization of extraction rather then development of an automated method.

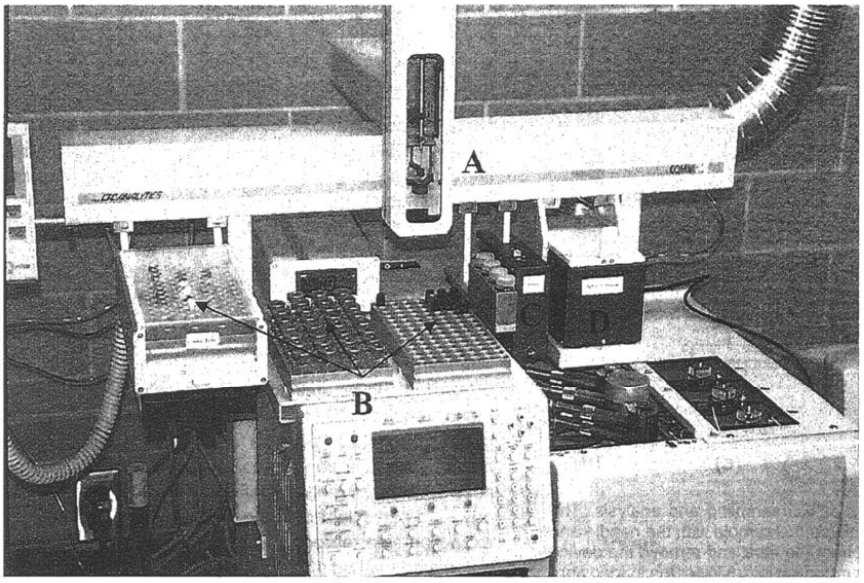


Figure 5. Commercial SPME-GC autosampler. A) sample preparation/injection arm, B) sample trays, C) needle heater, D) heater/agitator. (Reproduced from [12] with permission from Wiley© 2005).

In principle, any GC autosampler able to perform syringe injection can be modified to be capable of automated SPME-GC. With more advanced autosamplers (xyz robots, like the CombiPal™, see Figure 5), it is possible to perform agitation, temperature control, derivatization and other procedures fully automated. This allows faster nonequilibrium extractions. A nice example of a fully automated derivatisation and SPME procedure was

described by Parkinson et al [67] for the determination of organometallic compounds. A Twin PAL dual arm system was used with dedicated software. The upper arm was used to perform the derivatisation steps while, simultaneously, the lower arm performed the SPME procedure. The matrix here was water, but the same approach could be applied for the off-line SPME applications mentioned above for organotin and mercury. An excellent review on automation aspects of SPME, of both fiber and alternative configurations (e.g. in-needle) has been presented by O'Reilly et all. [12] and for details the reader is recommended to consult this paper.

4.1.3. Automated membrane based clean up

The use of membranes in extraction/clean up facilitates automation of classical LLE and can be used as an alternative to SPME. It aids in automation because emulsions are not formed and no phase separation step is required. In addition, membranes can add selectivity through their pore size (discrimination against molecular size) and enrichment of analytes can be achieved. Other advantages are the very small volumes of solvent required. On the other hand, the high selectivity inherently restricts its application to certain classes of analyte at a time. A general discussion on the topic is included in several reviews [2,10,68].

Miniaturised liquid-liquid extraction with separation of the two phases by a microporous membrane has been used for analysis of beverages. Schellin et al. [69] used 20 ml autosampler vials that contained a dense polypropylene membrane bag (illustrated in Figure 6).

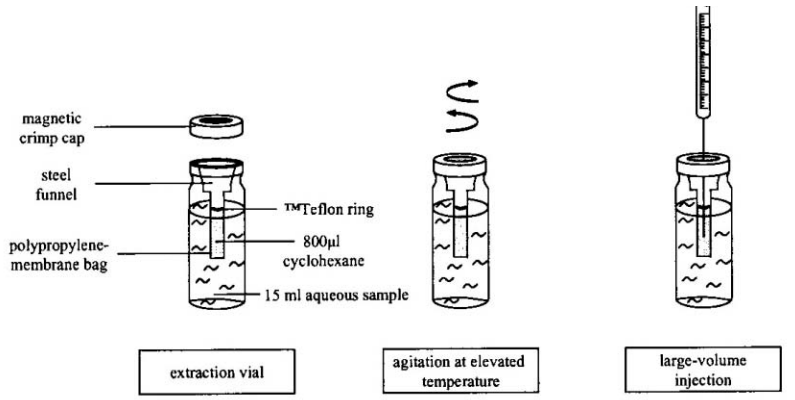


Figure 6. Device of membrane-assisted solvent extraction (Reprinted from [25] with permission from Elsevier© 2004).

After filling the vials with 15 ml of wine or apple juice and the membrane bag with 800 µl of organic solvent, the latter was emerged in the sample and the vial capped. Using a versatile multi purpose sampler, the vial was automatically agitated for 50 min and then organic phase is transferred into a standard autosampler vial. From there a large volume (100 µl) was introduced into the GC-MS. For a number of organophosphorus pesticides, absolute recoveries ranged between 47and 100%. Relative to reagent water calibration data, recoveries in wines and apple juice were between 75 and 124% indicating that the extraction efficiency was not greatly influenced by the matrices investigated. Clear extracts were obtained even for red wine showing that the membrane effectively prevented the (high molecular weight) pigments to end up in the organic extract, thereby adding to the selectivity of the technique compared to conventional LLE.

Another micro-extraction approach is membrane liquid-liquid extraction (MMLLE) which allows automation of classical LLE. Hyötyläinen et al. [70] coupled this technique to GC which was stated to be more straightforward then coupling of SPE to GC. The membrane is located in a teflon block and separates a donor flow (wine sample diluted in water to lower the ethanol content) from a stagnant acceptor phase (toluene). Although the optimum extraction time was rather long (40 min), the extraction could be performed while the GC analysis of the previous sample took place. Like SPME, the extraction is not exhaustive, but reproducible and can be used for quantitative analysis when using appropriate calibration procedures. In a subsequent paper by the same group [71] the same set up was used for determination of pesticides in grapes after pressurized hot water extraction (PHWE). Here, the PHW extract was the donor phase. Although the method yielded cleaner extracts than classical solvent extraction, the amount of sample that could be processed (50 mg) was unrealistically small for pesticide residue analysis in fresh produce (it would put very high constraints on sample homogenisation).

SDME

The ultimate miniaturisation probably is extraction of analytes into a single drop of organic solvent (so-called single drop micro extraction), hanging at the tip of the syringe needle, immersed into the aqueous sample in the autosampler vial. After the extraction period, the drop is withdrawn into the needle and injected into the GC. Batlle et al. [72] applied the technique for determination of phthalate esters in food simulants. However, drop dislodgment/stability is a problem [58] which probably prohibited the use of GC autosamplers. So far this extraction is not automated and requires careful and elaborate manual handling.

4.1.4. Automated clean up in the GC inlet

Instead of removing involatile matrix before injection into the GC, two approaches for clean up in the GC injector itself have been reported. Grob et al. [73] explored the use of a modified liner (with a kind of reservoir at the bottom) to separate organophosphorus pesticides from olive oil. The oil was diluted 1:1 with acetone and directly injected in the moderately heated split/splitless injector. The analytes evaporate from the oil film on the insert wall and are transferred into the GC column while the oil slowly flows along the wall to the bottom of the liner where it is retained. The injector temperature was a trade-off between transfer of higher boiling analytes and prevention of transfer of triglycerides. It could not be prevented that small amounts of triglycerides reached the GC column. Therefore, a daily thermal removal of triglycerates via a purge exit was done to prevent bandbroadening. Recently, eleven years after the initial publication, the approach was revisited by the same group [74].

Zehringer [75] applied a similar technique using a so-called laminar cup liner for the determination of pesticides in fat containing products like milk and avocado, after reducing the fat content in the extract by acetonitrile-hexane partitioning.

To prevent build up of involatile material in the GC liner, automatic replacement of liners, in a way similar to automated thermal desorption samplers, has been proposed for pesticide residue analysis in lettuce [76] and baby food [77]. Here, the sample extract, typically 10 µl, is transferred into a 30 µl microvial which is placed into a GC liner. The autosampler tray is loaded with such sample containing liners, and for each analysis the liner is automatically placed into the injector. After venting the bulk of the solvent at low temperature, the injector is heated and the analytes are transferred to the analytical column. Since a fresh liner is used for each sample, there is no accumulation of involatile matrix, thereby reducing the need for GC maintenance, i.e. replacement of liner and front section of GC column [76]. However, for optimal quantitative performance a straightforward dispersive clean up was still recommended [77].

FRAME: large volume injection in GC

Large volume injection in GC serves two main purposes: 1) it is a way of automated and fast evaporative concentration of off-line cleaned extracts, 2) is allows on-line coupling of extraction and/or clean up with GC. It is surprising how many laboratories still perform evaporation in tube heaters or, even more laborious, rotary evaporators. Especially for volumes up to 100 µl, large volume injection is well described and established. Evaporation of 100 µl only takes a minute or less which compares very favourably to evaporating 100 ml to a low volume using a rotary evaporator, then transfer into another tube and further evaporative concentration to 1 ml followed by injecting the traditional 1 µl into the GC. Note that the amount of matrix equivalent introduced into the GC and the LODs obtained are the same in this comparison.

There are several approaches for solvent evaporation in the GC which were mainly developed and fine-tuned in the late 1980s and 1990s. For a detailed description the reader is referred to a review by Teske et al. [78] and reviews on LC-GC [79, 80].

In LVI the solvent is evaporated and the vapour is generally vented before entering the analytical column. This can be done via the split outlet of a PTV injector (programmed temperature vaporizer) or through a so-called solvent vapour exit mounted through a T-piece between a capillary pre-column and the analytical column. For the latter approach, two concepts exist, i.e. loop-type injection and on-column injection. Loop-type injection and a later modification there-off (the wire interface) are typically part of an LC-GC system, whereas the on-column approach can also be used for large volume injection as such using a standard GC autosampler. By now, also in the interest of unification, the on-column technique is believed to be preferable, both for fully concurrent and partially concurrent solvent evaporation [81]. During elimination of the solvent, the analytes are retained in the liner (in case of PTV) or the pre-column (in case of on-column injection). After solvent elimination, in case of PTV injection the split exit is closed and the injector heated to transfer the analytes to the analytical column, as in conventional splitless injection. In case of on-column injection the vapour exit is closed and GC analysis proceeds as in conventional 1-2 μl on-column injection. Besides the two main stream techniques for LVI, on-column and PTV injection, a number of variations and adaptions have been described which will not be discussed in detail here.

On-column injection can be performed under concurrent solvent evaporation (CSE) conditions where the GC oven temperature is above the (pressure corrected) boiling point of the solvent, or under partially concurrent solvent evaporation (PCSE) conditions where the temperature is below the boiling point of the solvent. With CSE the introduction speed is adjusted such that it equals the evaporation speed. Only a short precolumn is needed since the liquid evaporates on introduction in the GC and there is no need to accommodate any solvent in the precolumn. Volatiles are typically lost together with the solvent vapours through the solvent vapour exit. With PCSE injection speed and temperature are such that the solvent is only partially evaporated during introduction in the GC. Excess solvent needs to be accommodated in a sufficiently long uncoated precolumn (e.g. 10 m x 0.53 mm ID). Wettability of the solvent in the precolumn is a prerequisite, otherwise the liquid film in the precolumn breaks up and is unevenly spread out over the entire precolumn, or even enters the analytical column which will result in distorted and/or double peaks. Compared to CSE, this technique effectively prevents losses of more volatile analytes, both due to the lower initial temperature and by solvent trapping in the solvent film in the precolumn.

In PTV injection in the most commonly used solvent vent mode, the solvent is introduced at a temperature below the boiling point with the split exit open. As long as the volume injected does not exceed the volume that can be accommodated in the liner (in packed liners

typically 20-100 µl [82,83], no optimisation of injection speed is required. In contrast to on-column injection with PCSE, wettability is not an issue in PTV injection. Loss of volatiles, on the other hand, is more critical.

Both on-column and PTV injection are fit for LVI, although for certain applications one may perform better then the other. With on-column injection there is little thermal stress imposed on the analytes and the deactivated precolumn is inert which avoids degradation of thermolabile compounds. High boiling compounds are, by definition, completely transferred onto the column. However, this is also true for involatile matrix which is deposited in the precolumn and may cause peak distortion after a certain number of injections, depending on the extract injected. With extensively cleaned extracts, as for example in LC-GC, this is less likely to be a problem then for extracts that were cleaned using more generic approaches.

With PTV injection, involatile matrix is retained in the liner and does not reach the analytical column which makes this technique more tolerant to “dirty” extracts. In the past, liners were often packed with a support material in order to retain the liquid [82,83]. Such materials lacked inertness which resulted in degradation of thermolabile compounds and extensive matrix effects of these and polar analytes, i.e poorer quantitative performance for such compounds. The commercial availability of liners with a deactivated porous glass surface on the inner wall has substantially improved the situation. Furthermore, degradation of thermolabile analytes, often not being very GC amenable anyway, is becoming less of an issue since today LC-MS is widely available as an alternative technique.

4.1.5. SPE-GC

Coupling of SPE to GC has been described extensively for water analysis. However, for food toxicant analysis, in contrast to LC-GC, see later, no publications were found. This is somewhat remarkable since off-line (automated) SPE is a very common clean up procedure. Apparently, the threshold for development of on-line procedures involving large volume injection into the GC is too high for the often relative straightforward off-line methods. This despite the commercially availability of automatic cartridge exchangers (Prospekt) which would be very convenient for this purpose.

4.1.6. GPC-GC

As mentioned earlier, GPC is a very effective and generic way for removing large molecular weight material, especially applicable to food matrices since fat is such a dominant co-extractant. It was early recognized that further miniaturization would enable an on-line coupling of GPC with GC [84, 85], although it should be mentioned that there are limitations with respect to sample capacity here. Inherent to GPC is that the fraction containing the analytes of interest is relatively large. Commercially available GPC columns usually have internal diameters not smaller than 4.6-7 mm. A 25 cm column then generates fractions of 1-3

ml. Such large volumes are not easily introduced into the GC which complicates direct coupling to the GC. Nevertheless, Jongenotter et al. [86] have demonstrated that it is possible to apply on-line GPC-GC routinely, after thorough investigation of the system and all parameters. They used their system for determination of organophosphorus pesticides olive oils. The oil was simply diluted 20 times in cyclohexane after which 20 µl was injected onto a 250 x 4.6 mm ID GPC column. The GPC eluent was carefully selected (azeotropic mixture of methyl acetate/cyclopentane with 7% nonane) which resulted in 1.3 ml fraction volumes that were introduced into the GC through an on-column-type interface. The high volatility of the solvent facilitated solvent elimination at low temperatures (allowing inclusion of the more volatile mevinphos) while the nonane co-solvent ensured sharp peaks for the volatiles through the solvent-effect. Unattended operation was possible with a total analysis time of 75 min and a LOD of 2 µg/kg, using FPD detection. Maintenance (replacement of the retention gap) was required after 90 injections.

David et al. [87] coupled GPC to GC indirectly through a flowcel and an autosampler. The eluent leaving the GPC column was sent to a T-flowcell. The flow entered the cell from the bottom and exits from the side. A GC syringe can be introduced into the cell and starts sampling the eluent at a speed equal to the LC flow rate. When the entire fraction of interest was taken up by the syringe (1 ml), the syringe leaves the flow cell and a large volume injection into the GC using a PTV was performed. As an example the removal of alkaloids from tobacco extracts was shown. Phenylurea were detected down to ppb levels using MS detection. Sofar, the complexities resulting from the very large volumes seems to have prohibited extensive application of GPC-GC.

An innovative recent development was presented by Kerkdijk et al. [88,89]. The technique is based on volume overload, rather then analytical chromatography. When injecting a very large volume of extract, the concentration of the compounds injected continues to increase and instead of a Gaussian shaped peak a frontal peak is obtained that reaches a plateau concentration which is the same as the concentration in the extract injected (see Figure 7).

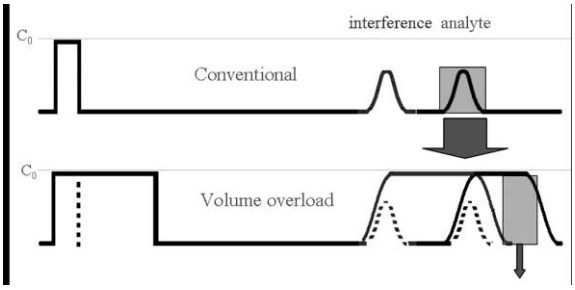


Figure 7. Schematic drawing of principle of clean up using volume overload chromatography

For compounds with different retention behavior the rise and fall of the plateaus starts at different times (the difference in time being the same as that for the peaks in analytical chromatography). Figure 7 depicts the situation for GPC where first the large molecular weight compounds (fats) reach the plateau and at a later moment in time the small molecules (residues/contaminants). After completion of the injection the concentration will decrease from the plateau values to zero. For the analytes, due to higher retention, this occurs later then for the fats. Consequently, there is a zone in the elution profiles where the fat concentration is virtually zero, while the analyte concentration is still at its plateau value being the initial concentration in the raw extract, i.e. in that zone the extract is cleaned without dilution of the analytes. Within the cleaned undiluted zone, any volume can be transferred into the GC. The advantage of this approach are very clear when comparing the volume that needs to be introduced here with that of the conventional set up described above: Jongenotter et al. [86] injected 20 µl of extract into the GPC which resulted in a cleaned fraction of 1.3 ml, with the volume overload approach only 20 µl from the cleaned undiluted zone would be needed for obtaining the same LODs. This greatly simplifies on-line coupling of GPC with GC. Kerkdijk et al. [89] applied the system for analysis of pesticides in cereals and peanuts. The schematic set up of the system is shown in Figure 8.

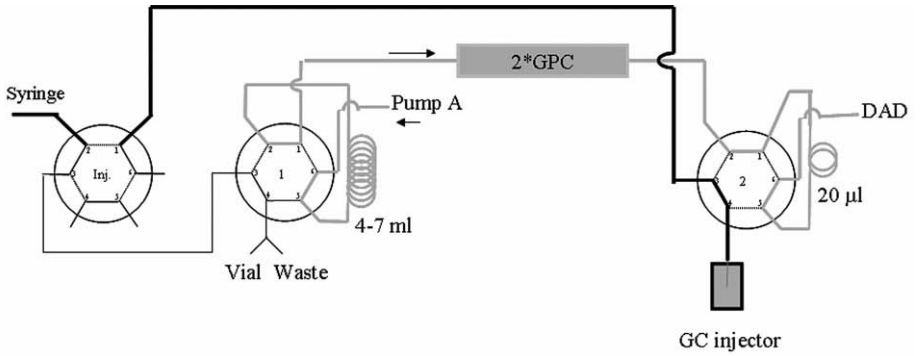


Figure 8. Schematic set up of system for on-line GPC-GC based on volume overload chromatography. P = pump, V = valve, GPC = two 30 cm x 7 mm ID GPC columns in series.

After extraction of the grinded sample with ethyl acetate/cyclohexane, 7 ml was injected onto the GPC (2x 30 cm x 7 mm ID) and 20 µl were transferred into the PTV-GC-MS system. An additional advantage of volume overload clean up is that it is much easier to perform another clean up step, e.g. SPE. This was demonstrated for peanuts. The defatted zone containing the analytes at the plateau concentration eluting from the GPC was switched to a NH_2-cartridge. This way, interferences like free fatty acids and monoglycerides were removed

and a much cleaner TIC chromatogram was obtained (see Figure 9). The NH_2-cartridge was automatically exchanged for each sample using a Prospekt system which was considered more straightforward then regeneration by backflush.

In principle the volume overload clean up approach also works for off-line automated GPC, however, there are two limitations. Firstly, depending on the application, the volume of the cleaned undiluted zone can be relatively small, e.g. in the order of a few hundred microliters in case of a 30 cm x 7 mm ID column. Secondly, it is essential that the volume injected into the GPC is large enough to allow all analytes of interest to reach the plateau value, with a 7 mm ID column already 7 ml of extract is needed. For larger cleaned fractions, which would be practical in off-line GPC, larger ID columns with corresponding larger extract volumes and longer run times would be required.

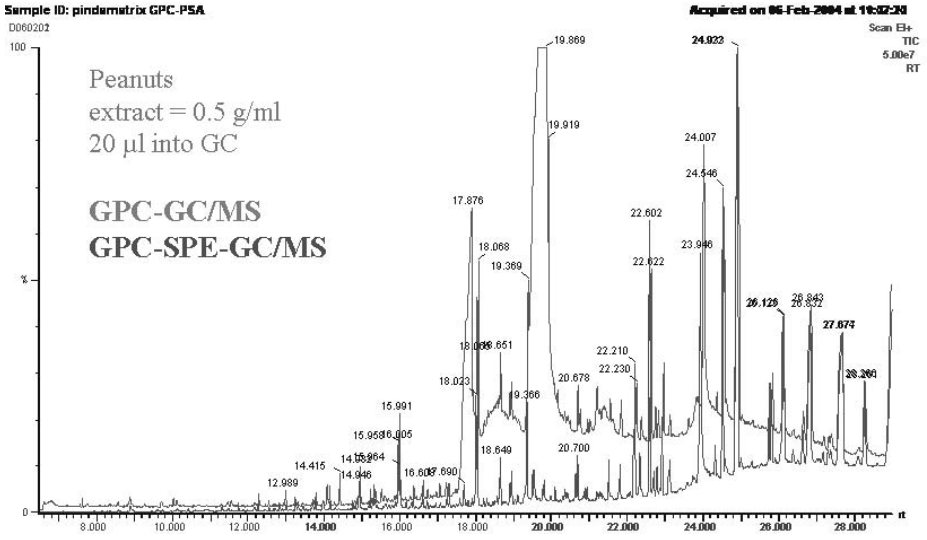


Figure 9. GC-MS TIC chromatograms of peanut extracts obtained after on-line GCP-GC based on volume overload. Without (upper trace) and with (lower trace) additional automated SPE (NH2) clean up.

4.1.7. LC-GC

On-line LC-GC basically is a fully automated version of the off-line LC fractionation described earlier. It offers the advantage of higher degree of automation, ability to transfer very sharply cut fractions, high accuracy, avoidance of contamination from external sources, and lower consumption of solvents because on-line systems inherently involves miniaturized

LC. On the other hand, LC conditions (eluent, flow rate) should be compatible with GC in general and large volume injection in particular. This understandably makes on-line LC-GC more complicated and less flexible. Two main reasons for justifying the development efforts and knowledge investment are high sample numbers and applications where extensive clean up is required to make up for lack of possibilities for selective detection. A typical example of the latter is the determination of hydrocarbons (mineral oils) in foods with GC-FID.

The general aspects of on-line LC-GC have been excellently described in two earlier reviews [79, 80]. Figure 10 shows a typical LC-GC instrument, which consists of a basic LC system, an interface valve with a transfer line into the GC, and a GC system which is suited for large volume injection (here on-column interface with solvent vapor exit, a PTV injector can be used alternatively, see also the frame on large volume injection).

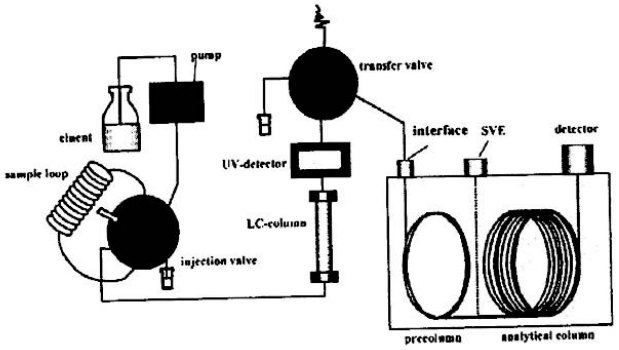


Figure 10. Basic instrumentation for on-line coupled LC-GC (Reprinted from [79] with permission from Elsevier© 2003).

In LC-GC method development, the LC method is the starting point but from the start the conditions for transfer and GC analysis have to be kept in mind. This means that NPLC, isocratic elution and smaller ID columns (fraction volume and flow rates more compatible with introduction into the GC) are most suited. As a compromise between LC capacity and compatibility with GC, 2 mm ID columns are considered a good choice [79]. In food analysis, LC-GC has been used quite a lot for determination of composition of edible oils and the determination of specific natural compounds therein. In the field of contaminants analysis the number of applications described in the recent literature is rather limited, despite its potential. This is probably due to a reputation of being complex and the fact that so far only one dedicated instrument is commercially available (Dualchrom 3000). For this to change it is necessary that large volume injection as such becomes a real standard tool in routine laboratories, because from there on-line LC-GC is only a little step further away. Below some typical examples of current on-line LC-GC applications are presented.

Several papers, mainly from Grob and associates, appeared on the determination of mineral oils in food (e.g. [90, 91], and references therein). Oil or animal fat were extracted or dissolved in pentane and 110-150 µl was injected onto two (four in case of egg yolk) 25 cm x 2 mm ID silica gel columns in series. The first LC column was back flushed with 1 ml of dichloromethane and could be reconditioned with pentane without the other column being in-line. The LC separation served two purposes, removal of the large amount of fat and separation of the mineral oil from natural sample constituents, mainly olefins. The latter could be achieved by sharply cutting the fraction transferred to the GC. For some of the feed samples this was not completely successful. In this case an additional pre-injection bromination was carried out to improve separation between saturated and unsaturated hydrocarbons. LC fractions transferred to the GC were 450-660 µl. Using FID detection, detection limits of about 2 mg/kg were obtained.

Another application from the same group was the determination of epoxidized soy bean oil (ESBO) in (oily) food. Diepoxy fatty acids, used as marker compounds for ESBO, were methylated in the food itself and separated from other fatty acid methyl esters by NPLC. A cyano column, rather then silica column was used because of more stable retention behaviour. 300 µl heart-cuts were transferred to the GC-FID system allowing LODs of 2-5 mg/kg which were sufficient in relation to the 60 mg/kg legal limit [81].

Van der Hoff et al. [92] rightly pointed out that LC-GC is very effective when it comes to a heart cut of a single analyte, but is more difficult to apply when it comes to multi-analyte applications involving analytes with different chromatographic behaviour, as they experienced during the determination of organochlorine pesticides and PCBs in fat containing matrices. Their aim was to develop a method for OCP/PCB in human milk. On-line LC-GC was considered the method of choice because this technique requires very small amounts of sample while still achieving low LODs. For the determination of PCBs in human milk the translation from off-line NPLC fractionation to on-line LC-GC was relatively straightforward: using a silica gel column the PCBs eluted closely together and were well separated from the triglycerides, and on-line coupling was achieved by scaling down the LC dimensions in order to make the fraction volume compatible with the GC transfer. For the organochlorine pesticides, dieldrin eluted closely to the triglycerides. Moreover, the retention behaviour was affected by the matrix [93]. The interrelationship between amount of fat injected, stationary phase and dimensions of the LC column was quite complex and studied in detail in another paper [92], but no robust on-line method including all OCPs could be achieved.

To avoid problems with triglycerides affecting retention behaviour in NPLC (SiOH), Sanchez et al. [94] used RPLC (C4 column) for separation of pesticides (organophosphorus and lindane) from matrix (sterols/squalene/triglycerides). A very good separation between analytes and matrix constituents was obtained. The method involved transfer of 2200 µl of methanol/water (70/30) to the GC. Since this solvent was not-compatible with standard on-

column interfaces, a modified PTV injector with a liner packed with Tenax was used as interface. The relevant fraction from the LC was transferred into the PTV (kept at 100°C) at reduced flow rate (100 µl/min). This way, the LC eluent was largely evaporated while the analytes were adsorbed on the Tenax. Trapping of the analytes was not quantitative for all analytes and recoveries varied from 19-92%. Nevertheless, due to full automation, RSD were better then 10%.

4.1.8. LCxGC

LC-GC typically isolates a group of compounds with similar physico-chemical properties from the rest, thereby limiting the application to one or a small number of analytes. A logical extension of the scope of heart cutting LC-GC would be the use of comprehensive LCxGC, where a continuous series of narrow time fractions are transferred to the GC column. The feasibility of this approach has been demonstrated in an off-line set up [95] by analysing a series of collected fractions which was then turned into a true on-line system and applied for characterisation of edible oils and fats [96] and mineral oil [97]. The NPLC as the first dimension was used for group separation (e.g. number of double bonds) while the GC as the second dimension separated on carbon number (volatility). No applications for food toxicant analysis have been reported so far.

4.1.9. GC-GC

Conventional two dimensional GC (2DGC) where one (up to a few) discrete fraction (heart cut) is diverted into the second GC column of different polarity can be looked upon as an automated way of clean up when it comes to separating analytes from interfering co-extractants. It has found limited application in general, and even less so in food toxicant analysis, due to relatively complex instrumentation and specific technical expertise. The subject has been reviewed by Bertsch in 1999 [98]. Very few recent applications were found, one example being the enantiomeric separation of chiral PCBs [99]. Conventional 2DGC has by now been superseded by a new form of 2DGC, being comprehensive GC, or GCxGC, that is now rapidly gaining interest, also in food analysis (see next section).

4.1.10. Comprehensive GCxGC

In comprehensive 2D GC, in contrast to heart-cut GC-GC where only a small region of the primary column is further separated, the entire effluent from the first column is segmented and analysed in a second column. It has high potential for characterisation of complex (food) products (petrochemicals, fat and oils, essential oils, head space analysis of food products), the determination of individual congeners of the complex organohalogen contaminant mixtures like PCBs, PBDEs and toxaphene, and the determination of multiple classes of food contaminants in complex matrices. Again, depending on the reader's point of view, GCxGC

can be regarded as the ultimate final separation system, or the ultimate automated clean-up technique. Either way, the technique can definitely affect the design of an analysis method, including clean up requirements, and therefore deserves attention in this chapter.

The principles and experimental set up of GCxGC will only briefly be discussed based on several excellent reviews [100-102] that provide much more details.

In GCxGC, two GC columns are coupled in series, the first column being a conventional GC column (e.g. 25 m x 0.25 mm id, 0.25 μm), the second column being a short narrow bore column (e.g. 1 m x 0.1 mm i.d., 0.1 μm). Analytes eluting from the first column need to be trapped, refocussed and released as narrow band into the second column for further separation. During the separation in the second column, which needs to be very fast (e.g. 1-10 sec), the next fraction from the first dimension is trapped again, etc. This is achieved by a so-called modulator located at the connection of both columns. There are several types of modulators, the more robust ones are based on cryogenic jets continuously switching on and off, thereby trapping and releasing refocussed fractions from first column.

In most cases, the first dimension is a non-polar column that separates basically on volatility using a relatively low ramp temperature program (typically 45-120 min), while the second column uses a polar column for separating compounds of similar volatility on polarity (usually isothermal, can be in same oven). The slow ramp in the first dimension comes from the requirement that the segments trapped from that column should be no larger than one quarter of the peak width in order to maintain the first-column separation. A schematic representation of the generation and visualisation of GCxGC chromatograms is shown in Figure 11.

Besides the very high peak capacity (i.e. improved separation power) another advantage of GCxGC is the improved sensitivity due to the refocusing and the narrow bore GC analysis in the second dimension. A drawback up to now is that user-friendly software for quantification, which is essential for efficient reviewing the complex data sets obtained, is hardly available.

Currently, in the field of food toxicant analysis, the focus is still more on exploring the possibilities of the technique then application in routine analysis. Quite a number of papers deal with the determination of organohalogen contaminants. Korytár et al. [103] optimised the choice of the second dimension column and temperature program for separation of PCBs with emphasis on the 12 WHO-list congeners (non- and mono-ortho PCBs). In optimised 1D GC, out of 90 congeners, 14 peaks contained two or three co-eluting PCBs, involving five priority PCBs. Two second dimension columns were found that enabled base line separation of all 12 priority PCBs. With one, an ordered structure of the two-dimensional chromatogram was observed which was based on the number of chlorine atoms. This is illustrated in Figure 12. As real-life sample a cod-liver sample was analysed, using μECD as detector. PCB-77, a non-ortho (coplanar) PCB was detected as individual congener, in presence of high levels of other

congeners, without the carbon column clean up that would normally be required in 1D GC-(HR)MS.

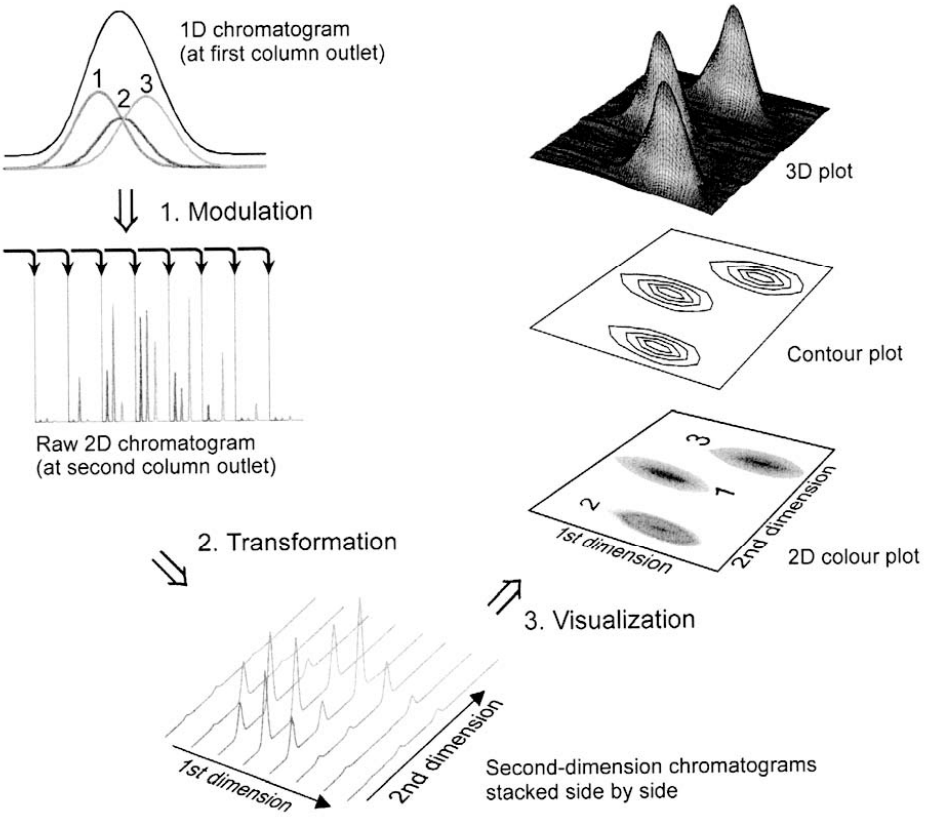


Figure 11. Generation and visualisation of a GCxGC chromatogram (Reprinted from [101] with permission from Elsevier© 2003).

In further publications, chiral columns were used for analysis of PCBs [104], the application was extended to include also dioxins [105] and tested for several food matrices. The comprehensive method was using μECD detection was compared with classical GC-HRMS [106]. GCxGC-μECD offered high potential as screening method although the LODs were still 10 times lower and quantification was time consuming due to lack of effective software. A similar comparison was made by Focant et al [107] using TOF-MS detection.

GCxGC-TOF-MS appeared to be a viable alternative to GC-HRMS, the poor mass resolution of the TOF-MS was partly compensated for by the GCxGC separation efficiency. Quantitative performance was similar while instrument cost and required operational skills were less for the comprehensive method. In contrast to the μECD paper, data processing was not reported to be tedious. Data archiving required extended storage capacity.

Further extension of the analyte scope with toxaphene, polychlorinated alkanes (PCAs), polybrominated and polychlorinated diphenylethers (PBDEs, PCDEs), polychlorinated naphthalenes (PCNs), polychlorinated dibenzothiophenes (PCDTs), polychlorinated terphenyls (PCTs), organochlorine pesticides (OCPs) and polybrominated biphenyls (PBBs) was also explored using both μECD and TOFMS detection (applicability was shown for environmental rather then food samples) [108]. Several phases for the second dimension were evaluated, each phase had unique characteristics and could or could not be used for specific purposes (i.e. either between group or within group separation was achieved.

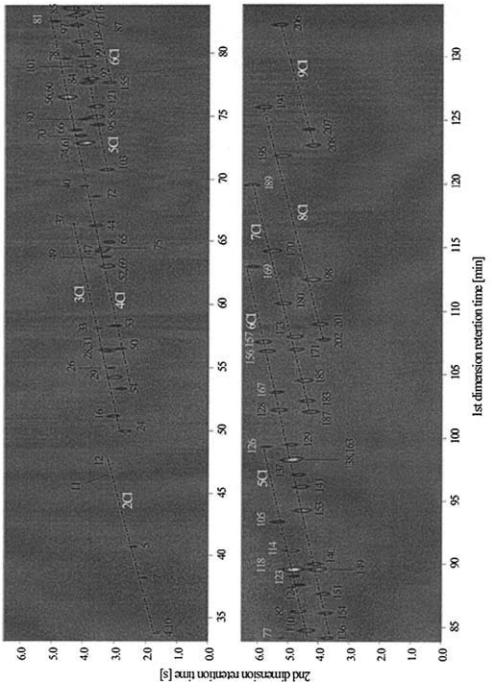


Figure 12. GCxGC-μECD chromatogram of cod liver extract. First dimension 30 m x 0.32 mm ID 0.25 μm HP-1, second dimension 1 m x 0.1 mm id 0.1 μm HT-8 (Reprinted from [103] with permission from Elsevier© 2004).

Again, up to now GCxGC research for organohalogens has been mainly explorative. For dioxins, the main objective of using GCxGC seems to be replacement of HRMS detection. Simplification of sample pretreatment, for example, replacing the carbon column clean up by separation based on planarity in GCxGC (which has been demonstrated, see papers cited above) has not been used for real samples so far.

Both Dallüge et al. [109] and Zrostlíková et al. [110] explored the potential of GCxGC-TOF-MS for pesticide residue analysis, using vegetable and fruit as example matrices. After extraction with no clean up at all or a generic GPC clean up, both groups identified situations in 1D GC-MS where matrix interfered with detection and/or proper identification of some of the pesticides. Using the enhanced separation power of GCxGC, these problems were solved and in addition better sensitivities were obtained.

4.2. Clean up coupled to LC

4.2.1. Combined extraction/clean up coupled to LC

Similar to the situation described for GC, the applications here mainly involve aqueous samples or extracts. Many of the techniques mentioned in the GC section can also be coupled to LC analysis.

Solid phase micro extraction (SPME)

SPME was initially introduced as sample preparation technique for GC, but has also found its way as automated sample pretreatment technique before LC analysis. Instead of thermal desorption, desorption is performed with a liquid, i.e. the mobile phase or another suitable solvent. At first special fiber desorption chambers mounted in a six port HPLC injector were used for this purpose, which involved manual extractions and desorptions. Later on, hollow fibers coated on the inside (instead of a solid fiber rod coated on the outside), called in-tube SPME, were introduced which were suited for automation. In this case, instead of a fiber in a syringe, a coated capillary (in fact a piece of GC column) is connected between the injection loop and the injection needle of an HPLC autosampler (see Figure 13).

Advantages of in-tube SPME over fiber SPME for LC are shorter equilibration times (typically 10-15 min), negligible carry over and easier automation. The only limitation is that the sample should be free of solid matter to prevent blocking of the flow channels and valves.

There are numerous papers on SPME combined with LC (see e.g. the reviews by O`Reilly et al [12], Katoaka [111] and Zambonin [112], here a selection is presented from applications involving food toxicants and full automation, thereby excluding most fiber publications.

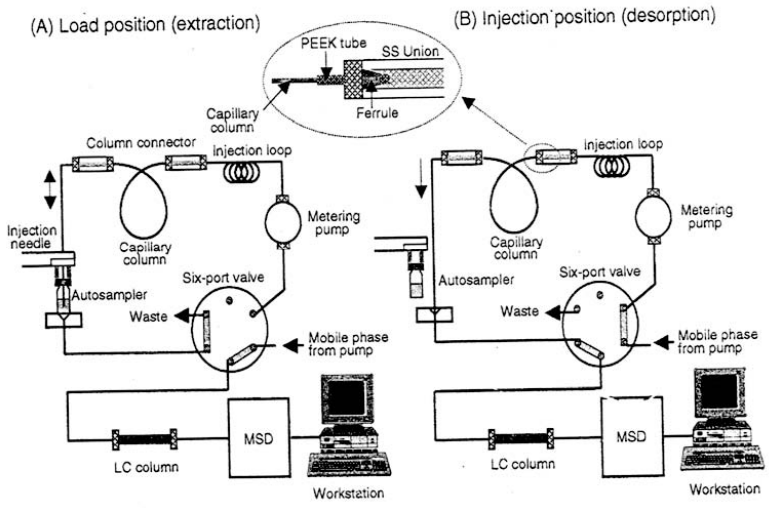


Figure 13. Schematic diagrams of in-tube SPME-LC-MS. A = load position (extraction), B = injection position (desorption) (Reprinted from [111] with permission from Springer-Verlag© 2002).

Wen et al. [113] applied in-tube SPME for the determination of sulfonamides in milk samples. After treatment with ethanol and phosphoric acid, the supernatant was injected onto an in-house coated monolithic capillary column (15 cm x 0.25 mm id). The stationary phase, which possessed a polar group (COOH) on a hydrophobic backbone resulted in adsorption of the analytes without irreversable adsorption of proteins and fats. These could be eluted by rinsing with buffer solution. The sulfonamides were desorbed with mobile phase (30% MeOH/buffer). While efficient extraction yields of around 60% were obtained for all five sulfonamides in water standards, this varied (11-96%) in case of the ethanol and fat containing milk extracts.

Wu et al. [114] applied in-tube SPME for the determination of phenylurea and carbamate pesticides in wine (and water) using LC-MS. Using a 60 cm capillary coated with a polypyrrole phase, 0.45 ml was sampled. Since ethanol lowered the extraction efficiency, wine samples were diluted 10-fold before analysis. The analysis time, including extraction was 25 min. Like fiber SPME, this is a non-exhausive extraction technique and use of internal standards or standard addition was recommended for accurate quantification.

Kataoka et al. [115] determined endocrine disruptors (phthalates, bisphenol-A, alkylphenols) in food simulants from migration experiments and from packed food products. Beverages like tea and fruit juice were centrifuged and directly analysed. Other samples

required sample pretreatment (e.g. extraction and/or fat removal and a solvent switch into 10% methanol/water) before analysis. For standard solutions detection limits of 0.1-4 µg/l were obtained using diode array detection. No LODs for the food samples were given. Difficulties with adsorption onto the walls of the glass vials and tubing of the most non-polar phthalates were encountered. This is a typical limitation in scope with on-line systems when analysing highly aqueous solutions. Addition of modifier (like the 10% methanol) reduces the problem but there always is a trade-off between solvation of non-polar compounds and lower extraction efficiency of polar compounds.

Automated membrane based clean up

Dialysis is a widely-used membrane based sample pretreatment technique and can be coupled on-line with LC. Dialysis membranes have a controlled pore structure providing a separation diffusion process based on molecular size. It is mainly used for biological matrices to separate the analytes from proteins and other macromolecular compounds. For trace analysis, continuous dialysis is performed, i.e. the sample located in a stagnant donor phase is dialyzed into a flowing acceptor solution. This dilutes the cleaned extract and an enrichment step, e.g. by SPE, is required before LC analysis. A commercial system for automated dialysis and SPE enrichment, ASTED, has been on the market since the late 1980s. The majority of applications for food toxicant analysis are in the field of veterinary drug residues. Following the introduction of the ASTED, a number of papers assessing this system appeared in the 1990s. After that, the reported use of dialysis for food toxicant analysis declined. Issues that need to be addressed with the use of dialysis for automated clean up include fouling of the tubings and dialysis membrane, and growth of bacteria and mold in the dialysis cell. Like SPME and membrane extraction in general, absolute recoveries are not quantitative.

Zurhelle et al. [116] successfully used the ASTED system for largely automated determination of tetracyclines in egg samples. No other manual pretreatment then homogenisation and dilution of the sample were necessary. Over a period of two years, more than 2000 samples (eggs and hen's plasma) were analysed. To address the issues mentioned above, several precautions were made to obtain a robust system. The pH was adjusted to prevent precipitation of proteins, automated rinsing steps were included to prevent clogging of the membrane and, in addition, valve contamination was regularly checked and the membrane was manually cleaned weekly. Furthermore, the ASTED system was modified to improve the SPE preconcentration/clean up of the 5 ml dialysate. To prevent epimerisation, the autosampler tray was cooled at 6°C. The same system was also used for determination of fluoroquinolones in chick liver [117] and sulphonamides in pork muscle [118]. In the latter case, an overall recovery of 53-56% was reported for the most of the sulphonamides, which is relatively high for dialysis clean up procedures. In a different on-line set up, using a hollow dialysis fiber, Yang et al [119] determined sulphonamides in milk samples.

Another type of membrane based extration/clean up uses porous impregnated membranes or non-porous membranes. Here, the analyte must dissolve in the solvent/polymer film in order to pass through. It can be relatively easily coupled to HPLC [2,10,58]. Up to now, it has found limited use in food toxicant analysis.

Zhu et al. [120] described the use of SLM (supported liquid membrane) for selective extraction of phenoxy herbicides from milk. A hollow fiber (3.5 cm), sealed on one end, was impregnated with 1-octanol. The fiber was filled with 7 µl of acceptor phase (0.1 M NaOH) and immersed 8 ml milk solution acidified with 0.5 M HCl. After extraction (60 min) the syringe-fiber assembly was taken out of the sample, 5 µl was withdrawn from the fiber and injected into the HPLC. Enrichment factors of 260-950 were obtained resulting in LODs of 0.5 µg/l using UV detection.

An example of an application of membrane separation for clean up of non-aqueous extracts was presented by Carabias-Martínez et al. [121]. An ASTED system (normally used for automated dialysis) was equipped with a nonporous silicon membrane. Pesticides in a fat-containing egg extract in hexane, being the donor phase, were extracted into a stagnant aqueous phase (methanol/water 70/30). Macromolecules (fat) remained in the donor phase. Acetonitrile (90% in water) was also investigated and showed very favourable extraction yields. It was not used because of co-extraction of compounds interfering with UV detection of some of the analytes. The set up could be interesting for automation of the classical hexane/acetonitrile liquid-liquid partitioning for fat removal when more selective detection techniques (MS) would be used.

4.2.2. SPE-HPLC

In contrast to GC where on-line coupling of SPE is virtually not used for food toxicant analysis, coupling of SPE to LC for automated clean up has been described in a number of papers.

For fully automated SPE-LC a key requirement is that the extract obtained after SPE is compatible (pH, salts, modifier content) with the subsequent LC separation. In contrast to off-line automated SPE, it is not possible (or at least not straightforward) to carry out a solvent switch. Since reversed phase LC is most commonly used, this means the SPE is also performed in the reversed phase mode and that applications involve aqueous samples or extracts in an aqueous or water-miscible solvent. Usually, the analytes are trapped on the SPE cartridge, then the interfering matrix is removed by rinsing with appropriate solvents, and then the analytes are eluted and transferred to the analytical column.

Fully automated SPE-LC can be performed by workstations, like the ASPEC mentioned in section 3.3. After collection of the SPE elute in a vial, the system injects an aliquot into the LC. Before injection, the system can carry out additional procedures like dilution with water to lower the modifier content of the extract, i.e. to make the extract composition compatible

with the HPLC conditions in order to prevent band broadening. Certain pre-column derivatisation procedures can also be automatically performed by such systems.

For true on-line SPE-LC several instrumental configurations can be used (see Figure 14). In its simplest form, the sample loop of the autosampler valve is replaced by the SPE cartridge and no additional hardware is required. Matrix is by-passing the column (discarded through the detector). In most cases, however, an additional valve and pump are required, which in terms of investment compare favorably to workstations, especially in cases where additional valves are already available on the autosampler or MS detector (e.g. the divert valve). Here, two configurations are possible, forward and backflush elution, the latter minimises band broadening of the transferred zone on the precolumn. In set ups as depicted in Figure 14 (B) and (C) it is possible to clean an extract during LC analysis, i.e. after transfer of the analytes from the SPE column to the HPLC column, the valve can be switched and the clean up for the next sample can be performed simultaneously. This way, the clean up adds little or no time to the HPLC analysis.

The main parameters to be optimised and investigated in the on-line systems are the composition of the loading, rinsing and elution phases and their volumes. These should be such that the analytes are retained (no breakthrough should occur) while matrix is a much as possible eliminated. Further, possible carry over should be investigated and, if present, solved by additional cleaning steps of the tubing, valves and SPE cartridge.

SPE-LC has been applied for well over 10 years. Here the focus is on the contributions from the last five years. Typical examples are included in the application table (Table 4). Earlier applications have been reviewed by Bovanová et al [7]. The currently wide spread use of MS/MS detection in LC has not eliminated the need for clean up, i.e. for prolonged life-time of the LC column, reducing MS maintenance, and reduction of matrix effects [14,15], automated SPE clean up procedures continue to be developed and successfully used.

The ASPEC workstation with automatic injection into the LC has been reported several times for the determination of mycotoxins. Brera et al. [122] isolated ochratoxin A from wine using immunoaffinity cartridges. After desorption with methanol, the eluate was diluted with water, mixed and then injected onto column. Using this sequential processing, the overall analysis time per sample was reduced from 70 to 45 min. Here, improvement in turnaround time was relatively limited, but the employment of personnel was strongly reduced: sample preparation of 20 samples took 8 hours with the manual method and only 90 min with the automated method. Dilkin et al [123] used the ASPEC for cleaning of extracts of corn and corn-based feed for the determination of fumonisins. After elution of the analytes, a derivatisation was automatically performed by transferring an aliquot of the eluate into a tube containing the reagents. The final method proved to be reliable and robust for routine analysis of over 1000 samples during 14 months. Chan et al. [124] developed a method for combined

determination of aflatoxins and ochratoxin-A using combined immunoaffinity columns. Automated clean up took 30 min during which HPLC analysis took place.

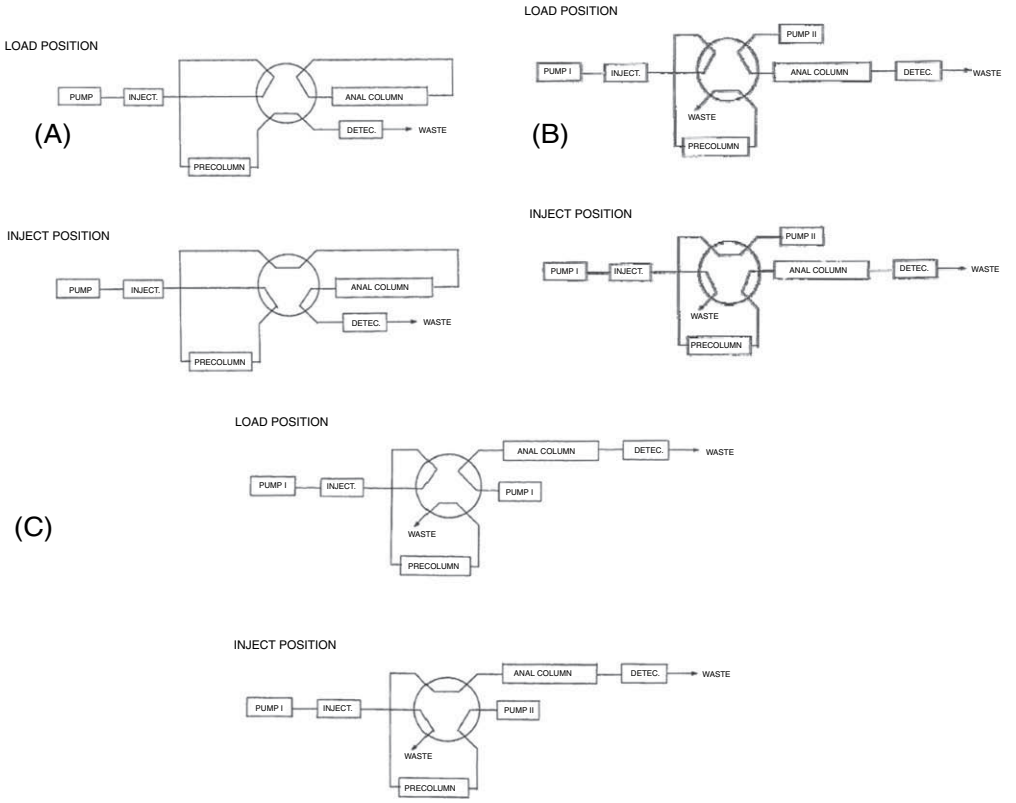


Figure 14. Instrumental configurations for on-line automated SPE-LC. (A) Replacement of sample loop by SPE cartridge, (B) With additional valve and pump, forward elution to LC, (C) with additional valve and pump, backflush elution to LC (Reproduced (modified) from [7] with permission from Elsevier© 2000).

Riediker et al. [125] used a device for automatic exchange of SPE cartridges (Prospekt) for clean up of flour, pear and tomato extracts for determination of chlormequat and mepiquat. Like with the ASPEC, a fresh cartridge is used for every sample, but here in a closed on-line configuration, i.e. the analytes are desorbed by the LC eluent and directly transferred to the LC column.

Table 4
Selected applications of semi- and fully automated clean up in food toxicant analysis

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
Pesticide residues							
Semi-automated clean up (a)							
OCP/pyrethroids	Fruits	Freeze-drying, extraction by shaking with hexane	SPE: 20 x 4 mm (50 mg) SiOH. Elut: 175 µl EtAc	Flow injection manifold	None (cleaning of SPE with 2-propanol)	GC-ECD [0.6-6 mg], 0.5-8 µg/kg	[142]
OCP/OPP	Butter, corn oil, soybean oil, olive oil	Mixing with hydromatrix	SFE coupled to 25 cm x 10 mm ID C1-column which is regenerated	ISCO SFX3560	Expansion in vial with hexane evaporation	GC-ECD, GC-FPD	[21]
OP pesticides	Wheat, maize	3.1-3.8 g, SFE, CO2, decompression on C18, elut. acetone	SPE : Envicarb 0.5 g/6 ml Elut ACN/toluene 3:1	ASPEC	evap. conc., add of acetone (2 ml)	GC-FPD 1 mg, 4-49 µg/kg	[143]
Acaricides	Honey	Dilution of 0.5 g sample in 5 ml buffer	SPE: C18 100 mg 4 ml extract, elut: 1 ml THF	ASPEC	Evap/reconst (0.1-0.2 ml)	LC-DAD (233-324 nm) 100-200 mg, 2-200 µg/kg	[144}
Pesticides	tobacco	7 g sample+hydromatrix, PLE, acetone Evaporation to 2 ml	OCP/DNA: SPE-1 Florisil, SPE-2 SiOH OPP/AA/OCP: SPE-1 SiOH, SPE-2 GCB N-methylcarbamates SPE-1 NH2, SPE-2 GCB Details see text	ASPEC (96 well)	None (1.6 ml) (0.8 ml) (0.5 ml)	GC-MS/MS 0.4-0.5 mg, 1-30 µg/kg LC-PCR-Flu (carbamates) 7 mg, 10-30 µg/kg	[35}
Fully automated clean up							
OP pesticides	Wine, apple juice	15 sample +5 g NaCl in vial, 800 µl Chex in membrane bag	Static membrane extraction, 50 min, 45°C	Multi purpose sampler (xyz sampler)	Transfer of organic phase into autosampler	LVI-GC-MS 0.001-0.023 µg/l	[69]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment vial	Analysis	Ref.
Pesticides	Wine	Dilution 1 :3 in water, filtration	MMLLE, porous polypropylene, acceptor phase = toluene	DualChrom 3000	LVI (230 µl)	GC-MS [8 ml], 0.3-3.5 µg/l	[70]
Pesticides	Wine	10-fold dilution	In-tube SPME (60 cm x 0.25 mm id , polypyrrole)	LC autosampler	none	LC-MS [0.045 mg], 0.1-4 µg/l	[114]
dithiocarbamates	Lettuce, black currant, onion	Putting sample + hydrolysis reagent in autosampler vial	Headspace analysis Preceded by automatic heating and agitation	Combi PAL	none	GC-ECD [50 mg], 20 µg CS_2/kg	[48]
Pesticides	lettuce	Turrax extraction, EtAc	In GC inlet: automatic replacement of liner with cup containing the extract	Focus DTD (automatic liner exchanger)	none	GC-TOF-MS [5 mg], < 10 µg/kg	[76]
Pesticides	grapes	Grinding of sample, loading PHWE extraction cel with sample and sand	PHWE-MMLLE, porous polypropylene, acceptor phase = toluene	Dualchrom 3000	LVI (150 µl)	GC-MS [50 mg], 0.3-1.8 µg/kg	[71]
Fungicides	Fruits	Turrax extraction with ace/dcm/pe and etac, evap. conc./solvent switch into ACN	LC (30 x 4 mm id C18) ACN/0.01 M NH4Ac (7/3)	ASPEC	Transfer of fraction to 2^{nd} LC column	LC (C18) -Flu [75 mg], < 10 µg/kg	[139]
Pesticides	Celeriac, carrot	Etac extraction	GC (15 m x 0.25 mm id 0.25 µm CP-Sill-5-CB)	modulator	Trapping, refocussing, reinjection	GCxGC-TOF-MS [0.5 mg], 6-46 µg/kg	[109]
Pesticides	Apple, peach	EtAc extr., GPC, evap/recon	GC (30 m x 0.25 mm id 0.25 µm DB-XLB)	modulator	Trapping, refocussing, reinjection	GCxGC TOF-MS [1 mg], 0.2-100 µg/kg	[110]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
Pesticides	Baby food	Extraction/partitioning into ACN (+optional dispersive SPE clean up)	In GC inlet: automatic replacement of liner with cup containing the extract	Combi PAL + DTD/DMI accessory	none	GC-MS [10 mg] < 10-100 µg/kg	[77]
N-methylcarbamates	Pear, apple, cucumber	Filling extraction cell with sample (5 g)	PHWE (75°C), filtration, SPE (50 x 4 mm id C18)	In-house build	Post column reaction detection (alkaline hydrolysis, OPA derivatisation)	LC-Flu [333 mg], not specified	[134]
N-methyl carbamates	Pear, apple, cucumber	Filling extraction cell with sample (5 g)	Ultrasound assisted extraction with water, filtration, SPE (50 x 4 mm id C18),	In-house build	Post column reaction detection (alkaline hydrolysis, OPA derivatisation)	LC-Flu [430 mg], 3-10 µg/kg	[145]
Pyrethroids	vegetables	Extraction with DCM, evap/reconst. in ACN/water (7/3)	ISRP column (50 x 4.6 mm id, 5 µm GFF-II) coupled to LC column, first dicard high molecular weight, then connect both columns	In-house built	none	LC-PIF-Flu [600 mg], 0.01-0.22 µg/kg	[129]
Chlormequat, mepiquat	Pear, tomato, wheat	Extraction by stirring with MeOH/H2O, centrifugation/filtration	SPE (10 x 2 mm ID SCX), 30 µl injection, rinsing cartridge, elution with 150 mM NH4COO in MeOH/H2O	Prospekt	none	LC(SCX)-MS/MS [30 mg], < 5 µg/kg	[125]
Glyphosate	cereals	Extraction with water, centrifugation, C18 SPE, derivatisation with FMOC, dilution	LC (30 x 4.6 mm C18), ACN/0.05 M phosphate buffer pH 5.5	ASPEC	Transfer of fraction to 2nd lc column	LC (NH2) – Flu [11 mg], 0.5 mg/kg	[138]
Pesticides	Milk,	Turrax extraction ace/PE	In GC inlet (laminar	Special liner	none	GC-ECD	[75]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
	avocado	Evap/recon, hex/ACN liquid-liquid partioning	cup liner)			[milk 25 mg, avocado 1 mg], LOD not specified	
Pesticides	Olive oil	filtration	RPLC (C4, 50 x 4.6 mm id), MeOH/water 7/3	In-house built	LVI (2200 µl) PTV interface	GC-FID [20 mg], 0.1-0.3 mg/kg	[94]
OP pesticides	Veg. oil	Dilution in GPC eluent	GPC (25 cm x 4.6 mm ID PLgel) meac/cpen/c9	DualChrom	LVI (1300 µl) On-column interface	GC-FPD [1 mg], 2 µg/kg	[86]
Pesticides	Cereals, peanuts	Turrax extraction EtAc/cyclohexane	GPC (2x 30 cm x 7 mm ID Plgel) etac/chex 1:1 GPC-SPE (GPC as above, SPE = 10 x 2 mm ID NH2	In-house built	LVI (20 µl) PTV interface	GC-MS [5-10 mg], 1-20 µg/kg	[89]
Organophosphorus pesticides	Orange oil	Dilution in hexane	NPLC (diol, 25 cm x 4.6 mm ID), hexane/isopropanol gradient	MultiPurpose Sampler (xyz robot)	LVI (500 µl) PTV injection	GC-NPD [2 mg]	[87]
Phenylurea	tobacco	Extraction with EtAc Evap/recon in ace/chex	GPC (30 cm x 7 mm ID Phenogel) ace :chex 1 :2	MultiPurpose Sampler (xyz robot)	LVI (1000 µl) PTV injection	GC-MS [1000 mg]	[87]
Pesticides	Eggs	Drying with Na2SO4, Soxhlet extr. with hexane, evap. conc.	Membrane separation, nonporous silicon sheet. Contineous donor phase (total 6 ml) = hexane extract, stagnant acceptor phase = 100 µl MeOH/H2O 7/3	Modified ASTED	none	LC-DAD [1920 mg], 2-18 µg/kg	[121]
Veterinary drug residues							
Semi-automated clean up							
nicarbazin	Eggs, meat, poultry feed	mixing Na2SO4 with sample in extraction cell with small spatula	SFE in-situ clean up, Na2SO4/1 g of sample/Na2SO4 packed	ISCO 260D	Add MeOH to mark	LC-UV (344 nm) 1 mg/400 µg/kg	[20]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
			onto 3 g Al2O3, expansion into methanol				
sulfonamides	meat, infant food	blending of homogenised sample with C18 (1:2)	PLE/in situ clean up, super heated water	ACE200	freezing out of co-extracted fat/matrix	LC-MS/MS 10 mg/0.4-2.6 µg/kg	[29]
corticosteroids	bovine liver	grinding with hydromatrix	PLE, fat removal by pre-extraction with hexane	ASE200	Evaporation to dry Solvent switch	LC-MS/MS 50 mg, 1 µg/kg	[27]
anabolic steroids	kidney fat	Melting the fat	PLE, Al2O3 packing, fat removal by pre-extraction with hexane, then desorption of steroids from packing with ACN	ASE200	Freezing out remaining fat in ACN extract, solvent switch, clean up on C18 SPE, solvent switch	LC-MS/MS [1000 mg] 0.3-0.9 µg/kg	[28]
Anabolic steroids	Urine	5 ml, centrifugation, enzymatic hydrolysis	SPE-1: 5 ml urine, C18 0.5g/3ml, elution 3 ml MeOH/EtAc SPE-2 : 3 ml extract, NH2	ASPEC	Evap/reconst. (0.1 ml)	LC-MS/MS 4.5 ml, CC_{α} = 0.2-0.5 µg/l	[146]
Malachite green, leuco-MG	Fish	5 g + 5 ml acetate buffer, maceration shaking with ACN partitioning into DCM evap./solvent switch to ACN/MeOH/DCM	SPE-1: filter through Al2O3 1 g/6 ml SPE-2: propylsulfonic acid, 0.5 g/3 ml Elut: amm. Acetate/ACN	ASPEC	Addition of ascorbic acid and LC eluent (3 ml)	LC-post column oxidation-UV (600 nm) 250 mg, 0.6-0.8 µg/kg LC-post column oxidatio - MS/MS 17 mg, 1-2.5 µg/kg	[147]
Carbadox and	Liver	5 g, enzymatic hydrolysis	SPE: SCX, 1 g/3 ml	ASPEC	+HCl, LLE,	LC-MS/MS	[148]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
olaquidox metabolites		overnight, LLE, acid/base partioning	Elut: NaOH/MeOH		evap./reconst. (0.1 ml)	375 mg, CC_α = 0.4-0.7 µg/kg	
Polyether ionophore antibiotics	Liver, eggs	5 g, extraction with 87% MeOH	SPE : C18 100 mg, Elut : MeOH	ASPEC	none	LC-MS/MS [50 mg], 0.03 µg/kg	[149]
benzimidazoles	Liver	3g, extraction with EtAc basic cond., evap/recon EtOH/HCl, LLE hex (fat removal) dilution with K2HCO3	SPE : C18, 0.5 g/3 ml Elut. EtAc	ASPEC	evap/reconst (0.4 ml)	LC-DAD (298 nm) 186 mg, 116-1303 µg/kg (CC_α = MRL+12-25%)	[150]
Quinolones	Feed	1 g, PLE H2O/ACN pH 2.6; evap. of ACN, filtration	SPE : HLB cartr. Elut. MeOH	ASPEC	Evap/reconst. (5 ml)	LC-DAD (278 nm), Flu 4 mg, 1000 µg/kg	[151]
Quinolones/ fluoroquinolones	Fish, seafood	Extraction with ACN, evap. Conc.	SPE-1 : ENVI Chrom P, 'filter through' SPE-2 : AG MP-1 resin (SAC)	ASPEC	none	LC-MS/MS [5 mg], 1-3 µg/kg	[152]
Fully automated clean up							
sulfonamides	Milk	Addition of ethanol, Na2HPO4, centrifugation	In-tube SPME (15 cm x 0.25 mm id, monolythic poly MAA-EFDMA)	LC autosampler	None	LC-UV [640 mg], 2-22 µg/l	[113]
Sulphonamides	Milk		Hollow dialysis fiber (cellulose), stagnant donor (5 µl), acceptor 1 µl/min 1 M NaCl, pH 6.5 (15 min)	In-house built, (acceptor flow through LC autosampler loop)	None	LC-UV [0.002-0.15 µg/kg	[119]
Sulfonamide and metabolites	Bovine milk	Extraction with EtOH/HAc (97/3), centrifugation	none	HPLC	None	HPLC using Hisep Shielded Hydrophobic Phase (SHP) column, DAD	[153]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
sulfonamides	Bovine milk	Extraction with EtOH	ultrafiltration	centrifuge	Non	[15 mg], 9-25 µg/kg LC-DAD [25 mg] 3-5 µg/kg	[154]
sulfonamides	Bovine kidney	Vortex/ultrasonic extraction with MeOH, centrifugation	SPE (50 x 1 mm id Oasis HLB), 100 µl injection, rinsing with formic acid, desorption by gradient	HPLC system with SPE column instead of LC column	Splitting of eluate (10% to MS)	SPE-MS/MS [5 mg], 5-13 µg/kg	[135]
sulphonamides	Pork muscle	Blending of sample with saline solution	Dialysis, cellulose membrane. Stagnant donor phase = sample in buffer (100 µl), contineous acceptor = buffer (total 2 ml), SPE C18	ASTED	None	LC-UV [50 mg], 40 µg/kg	[118]
sulfonamides	Honey	Dissolving in 1 M HCl, acid hydrolysis by shaking, filtration	SPE (20 mm x 2.1 mm id Oasis HLB)	Discard valve of MS	None	SPE-MS/MS [20 mg], 0.5-2 µg/kg	[131]
Tetracylines	Eggs	Dilution with buffer	Dialysis, cellulose membrane. Stagnant donor phase = sample in buffer (740 µl), contineous acceptor = buffer (total 5 ml), SPE 20x4 mm PLRP-S	Modified ASTED	None for UV detection, adjustment of pH for fluorescence detection	LC-UV, LC-Flu [220-370 mg], 11-15 µg/kg	[116]
Fluoroquinolones	Milk	Centrifugation, cooling to solidify fat, proceed with skim milk	SPE = immuno affinity, elution with 2% acetic acid onto LC column	Micro analytical workstation	Refocussing on head LC column	LC-Flu [100 mg], 0.3-0.8 µg/kg	[132]
fluoroquinolones	Bovine milk	Deproteination with HPO4, centrifugation,	SPE (30 x 2 mm id Oasis HLB)	LC autosampler (replacing loop	None	LC-Flu [not specified],	[128]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
		filtration		by SPE column)		16-27 µg/l	
fluoroquinolone	Chicken liver	Turrax extraction/deproteination/ debinding (ACN, NH4OH) using, defatting by LL portioning H2O/ACN:DEE/hex	Dialysis, cellulose membrane. Stagnant donor phase = aqueous phase from extraction (370 µl), contineous acceptor = phosphate buffer pH 5 (3x650 µl). Two cycles for each sample were concentrated on SPE C18 5.8 x 4.6 mm id.	ASTED	None	LC-Flu [165 mg] 0.2 µg/kg	[117]
Environmental contaminants							
Semi automated clean up							
PCBs	mussel, bivalves, fish, eggs of spoonbill	grinding/mixing with Florisil	PLE/in situ clean up, Florisil packing	ASE200	concentration (rotary evaporation)	GC-MS/MS 20 mg, 0.002/0.07 µg/kg GC-ECD 60 mg, 0.001-0.03 µg/kg	[24]
PCBs	Feed	grinding/mixing with Na2SO4	PLE/in situ clean up, sulfuric acid-silica packing	ASE300	concentration (rotary evaporation)	GC-µECD 0.25 mg (fat/oil) 2.5-5 mg (feed) 1-10 µg/kg	[23]
PCBs	fish meal, cod liver oil	Grinding of sample with sand/Na2SO4	PLE/in situ clean up, 2 g fish meal, 0.5 g oil several sorbents, see text	ASE200	concentration	GC-MS (SIM) 2 mg fish meal 0.5 mg oil	[22]
Organohalogen compounds (PBDE, PCN, PCB, PCB metabolites, OCP, TBBPA, PCP)	Fat, kidney, liver, heart	PLE, 5 g (fat 1 g), DCM/ace, evap; LLE, evap; GPC, evap	SPE: SiOH, 1 g/3 ml A: 5% DCM/hex (PBDE, PCB, PCN, OCP) B: 10% MeOH/DCM (PCB metabolites,	RapidTrace	A: evap. (0.2 ml) B: deriv. Diazomethan, evap	GC-NCI (SIM) 50 mg (fat: 10 mg) < 1 µg/kg (fat: < 5 µg/kg)	[155]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
			OCP, PCP, TBBPA)		(0.2 ml)		
PBDE, PCB, PBB	Breast milk	Filling SPE –1 cartridge with diatomaceous earth and 1 g of milk	SPE-1: drying with N2 Elution through SPE-2: 0.1 g SiOH/1g SiOH-Sulf.acid with hexane	RapidTrace 20 sample/11.5 h 20 extracts/2.5 h	Evap. (10 µl)	GC-HRMS 100 mg 0.004-0.12 µg/kg (0.1-1.2 µg/kg fat)	[156]
Perfluoro compounds	Breast milk (serum)	Mixing of 1 ml sample with 3 ml 0.1 M formic acid	Oasis-HLB 60 mg/3 ml Elut: 1 ml 1% NH4OH/ACN	RapidTrace (100 sample/4h)	Evaporation, addition of acetic acid/MeOH (0.3 ml)	LC-MS/MS 40 mg, 0.1-1 µg/kg	[157]
PCB, OC pesticides	Fish feed, fish	8 g, dry with Na2SO4 Reflux in hexane (feed: fat destruction by shaking with H2SO4 solution) evaporate	NPLC 150 x 3.9 mm ID 4 µm SiOH Fraction A PCB, part OCP Fraction B: other OCP Regeneration with EtAc	Fraction collector	Evap/conc (1 ml)	GC-MS/MS 8 mg, 0.1-1 µg/kg	[43]
Dioxins/furans, coplanar PCBs	Poultry, eggs, mackerel	Freeze-drying PLE, hexane Evap. to dry => 4 g fat Dissolve in 50 ml hexane	SPE-1: SiOH a/b/n 50 g SPE-2: SiOH a/b/n 7.5 g SPE-3: Al2O3 8 g SPE-4: carbon PX-21 2 g Details see text	PowerPrep	Evap of 60 ml toluene (4 µl)	GC-HRMS 2000 mg fat eq., 0.1 ng/kg fat	[36]
Dioxins/furans, coplanar PCBs	Milk	20-100 ml, Protein precipitation with ACN (1:1), dilution with water (1:1)	SPE-1: C18 20 g SPE-2: SiOH a/b/n 50 g SPE-3: carbon PX-21 2 g Details see text	PowerPrep	Evap of 60 ml toluene (4 µl)	GC-HRMS 10.000 mg/300 mg fat eq., 0.1 ng/kg fat	[37]
Fully automated clean up							
PCBs	Human milk	Extraction with MeOH/DEE/PE	NPLC (SiOH, 30 x 2.1 mm id), hexane	Dualchrom	LVI (240 µl) On-column interface	GC-ECD [4.5 mg fat], 1-5 ng/kg fat	[93]
OCPs	Human milk	Extraction with	NPLC (SiOH, 50 x 1	Dualchrom	LVI (240 µl)	GC-ECD	[93]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
		MeOH/DEE/PE	mm id), hexane		On-column interface	[1.6 mg fat]	
PCBs/coplanar PCBs	Cod liver	Al2O3 clean up Evap conc. SiOH clean up	GC (30 m x 0.25 mm id 0.25 µm HP-1)	modulator	Trapping, refocussing, reinjection	GCxGC-µECD [?], abs. LOD 10-20 fg	[103]
Dioxins, coplanar PCBs	Fish	HCl treatment, extr. Hex, multi-step column chrom., incl. Carbon column	GC (60 m x 0.25 mm id 0.25 µm Rtx-dioxin)	modulator	Trapping, refocussing, reinjection	GCxGC-TOF-MS [?], abs. LOD 0.5 pg	[107]
Dioxins, coplanar PCBs	Milk fat, fish oil	extr. Hex, multi-step column chrom., incl. Carbon column	GC (30 m x 0.25 mm id 0.25 µm DB-XLB)	modulator	Trapping, refocussing, reinjection	GCxGC-µECD [100 mg fat], 3 ng/kg fat	[106]
Natural contaminants							
Semi-automated clean up							
Ochratoxin A	cereals	Extr. with ACN/water (4/6) (shaking 1 hr), filtration, dilution in PBS	Ochraprep immunoaffinity column, rinsing PBS, elut. 2 ml MeOH/HAc (98/2)	ASPEC	Evap/reconst (0.2 ml mobile phase), filtration	HPLC-Flu [160 mg] 0.1 µg/kg	[158]
Zearalenone	cereals	Extr. with ACN/water (4/6) (shaking 1 hr), filtration, dilution in PBS	Easi-extract immunoaffinity column, rinse with PBS, elut. 2 ml ACN	ASPEC	Evap/reconst (0.2 ml MeOH), filtration	HPLC-Flu [160 mg] 3 µg/kg	[158]
Fully automated clean up							
Ochratoxin A	Wine	Dilution with buffer, filtration	SPE = immuno affinity column, elution with 1.25 ml MeOH into vial	ASPEC	Dilution of eluate, injection into LC	LC-Flu [126 mg], 0.1 µg/l	[122]
Fumonisine B1, B2	Corn, corn based feed	Extraction with ACN/H2O (blender), dilution with water/pH adjustment	SPE = 300 mg C18, elution with 2 ml ACN/H2O 7/3	ASPEC	Derivatisation with OPA in vial, injection into LC	LC-Flu [4 mg], 30-50 µg/kg	[123]
Ephedrine alkaloids	Dietary supplements	Extraction by stirring with Ace/H2O 12 mM HCl (1 g in 100 ml)	SPE = 30 x 4.6 mm id SCX	LC autosampler (replacing loop by SCX column)	None	LC-DAD [0.2-5 mg], 6 mg/kg	[126]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
Aflatoxin, ochratoxin	Maize, peanut butter	20-36 g, turrax extraction with ACN/H2O, filtration, dilution with buffer	50 ml, "AflaOchra" immuno affinity column Elut: MeOH (2 ml)	ASPEC	Dilution with 2% acetic acid, injection of aliquot into LC	LC-PCR-Flu [not specified], 0.2 µg/kg	[124]
Ochratoxin A	Beer, wine	Degassing of beer, filtration	SPE (4 x 4 mm id C18), 200 µl injection, sorption/cleaning with 15%EtOH/H2O 10 mM formic acid, backflush elution to LC with 60% MeOH/H2O 10 mM formic acid	Discard valve of MS	None	LC-MS/MS [200 mg], 0.008-0.01 µg/l	[130]
cannabinoids	Hemp food products	50-500 mg + 1 ml NaOH + 0.5 g NaCO3	HS-SPME: 100 µm PDMS 25 min, 90°C	CTC combi-PAL (xyz sampler)	silylation	GC-MS 50-500 mg 10-170 µg/kg	[55]
Biogenic amines	Fish, chicken, wine	Blending with TCA, centrifugation, filtration, dilution (wine: dilution)	SPE (10 x 4.6 mm id C18) loading cartridge with 100 µl benzyol-Cl, then 10 ml of extract	HPLC with guard column for SPE	None	Micellar LC-UV [500 mg], 1-8 µg/kg	[127]
Acetaldehyde, acetone	Yoghurt, fruit juices	none	Pervaporation, 10 min at 80°C	Home-built	None	GC-FID [?], 30-300 µg/l	[57]
Processing and packaging contaminants							
Semi-automated clean up							
Acrylamide	Coffee (beverage)	none	SPE Multimode (C18-SAX-SCX), 300 mg/3 ml Collection of fraction 0.5-0.9 ml	ASPEC	None	LC-MS/MS, 10 mg, 2 µg/l	[159]
Phthalate metabolites	Breast milk	1 ml + H3PO4, enzym. deconjug. Dilution to 3 ml	SPE HLB 60 mg/3 ml Elut. 0.5 ml ACN	RapidTrace	Evap/reconst. (0.2 ml)	LC-MS/MS 100 mg, 0.1-1.9 µg/l	[160]
Fatty acid aniline	Vegetable	Dissolving in	SPE (10 x 2 mm ID	Prospekt, elution	Evap/reconst.	LC-MS/MS	[34]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
derivatives	oil	IPA/EtAc/Hexane	SCX), 200 µl injection, multiple rinsing, elution with Tol/IPA/ACN	into fraction collector		[1.6 mg], 200-400 µg/kg	
Fully automated clean up							
Residual solvents	Natural food additives	Liquids: none Solid: dissolve in suitable solvent	Static headspace Equil. 20 min, 70°C	HS-40 (Perkin Elmer), split injection	none	GC-FID 1 g, < 7.5-12.5 mg/kg	[49]
1,3-dichloropropanol (1,3-DCP)	Soy sauce	1.5 g sample + 1.5 g (NH4)2SO4 in headspace vial	Static headspace, Equil. 20 min, 80°C	HS2000 (Thermo), cryotrap refocussing	none	GC-MS 1.5 g, 3 µg/kg	[50]
Dimethyldisulfide, dimethyltrisulfide (off-flavors from irradiation of meat)	turkey	none	Dynamic headspace (Purge&trap) 15 min, 40°C, 40 ml He/min Tenax/charcoal trap	Solatek 72 (Tekmar) Cryotrap refocussing	none	GC-MS (LOD not specified)	[51]
Hexanal	Pork meat	Transfer of 1.5 g ground meat + 3 ml water in vial, heating in water bath (75°C) 30 min	HS-SPME 85 µm Carboxen/PDMS 20 min, room temp.	Varian autosampler in SPME mode	None	GC-FID [1.5 g], < 100 µg/kg	[56]
Furan	Baby food, coffee, canned food, fruit juice	For solid samples homogenisation under subambient or cryogenic conditions. Dilution with NaCl	HS-SPME: 75 µm carboxen/PDMS 20 min, 50 °C	CTC combi-PAL (xyz sampler)	Cryofocussing (-20°C)	GC-MS [500 mg], 0.03 µg/kg	[54]
Mineral oil	Feed, eggs, animal fat, oils	Melting/dissolving in pentane (oil/fat), extraction with pentane (feed, egg yolk after drying with Na2SO4)	NPLC (SiOH 2-4x 25 cm x 2 mm ID), pentane	Dualchrom	LVI (450-660 µl) Wire interface	GC-FID [22-30 mg], 2 mg/kg	[90]
ESBO	(Oily) food from jars	Transesterification in the food with	NPLC (cyano, 25 cm x 2 mm ID), 20%	Dualchrom	LVI (300 µl) On-column	GC-FID [0.2-0.4 mg], 2-5	[81]

Analyte	Matrix	Pretreatment	Automated clean-up	Device	Post treatment	Analysis	Ref.
		metoxide/methanol, extraction into MTBE/hexane	MTBE/pentane		interface	mg/kg	
Phthalates, alkylphenols, bisphenol-A	Food simulants (migration studies) Beverages Canned food	LLE with DEE, evap/recon Centrifugation Extr. ACN, ACN/hex partitioning, evap/recon	In-tube SPME (60 cm x 0.32 mm id, 12 µm Supel-Q PLOT)	LC autosampler	none	LC-DAD [160-800 mg], 0.1-4 µg/l (in standard solutions)	[115]
Phthalates, alkylphenols, bisphenol-A	Liquid medicines and intravenous solutions	none	In-tube SPME (60 cm x 0.32 mm id, 12 µm Supel-Q PLOT)	LC autosampler	none	LC-DAD [800 mg], 1-10 µg/l (in standard solutions)	[161]

(a) examples of off-line automated GPC are not included but is well established and widely used.
Matrix eq. = matrix equivalent injected into the chromatographic system
LOD = limit of detection
ACN = acetontrile, hex = hexane, ace = acetone, EtAc = ethyl acetate, PLE = pressurized liquid extraction, SFE = supercritical fluid extraction

In other on-line SPE-LC configurations, the same SPE column is used for clean up of a large number of samples. Automation is realised through the LC system, by replacing the loop by the SPE cartridge or by using an additional valve and pump. Examples where this set up was used include the determination of ephedrine alkaloids in dietary supplements [126], biogenic amines in fish, chicken, and wine [127], fluoroquinolones in milk [128], pyrethroids in vegetables [129]. Examples where the divert valve of the MS detector was used for automation were given by Bacaloni et al. (ochratoxin A in beer and wine [130]) and Thompson et al (sulfonamides in honey [131]).

Prevention of carry-over and deterioration of system performance is important in on-line SPE-LC and several authors reported the need for additional (automated) rinsing steps [132, 131, 128] or adjustment of the mobile phase pH (to prevent clogging of the SPE cartridge, [130]).

In several contributions other sample pre-treatment procedures were included in the on-line automated set up. A combined derivatisation/clean up was described by Paleologos et al. [133] for determination of biogenic amines. After conditioning of the SPE cartrigde, benzoyl-Cl was loaded onto the cartridge and then 10 ml of alkaline extract was passed through the SPE. The amines reacted with the benzoyl-Cl and remained on the cartridge. The NaOH was washed off to ensure compatibility with the HPLC analysis. Compared to batch techniques, sample loss and random errors were reduced, but the analysis time per sample was higher (3 samples/hour).

Parrilla Vazquez [129] determined pyrethroid pesticides in vegetables. After removal of higher molecular weight matrix by an internal surface reversed phase (ISRP) column, the analytes were separated on the analytical column and degraded into fluorescent products using post-column photolysis.

A highly automated, but highly complex set up was described for the determination of N-methylcarbamates [134]. Pressurized hot water extraction, filtration, SPE concentration of the aqueous extract, backflush desorption, LC separation and post-column derivatisation were all coupled and automated. Given the number of interrelated variables, a multivariate optimization was employed. Despite the high degree of automation, it is questionable whether the advantages of automation here justify the effort put in to the development.

On the other extreme, a very straightforward approach, the direct coupling of clean up with detection, has also been described. In two papers reporting on the determination of sulfonamides, in kidney [135] and honey [131], SPE was directly coupled to MS/MS detection, i.e. no LC separation was performed. In both cases the sulfonamides were trapped on an Oasis HLB SPE column, then the matrix was removed by rinsing after which the analytes were desorbed and transferred to the MS/MS detector. In the kidney application, the eluate was split 1:10 to the detector, all analytes co-eluted but could be individually detected due to the selectivity of MS/MS detection. In the honey application, the SPE column was

packed with 5 µm particles and separation of two sulfonamides having the same major MRM transition was possible. The system could be used without maintenance for 600 samples over a period of 6 months.

4.2.3. LC-LC (column switching)

The systems used for LC-LC are basically the same as described for SPE-LC in Figure 14. Here, an analytical column (rather then a low resolution cartridge) is used for a more efficient separation of analytes from interferences. At the same time this means, like in LC-GC and GC-GC, that the method is usually dedicated to one or a few analytes that are cut from the first dimension and transferred to the second analytical column. Traditionally, LC-LC was typically used to compensate for limitations in selectivity which, besides the relatively low separation power of LC, was due to the non-specific detection (UV, or fluorescence in case co-derivatisation of matrix constituents). With the increased use of MS/MS as detector for HPLC, these limitations have largely disappeared, and so have the number of applications described in literature for food toxicant analysis, despite the fact that LC-LC can be a very effective way to eliminate matrix-induced suppression of ionization in MS detection [136].

The majority of applications stem from before 2000 and for a more extensive overview of examples the reader is referred to earlier reviews [6,7]. Both Hogendoorn and co-workers and Hernandez and co-workers have many contributions on LC-LC for pesticide residues in several matrices, including food, and also presented an overview of the versatility of the technique in general [137]. A nice example of an application of LC-LC is the determination of glyphosate in cereals [138]. Derivatisation of glyphosate with FMOC to increase LC retention and enable fluorescence detection, also resulted in a large number of intense interfering peaks. To solve this, 400 µl of the derivatised diluted extract was injected onto a C18 column using acetonitrile/phosphate buffer pH 5.5 as eluent. The glyphosate derivative showed little retention on this column and the appropriate 300 µl fraction was transferred to a second analytical column (NH_2) where it was retained and could be separated from the remaining interferences. An example of a more recent application is the determination of o-phenylphenol and bitertanol in oranges and bananas with LC-LC-Fluorescence [139]. In both examples mentioned above, an ASPEC system was used for controlling the flows and switching valves.

4.2.4. LCxLC

Comprehensive multi-dimensional LC is being used for characterization of sample constituents on a large or global scale [140]. Its main application lies in the field of protein and peptide mapping (i.e. proteomics) but there are also applications in food analysis (triacylglycerol profiling [141]). Unlike GCxGC, applications for food toxicant have not been reported. For target analysis the usefulness of LCxLC does not seem to be very obvious (or it

would be the reduction of matrix effects in multi-residue/contaminant analysis). For screening for unknown toxicants, the technique offers great potential but this has yet to be explored.

5. Concluding remarks

In Table 4 an overview is given of selected applications of automated sample clean up in food toxicant analysis. The applications are categorized by the type of residue/contaminant since analyses are still basically focusing on certain groups (pesticides or mycotoxins or PAHs, or veterinary drugs etc) rather than combining such methods which for many food products would certainly be relevant and beneficial. In the table, semi automated and fully automated clean up/chromatographic analysis are distinguished. The extend to which clean up is required depends on the matrix, the target LOD and the technique for final separation and detection and these items are included in the Table as much as possible. To put things in perspective, the amount of matrix equivalent introduced onto the analytical column is also reported in the table.

Automation of clean up procedures in food toxicant analysis can considerably increase sample throughput and reduce costs of analysis, although a cost-benefit analysis needs to be made for each situation. Method performance often improves since all steps can be carried out in a very reproducible manner and the reduction in manual sample handling is beneficial for the integrity of the sample(extract). Once implemented and thoroughly tested and validated, it has been demonstrated by several authors that automated systems can be used successfully for a long time and the analysis of thousands of samples.

Some of the well established methods, like off-line automated GPC and headspace analysis continue to be widely used. Other techniques like on-line dialysis and HPLC column switching seem to have lost attractiveness in today's practice. The latter can be explained by the improvement in selectivity and sensitivity of detection in HPLC through MS/MS. Another well established technique, SPE, currently remains to be the most popular procedure in automated clean up, both off-line through workstations, as well as in on-line configurations with HPLC. The commercial availability of a range of workstations and the ease of building-in a SPE cartridge in an existing HPLC set up certainly have contributed to this.

A variety of miniaturized and automated extraction/clean up methods have emerged over the past decade (SPME, membrane based techniques), but in many cases they still suffer from limited applicability (aqueous samples/extracts, only non-polar analytes), troublesome quantification due to influence of matrix on extraction efficiency and/or relatively long extraction times. From these techniques, HS-SPME (preferably with stable isotope internal standards) is a valuable tool for specific applications, as an alternative for headspace analysis.

Another relatively new development is the increasing use of PLE. With in-situ clean up in the extraction cell, an efficient combined extraction/clean up can be performed automatically.

Automation of clean up connected to GC is mostly facilitated through versatile xyz autosampler robots which can also perform additional procedures like heating, agitation and derivatisation. GPC and LC coupled on-line to GC still are routinely used by a happy few only, although improvements are being made that simplify transfer into the GC.

Comprehensive chromatography, especially GCxGC, has been demonstrated to offer potential for the analysis of complex mixtures of halogenated hydrocarbons.

Despite the advances in separation and detection of the chromatographic systems, clean up remains important for obtaining reliable quantitative data. The need for speeding up the analysis and reducing cost will also remain. Automation is one way of achieving that goal, as is evidenced by the large number of recent applications and the on-going presentation of new approaches.

References

[1] R.M. Smith, J. Chromatogr. A 1000 (2003) 3.
[2] P.L. Buldini, L. Ricci, J.L. Sharma, J. Chromatogr. A 975 (2002) 47.
[3] D.T. Rossi, N.Y. Zhang, J. Chromatogr. A 885 (2000) 97.
[4] R.F. Venn, J. Merson, S. Cole, P. Macrae, J. Chromatogr. B 817 (2005) 77.
[5] M.J. Shepherd in "Progress in food contaminant analysis, J. Gilbert (Ed), Blackie Academic & Professional, London, 1996, Ch. 2 p 32-64.
[6] A.A.M. Stolker, S.S. Sterk, L.A. van Ginkel in “Residue analysis in Food, Principle and applications”, M O'Keeffe (Ed), Harwood Academic Publ., Amsterdam, Ch. 4 p 73-105.
[7] L. Bovanova, E. Brandsteterova, J. Chromatogr. A 880 (2000) 149.
[8] M.C. Hennion, V. Pichon, J. Chromatogr. A 1000 (2003) 29.
[9] E.C. Goosens, D. de Jong, G.J. de Jong, U.A.Th. Brinkman, Chromatographia 47 (1998) 313.
[10] T. Hyotylainen, M.L. Riekola, Anal. Bioanal. Chem. 378 (2004) 1962.
[11] L. Ramos, J.J. Ramos, U.A.T. Brinkman, Anal. Bioanal. Chem. 381(2005) 119.
[12] J. O´Reilly, Q. Wang, L. Setkova, J.P. Hutchinson, H.L. Lord, C.M. Linton, J. Pawliszyn, J. Sep. Sci. 28 (2005) 2010.
[13] J.F. Focant, C. Pirard, E. de Pauw, Talanta 63 (2004) 1101.
[14] J. Hajslova, J. Zrostlikova, J. Chromatogr. A 1000 (2003) 181.
[15] J.-P. Antignac, K. de Wasch, F. Monteau, H. de Brabander, F. Andre, B. le Bizec, Anal. Chem. Acta 529 (2005) 129.
[16] M. Anastassiades, S. Lehotay, D. Stajbaher, F.J. Schenck, J. AOAC 86 (2003) 412.
[17] V. Camel, Analyst 126 (2001) 1182.
[18] M. Zougagh, H. Redigolo, A. Rios, M. Valcarcel, Anal. Chim. Acta 525 (2004) 265.

[19] R. Carabias-Martinez, E. Rodrigez-Gonzalo, P. Revilla-Ruiz, J. Hernandez-Hernandez, J. Chromatogr. A 1089 (2005) 1.
[20] D.K. Matabudul, N.T. Crosby, S. Sumar, Analyst 124 (1999) 499.
[21] M.L. Hopper, J. Chromatogr. A 840 (1999) 93.
[22] E. Bjorklund, A. Muller, C. von Holst, Anal. Chem. 73 (2001) 4050.
[23] S. Sporring, E. Bjorklund, J. Chromatogr A. 1040 (2004) 155.
[24] J.L. Gomez-Ariza, M. Bujalance, I. Giraldez, A. Velasco, E. Morales, J. Chromatogr. A 946 (2002) 209.
[25] Th. Bernsmann, P. Fuerst, Organohalogen Compounds 60 (2003) 408.
[26] S. Sporring, K. Wiberg, E. Bjoerklund, P. Haglund, Organohalogen Compounds 60 (2003) 1.
[27] R. Draisci, C. Marchiafava, L. Palleschi, P. Cammarata, S. Cavalli, J. Chromatogr. B 753 (2001) 217.
[28] H. Hooijerink, E.O. van Bennekom, M.W.F. Nielen, Anal. Chim. Acta 483 (2003) 51.
[29] A. Gentili, D. Perret, S. Marchese, M. Sergi, C. Olmi, R. Curini, J. Agric. Food Chem. 52 (2004) 4614.
[30] R.M. Smith, J. Chromatogr. A 975 (2002) 31.
[31] R.C. Tindle, D.L. Stalling, Anal. Chem. 44 (1972) 1768.
[32] F. Behre, W. Jodl, GIT Labor Fachzeitschrift 44 (2000) 409.
[33] G.G. Rimkus, M. Rummler, I. Nausch, J. Chromatogr. A 737 (1996) 9.
[34] R.E. Calaf, J. Pena, S. Paytubi, M. Carrascal, M. Posada, E. Gelpi, J. Abian, J. Agric. Food Chem. 49 (2001) 5085.
[35] J. Haib, I. Hofer, J.M. Renaud , J Chromatogr A. 1020 (2003) 173.
[36] J.-F. Focant, G. Eppe, C. Pirard, E. de Pauw, J. Chromatogr. A 925 (2001) 207.
[37] J.-F. Focant, E. de Pauw, J. Chromatogr. B 776 (2002) 199.
[38] C. Pirard, E. de Pauw, J.-F. Focant, J Chromatogr A. 998 (2003) 169.
[39] E. Daeseleire, R. Vandeputte, C.V. Peteghem, Analyst 123 (1998) 2595.
[40] E. Daeseleire, A. de Guesquiere, C.V. Peteghem, J. Chromatogr. Sci. 30 (1992) 409.
[41] C. van Poucke, M. van de Velde, C. van Peteghem, J. Mass Spectrom. 40 (2005) 731.
[42] G.R. van der Hoff, A.C. van Beuzekom, U.A.T. Brinkman, R.A. Baumann, P.J. van Zoonen, Chromatogr A. 754 (1996) 487.
[43] R. Serrano, M. Barreda, E. Pitarch, F. Hernandez, J. Sep. Sci. 26 (2003) 75.
[44] N.H. Snow, G.C. Slack, Trends in Anal. Chem. 21 (2002) 608.
[45] L.A. Pranoto-Soetardhi, M.A.H. Rijk, N. de Kruijf, R.H. de Vos, Int. J. Environm. Anal. Chem. 25 (1986) 151.
[46] J. Gilbert, J.R. Startin, C. Crews, Food Addit. Contam. 2 (1985) 55.
[47] B.D. Page, R.J. Avon, J. AOAC 72 (1989) 815.

[48] A. Royer, M. Menand, A. Grimault, P.Y. Communal, J. Agric. Food Chemistry 49 (2001) 2152.
[49] Y. Uematsu, K. Hirata, K. Suzuki, K. Iida, K. Kamata, Food Addit. Contam. 19 (2002) 335.
[50] C. Crews, G. LeBrun, P.A. Brereton, Food Addit. Contam. 19 (2002) 343.
[51] K.C. Nam, J. Cordray, D.U. Ahn, J. Agric. Food Chem 52 (2004) 1735.
[52] V.G. Zuin, A.L. Lopes, J.H. Yariwake, F. Augusto, J. Chromatogr. A 1056 (2004) 21.
[53] M.A. Stack, S. O'Connell, K.J. James, Anal Bioanal Chem. 373 (2002) 147.
[54] T. Goldmann, A. Perisset, F. Scanlan, R.H. Stadler, Analyst. 130 (2005) 878.
[55] D.W. Lachenmeier, L. Kroener, F. Musshoff, B. Madea, Anal. Bioanal. Chem. 378 (2004) 183
[56] L.N. Fernando, E.P. Berg, I.U. Grun, J. Food Comp. Analysis 16 (2003) 179.
[57] E .Priego-Lopez, M.D. Luque de Castro, J. Chromatogr. A 976 (2002) 399.
[58] E. Psillakis, N. Kalogerakis, Trends in Anal. Chem. 22 (2003) 565.
[59] K.E. Rasmussen, S. Pedersen-Bjergaard, Trends Anal. Chem. 23 (2004) 1.
[60] J. Yu, C. Wu, J. Xing, J. Chromatogr. A 1036 (2004) 101.
[61] C. Basheer, H.K. Lee, J Chromatogr A. 1047 (2004)189.
[62] L. Cai, J. Xing, L. Dong, C. Wu, J. Chromatogr. A 1015 (2003) 11
[63] N. Fidalgo-Used, G. Centineo, E. Blanco-Gonzalez, A. Sanz-Medel, J Chromatogr A. 1017 (2003) 35.
[64] M. Azenha, M.T. Vasconcelos, J Agric Food Chem. 50 (2002) 2713.
[65] J.Y. Liu, G.B. Jiang, J Agric Food Chem. 50 (2002) 6683.
[66] S. Tutschku, M.M. Schantz, S.A. Wise, Anal. Chem. 74 (2002) 4694.
[67] D.-R. Parkinson, I. Bruheim, I. Christ, J. Pawliszyn, J. Chromatogr. A 1025 (2004) 77.
[68] J.A. Jonsson, L. Mathiasson, J Chromatogr A. 902 (2000) 205.
[69] M. Schellin, B. Hauser, P. Popp, J. Chromatogr. A 1040 (2004) 251.
[70] T. Hyotylainen, K. Luthje, M. Rautiainen-Rama, M.-L. Riekola, J. Chromatogr. A 1056 (2004) 267.
[71] K. Luthje, T. Hyotylainen, M. Rautiainen-Rama, M.-L. Riekkola, Analyst 130 (2005) 52.
[72] R. Batlle, C. Nerin, J. Chromatogr. A 1045 (2004) 29.
[73] K. Grob, M. Biedermann, A.M. Giuffre, Z. Lebensm. Unters. Forsch. 198 (1994) 325.
[74] M. Biedermann, K. Fiselier, K. Grob, J. Sep. Sci. 28 (2005) 1550.
[75] M. Zehringer, Food Addit. Contam. 18 (2001) 859.
[76] K. Patel, R.J. Fussell, D.M. Goodall, B.J. Keely, Analyst 128 (2003) 1228.
[77] T. Cajka, K. Mastovska, S.J. Lehotay, J. Hajslova, J. Sep. Sci. 28 (2005) 1048.
[78] J. Teske, W. Engewald, Trends in Anal. Chem. 21 (2002) 584.
[79] T. Hyotylainen, M.-L. Riekola, J. Chromatogr. A 1000 (2003) 357.

[80] K. Grob, J. Chromatogr. A 892 (2000) 407.
[81] A. Fankhauser-Noti, K. Fiselier, S. Biedermann-Brem, K. Grob, J. Chromatogr. A 1082 (2005) 214.
[82] H.G.J. Mol, H-G Janssen, C.A. Cramers, U.A.Th. Brinkman, J. High Res. Chromatogr. 18 (1995) 19.
[83] H.G.J. Mol, P.J.M. Hendriks, H-G Janssen, C.A. Cramers, U.A.Th. Brinkman, J. High Res. Chromatogr. 18 (1995) 124.
[84] J.A. van Rhijn, L.G.M.Th. Tuinstra, J. Chromatogr. 552 (1991) 517.
[85] K. Grob, I. Kälin, J. Agric. Food Chem. 39 (1991) 1950.
[86] GA Jongenotter, MAT Kerkhoff, HCM van der Knaap, BGM Vandeginste, J. High Resol. Chromatogr. 22 (1999) 17.
[87] F. David, A. Hoffmann, P. Sandra, LC-GC int. 12(9) 1999, 550-558.
[88] H. Kerkdijk, PCT Int. Applic. WO 2005/111600 A1, 24.11.2005
[89] H. Kerkdijk, H.G.J. Mol, B. Nagel, submitted for publication (2006)
[90] K. Grob, M. Vass, M. Biedermann, H.-P. Neukom, Food Addit. Contam. 18 (2001) 1.
[91] S. Moret, T. Populin, L.S. Conte, K. Grob, H.-P. Neukom, Food Add. Contam. 20 (2003) 417
[92] G.R. van der Hoff, R. Hoogerbrugge, R.A. Bauman, U.A.T. Brinkman, P. van Zoonen, Chromatographia 53 (2000) 433.
[93] G.R. van der Hoff, R.A. Baumann, P. van Zoonen, U.A.T. Brinkman, J. High Resolut. Chromatogr. 20 (1997) 222.
[94] R. Sanchez, A. Vazquez, J.C. Andini, J. Villen, J. Chromatogr. A 1029 (2004) 167.
[95] H.G. Janssen, W. Boers, H. Steenbergen, R. Horsten, E. Floter, J Chromatogr A. 1000 (2003) 385.
[96] S. de Koning, H.G. Janssen, M. van Deursen, U.A.T. Brinkman, J. Sep. Sci. 27 (2004) 397.
[97] S. de Koning, H.G. Janssen, U.A.T. Brinkman, J. Chromatogr A. 1058 (2004) 217.
[98] W. Bertsch, J. High Resolut. Chromatogr. 22 (1999) 647.
[99] L.R. Bordajandi, P. Korytar, J. de Boer, M.J. Gonzalez, J. Sep. Sci. 28 (2005) 163.
[100] Ph. Marriott, R. Shellie, Trends Anal. Chem. 21 (2002) 573.
[101] J. Dallüge, J. Beens, U.A.Th. Brinkman, J. Chromatogr. 1000 (2003) 69.
[102] P.Q. Tranchida, P. Dugo, G. Dugo, L. Mondello, J. Chromatogr. A 1054 (2004) 3.
[103] P. Korytar, P.E.G. Leonards, J. de Boer, U.A.T. Brinkman, J. Chromatogr. A 958 (2002) 203.
[104] L.R. Bordajandi, L. Ramos, M.J. González, J. Chromatogr. A 1078 (2005) 128.
[105] P. Korytar, C. Danielsson, P.E.G. Leonards, P. Haglund, J. de Boer, U.A.T. Brinkman, J. Chromatogr. A 1038 (2004) 189.

[106] C. Danielsson, K. wiberg, P. Korytár, S. Bergek, U.A.Th. Brinkman, P. Haglund, J. Chromatogr. 1086 (2005) 61.
[107] J.-F. Focant, E.J. Reiner, K. MacPherson, T. Kolic, A. Sjodin, D.G.-Jr. Patterson, S.L. Reese, F.L. Dorman, J. Cochran, Talanta 63 (2004) 1231.
[108] P. Korytar, P.E.G. Leonards, J. de Boer, U.A.Th. Brinkman, J. Chromatogr. A 1086 (2005) 29.
[109] J. Dalluge, M. van Rijn, J. Beens, R.J.J. Vreuls, U.A.T. Brinkman, J. Chromatogr. A 965 (2002) 207.
[110] J. Zrostilikova, J. Hajslova, T. Cajka, J. Chromatogr. A 1019 (2003) 173.
[111] H. Kataoka, Anal. Bioanal. Chem. 373 (2002) 31.
[112] C.G. Zambonin, Anal. Bioanal. Chem. 375(2004) 73.
[113] Y. Wen, M. Zhang, Q. Zhao, Y.Q. Feng, J. Agric. Food. Chem. 53 (2005) 8468.
[114] J.C. Wu, C. Tragas, H. Lord, J. Pawliszyn, J. Chromatogr. A 976 (2002) 357.
[115] H. Kataoka, M. Ise, S. Narimatsu, J. Sep. Sci. 25 (2001) 77.
[116] G. Zurhelle, E. Mueller-Seitz, M. Petz, J. Chromatogr. B 739 (2000) 191.
[117] E. Cohen, R.J. Maxwell, D.J. Donoghue, J. Chromatogr. B 724 (1999) 137.
[118] M. McGrane, M. O'Keeffe, M.R. Smyth, Anal. Lett. 32 (1999) 481.
[119] Th. C-C. Yang, I-L. Yang, L-J. Liao, J. Liq. Chromatogr. Relat. Techn. 27 (2004) 501.
[120] L. Zhu, K.H. Ee, L. Zhao, H.K. Lee, J. Chromatogr. 963 (2002) 335.
[121] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P.H. Paniagua-Marcos, J. Hernandez-Mendez, J. Chromatogr. A 869 (2000) 427.
[122] C. Brera, S. Grossi, B. de Santis, M. Miraglia, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 119.
[123] P. Dilkin, C.A. Mallmann, C.A.A. de Almeida, B. Correa, J. Chromatogr. A 925 (2001) 151.
[124] D. Chan, S.J. MacDonald, V. Boughtflower, P. Brereton, J. Chromatogr. A 1059 (2004) 13.
[125] S. Riediker, H. Obrist, N. Varga, R.H. Stadler, J. Chromatogr. A 966 (2002) 15.
[126] R.A. Niemann, M.L. Gay, J. Agric. Food Chem. 51 (2003) 5630.
[127] E.K. Paleologos, M.G. Kontominas, Anal. Chem. 76 (2004) 1289.
[128] C. Ho, D.W. Sin, H.P. Tang, L.P. Chung, S.M. Siu, J Chromatogr A. 1061 (2004) 123.
[129] P. Parrilla-Vazquez, M.D. Gil-Garcia, D. Barranco-Martinez, M. Martinez-Galera, Anal. Bioanal. Chem. 381 (2005) 1217.
[130] A. Bacaloni, C. Cavaliere, A. Faberi, E. Pastorini, R. Samperi, A. Lagana, J. Agric. Food Chem. 53 (2005) 5518.
[131] Th.S. Thompson, D.K. Noot, Anal. Chim. Acta 551 (2005) 168.
[132] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. AOAC 82 (1999) 607.
[133] E.K. Paleologos, M.G. Kontominas, Anal Chem. 76 (2004) 1289.

[134] M.C. Herrera, R.C. Prados-Rosales, J.L. Luque-Garcia, M.D. Luque de Castro, Anal. Chim. Acta 463 (2002) 189.
[135] N. van Eeckhout, J. Castro-Perez, C. van Peteghem, Rapid Commun. Mass Spectrom. 14 (2000) 2331.
[136] R. Pascoe, J.P. Foley, A.I. Gusev, Anal. Chem. 73 (2001) 6014.
[137] E. Hogendoorn, P. van Zoonen, F. Hernandez, LC-GC Europe, 16 (2003) 44.
[138] EA Hogendoorn, FM Ossendrijver, E Dijkman, RA Baumann, J. Chromatogr. A 833 (1999) 67.
[139] T. Zamora, C. Hidalgo, F.J. Lopez, F. Hernandez, J. Sep. Sci. 27 (2004) 645.
[140] C.R. Evans, J.W. Jorgenson, Anal. Bioanal. Chem. 378 (2004) 1952.
[141] L. Mondello, P.Q. Tranchida, V. Stanek, P. Jandera, G. Dugo, P. Dugo, J. Chromatogr. A 1086 (2005) 91.
[142] A Colume, S Cardenas, M Gallego, M Valcarcel, J. Agric. Food Chem. 49 (2001) 1109.
[143] K.N.T. Norman, S.H.W. Panton, J. Chromatogr. A 907 (2001) 247.
[144] E. Korta, A. Bakkali, L.A. Berrueta, B. Gallo, F. Vincente, J. Chromatogr. A 930 (2001) 21.
[145] A. Caballo-Lopez, M.D. Luque de Castro, J. Chromatogr. A, 998 (2003) 51.
[146] S.A. Hewitt, M. Kearney, J.W. Currie, P.B. Young, D.G. Kennedy, Anal. Chim. Acta 473 (2002) 99.
[147] K. Halme, E. Lindfors, K. Peltonen, Food Addit. Contam. 21 (2005) 641.
[148] M.J. Hutchinson, P.B. Young, D.G. Kennedy, J. Chromatogr B 816 (2005)15.
[149] J. Rosen, Analyst, 126 (2001) 1990.
[150] G. Dowling, H. Cantwell, M. O'Keeffe, M.R. Smyth, Anal. Chim. Acta 529 (2005) 285.
[151] I. Pecorelli, R. Galarini, R. Bibi, A. Floridi, E. Casciarri, A. Floridi, Anal. Chim. Acta 483 (2003) 81.
[152] L. Johnston, L. Mackay, M. Croft, J. Chromatogr. A 982 (2002) 97.
[153] K. Kishida, N. Furusawa, J. Chromatogr. A 1028 (2004) 175.
[154] N. Furusawa, J. Chromatogr. A 898 (2000) 185.
[155] K. Saito, A. Sjodin, C.D. Sandau, M.D. Davis, H. Nakazawa, Y. Matsuki, D.G. Jr. Patterson, Chemosphere 57 (2004) 373.
[156] A. Sjodin, E.E. McGahee, J.-F. Focant, R.S. Jones, C.R. Lapeza, Y.L. Zhang, D.G.-Jr. Patterson, Anal. Chem. 76 (2004) 4508.
[157] Z. Kuklenyik, J.A. Reich, J.S. Tully, L.L. Needham, A.M. Calafat, Environ Sci Technol. 38 (2004) 3698.
[158] M. Eskola, M. Kokkonen, A. Rizzo, J. Agric. Food Chem. 50 (2002) 41.
[159] K. Granby, S. Fagt, Anal. Chim. Acta 520 (2004) 177.

[160] A.M. Calafat, A.R. Slakman, M.J. Silva, A.R. Herbert, L.L. Needham, J. Chromatogr. B 805 (2004) 49.
[161] K. Mitani, S. Narimatsu, F. Izushi, H. Kataoka, J. Pharm. Biomed. Anal. 32 (2003) 469.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 12

Gas chromatography–mass spectrometry (GC–MS)

Jana Hajšlová, Tomáš Čajka

Institute of Chemical Technology, Faculty of Food and Biochemical Technology, Department of Food Chemistry and Analysis, Technická 3, 166 28, Prague, Czech Republic

1. Introduction

Gas chromatography (GC) is undoubtedly one of the key techniques used for screening / identification / quantification of many groups of non-polar and/or semi-polar food toxicants (or their GC amenable derivatisation products). The high attainable separation power (potential number of theoretical plates) in combination with a wide range of the detectors employing various detection principles to which it can be coupled makes GC an important, often irreplaceable tool in the analysis of (ultra)trace levels of toxic food components that may occur in such complex matrices as foods and feeds.

In practice, the methods used for analyses of food toxicants typically consist of basic steps as shown in Figure 1: (*i*) isolation from a representative sample (extraction step); (*ii*) separation from bulk co-extracted matrix components (clean-up step); (*iii*) identification and quantification (determinative step). The last step is often optionally followed by (*iv*) confirmation of results.

Although the main accent of this chapter is focused on the optimisation of operations involved in the final phase of food toxicants analysis, *i.e.*: (*i*) introduction of (purified) sample extract onto the GC column, (*ii*) separation of its components on an analytical column and (*iii*) detection of target analytes by currently the most commonly used device—mass spectrometric (MS) detector; it should be noted that the sample preparation practice plays a crucial role in the obtaining required parameters of particular analytical method. In any case, all the bulk, non-volatile co-isolated matrix components such as lipids (waxes, triacylglyceroles, phospholipids, *etc.*), various pigments (chlorophylls, carotenoids, melanoidines, *etc.*) and other higher molecular weight components (*e.g.* plant resins) should be removed from crude extract prior the GC analysis to avoid various problems. Not only the method performance characteristics such as detection limits, accuracy and ruggedness, but also its speed, labour demands and (consequently) the cost of an analytical procedure depend on selected clean-up strategy and its efficiency. Since the complete removing of all the matrix components is not a realistic concept, the discussion of matrix effects, the issue closely associated with sample handling practice, is involved as an opening part in this chapter.

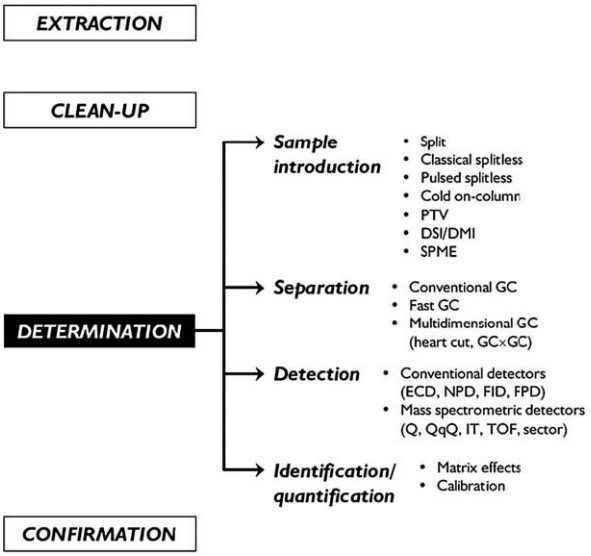


Figure 1. Basic steps typically involved in GC analysis of organic food toxicants, alternative approaches applicable in determinative step shown in a greater detail.

2. Matrix effects

Under the real-world conditions, specifically when multiple analytes possessing a wide range of physico-chemical properties are to be analysed in a single run, *i.e.* on condition when the selectivity of sample preparation step has to be compromised with obtaining good recoveries, some residues of matrix co-extracts unavoidably penetrate into the purified sample prepared for examination by GC analysis. Supposing trace levels of target toxicants are to be determined, various difficulties, both at the injector and detector site, may occur due to the presence of these interferents. Inaccurate quantification, decreased method ruggedness, low analyte detectability, and even reporting of false positive or negative results are the most serious matrix-associated problems which can be encountered [1].

Matrix-induced chromatographic response enhancement, first described by Erney *et al.*, is presumably the most discussed matrix effect adversely impacting quantification accuracy of certain, particularly more polar analytes [2]. Its principle is as follows: during injection of calibration standards in neat solvent, adsorption and/or thermo-degradation of susceptible analytes on the active sites (mainly free silanol groups) present in the injection port and in chromatographic column may occur; on this account the amount of molecules reaching GC

detector is reduced. This is, however, not the case when a real sample is analysed. Co-injected matrix components tend to block active sites in GC system thus reducing the analyte losses and, consequently, enhancing their signals (Figures 2 and 3). As far as these facts are ignored and calibration standards in solvent only are used for calculation of target analytes concentration, recoveries as high as even several hundred percent might be obtained [1]. Worth to notice that hydrophobic, non-polar substances such persistent organochlorine contaminants (with some exceptions such as DDT that may thermally degrade in a dirty hot injector) are not prone to these hot injection related problems.

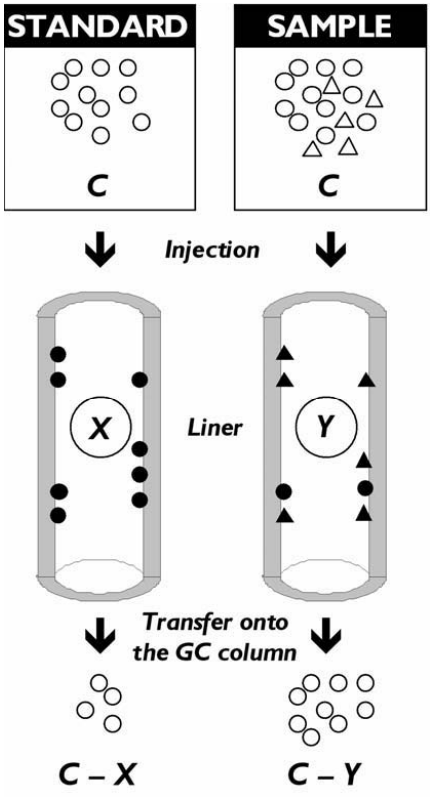


Figure 2. Illustration of the cause of matrix induced chromatographic enhancement effect; *C*—number of injected analyte molecules; *X*, *Y*—number of free active sites for their adsorption in injector; ○—molecules of analyte in injected sample; ●—portion of analyte molecules adsorbed in GC injector; △—molecules of matrix components in injected sample; ▲—portion of matrix compounds adsorbed in GC liner; $(C - X)<(C - Y)$ (Reproduced (modified) from [1] with permission from Elsevier© 2003).

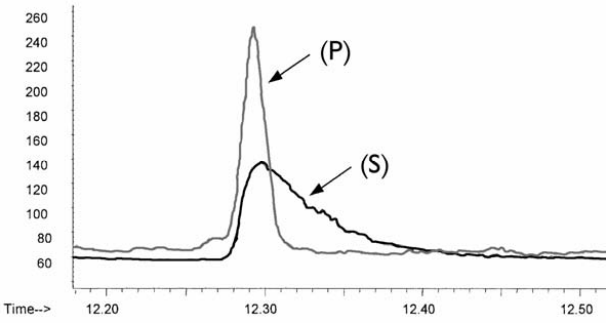


Figure 3 Matrix-induced enhancement response effect: 1 pg of 2-nitronaphthalene (*m/z* 173) injected in pure solvent (S) and in purified sample of pumpkin seed oil (P) (Reproduced (modified) from [3] with permission from Elsevier© 2002).

Repeated injections of non-volatile matrix components, which are gradually deposited in the GC inlet and (in case of hot splitless injection) front part of separation column, can give rise to successive formation of new active sites, which might be responsible for the effect, sometimes called *matrix-induced diminishment* [4]. Gradual decrease in analyte responses associated with this phenomenon together with distorted peak shapes (broadening, tailing) and shifting the retention times negatively impact ruggedness, *i.e.* long-term repeatability of analyte peak intensities, shapes, and retention times (Figure 4), performance characteristic of high importance in routine residue analysis [5].

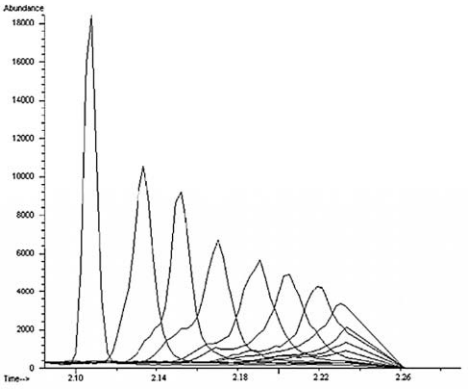


Figure 4. Overlay of 10 extracted ion chromatograms of lindane (*m/z* 181) in toluene solution. Between each standard analysis sample equivalents of 25 mg (carrot extracts) introduced onto the column (10 m × 0.25 mm × 0.25 µm). Acetone extraction followed by partitioning (mixture of dichlormethane–light petroleum) with solvent exchange to toluene used for sample preparation (Reproduced from [1] with permission from Elsevier© 2003).

Considering a complex nature of matrix effects (both the above phenomena occur simultaneously), intensity and extent of which is varying both with sample preparation strategy, type of GC–MS system employed and matrix/analyte combination examined, then a lack of general strategy for their complete elimination is not surprising. However, based on a deep understanding of this issue and taking into account all the until now existing knowledge, the adverse impact of matrix effects on the quality of generated data can be controlled within the acceptable range. Three basic approaches and/or their combination should be considered as way to improved quality assurance [1]:

(*i*) Elimination of primary causes
(*ii*) Optimisation of calibration strategy enabling compensation
(*iii*) Optimisation of injection and separation parameters

Unfortunately, the first concept of the GC system free of active sites, is in principle hardly viable—not only because of commercial unavailability of virtually inert materials stable even under long-term exposure to high temperatures that typical occur in a GC inlet port, but also due to impossibility to control formation of new active sites from deposited non-volatile matrix. More conceivable alternative might be based on avoiding sample matrix to be injected into the GC system. Unfortunately again, none of the common isolation and/or clean-up techniques are selective enough to avoid the presence of residual sample components in the analytical sample.

Since an effective elimination of the sources of the matrix effects is not likely in practice, their compensation by using alternative calibration methods is obviously the most feasible option. Several strategies are conceivable for this purpose: (*i*) addition of isotopically labelled internal standards; (*ii*) the use of standards addition method; (*iii*) masking of active sites in GC system (matrix-matched standards or analyte protectants).

The use of *isotopically labelled standards* that are affected by analytical procedure in the same extent as native analytes is a common practice in GC–MS analysis of various groups of persistent environmental pollutants (POPs) such as PCBs, PCDDs/PCDFs, PAHs *etc.* and several other priority food toxicants such as acrylamide, phthalates. However, only limited number of certified isotopically labelled standards is currently commercially available for effective compensation of matrix effects in susceptible representatives of other food contaminants such as modern pesticides. Considering hundreds compounds representing this group, even if available (what is not the case) the cost of isotopically labelled standards would be prohibitive in terms of routine monitoring. It should be also taken into account that using such standards for each target analyte (what is the ideal scenario) doubles the number of compounds for which optimisation of separation/detection conditions has to be carried out.

The *method of standard additions* may improve quantification of susceptible compounds in matrix, but this approach requires much extra effort and still leads to inaccuracies because the

matrix effect is concentration dependent. The use of this approach in multiresidue methods is hardly feasible.

Another apparently straightforward approach to deal with "matrix-induced chromatographic response enhancement" (lower signals of external standards in neat solvent as compared to those in real-world samples) is based on "masking" active sites in the GC system by intentional introduction of natural matrix components and/or matrix mimicking compounds together with calibrants. In practice, *matrix-matched standards* prepared in blank matrix extracts in order to provide the same amount of matrix-induced enhancement as in the sample extracts [6]. This concept works reasonably well and is widely used in pesticide residue analysis and will undoubtedly find application in many other areas of toxicants analysis, where the regulated levels of (medium-polar) target analytes are very low hence matrix effects potentially leading to overestimation of results are more pronounced.

However, choosing this calibration strategy its following disadvantages / limitations should be taken into consideration, too: (*i*) the stability of pesticides during a long-term storage in the presence of matrix components is limited; (*ii*) obtaining blank (toxicants free) samples of some commodities is difficult; (*iii*) demand for instrumentation maintenance is increased due to greater amount of matrix material injected onto the column in a sequence; (*iv*) additional workload when realising this type of calibration, and, consequently, increased cost of analyses is introduced. With regards to a heavy burden created by the use of matrix-matching standardisation, various short-cuts in their procedures are taken in routine laboratories. Although in a sequence of samples, there may be several different types of sample extracts, blanks for only one commodity to substitute for another are often used. This approach is more convenient, but it can lead to inaccuracies because the degree of the matrix-induced enhancement is commodity dependent (and sometimes sample dependent of the same matrix). In Europe, the regulatory guidelines for pesticide analysis call for the use of matrix-matched standards unless matrix effects are demonstrated not to affect the signal [7]. Worth to notice that this approach has not been approved in the US regulation agencies (Environmental Protection Agency and Food and Drug Administration) as yet.

Recently, a novel strategy simplifying solution of problems associated with risk of inaccurate calibration in routine trace analysis has been developed. So called *analyte protectants* (masking agents) are represented by compounds that strongly interact with active sites in the GC system, thus decreasing degradation and/or adsorption of co-injected analytes are used as additives [8]. The analyte protectants are added both to sample extracts and matrix-free standards to induce response enhancement in both instances, resulting in maximisation and equalisation of the matrix-induced response enhancement effect [9]. A wide range of compounds containing multiple polar/ionisable groups such as various polyols and their derivatives, carboxylic acids, amino acids, and derivatives of basic nitrogen containing heterocycles have been evaluated as analyte protectants [8]. In addition to their (protective) function, the degree in which following criteria are met was the basis for final selection:

(*i*) unreactiveness with analytes in solution or the GC system; (*ii*) sufficient stability under the GC conditions; (*iii*) no deterioration of the GC column or detector performance, *e.g.* due to accumulation; (*iv*) no interference with the detection process of analytes, *i.e.* low intensity, low mass ions in its MS spectra; (*v*) good availability, low cost, non-toxic; (*vi*) good solubility in solvent of interest. In particular case, a mixture of 3-ethoxypropane-1,2-diol, L-gulonic acid γ-lactone, and D-glucitol (in acetonitrile extracts) was found to most effectively cover a wide volatility range of GC-amenable pesticides [9, 10]. Figure 5 shows chromatograms for three pesticides lindane, phosalone, and *o*-phenylphenol obtained by hot splitless injection in matrix-matched and solvent standards, both with analyte protectants. Also, injection in solvent standards without analyte protectants is shown for comparison, demonstrating dramatic improvement in peak shapes and intensities with the use of analyte protectants [9].

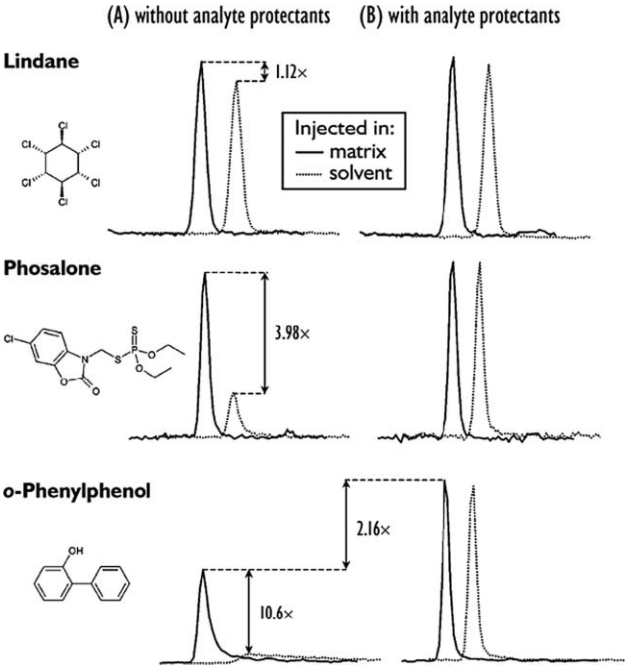


Figure 5. Comparison of peak shapes and intensities of 100 ng/ml lindane (*m/z* 219), phosalone (*m/z* 182), and *o*-phenylphenol (*m/z* 170) obtained by injection in matrix (mixed fruit extract) and solvent (MeCN) solutions (A) without and (B) with the addition of analyte protectants (3-ethoxypropane-1,2-diol, L-gulonic acid γ-lactone, and D-glucitol at 10, 1, and 1 mg/ml in the injected sample, respectively) (Reproduced from [9] with permission from American Chemical Sociey © 2005).

Besides of the above compensation approaches also careful optimisation of injection and separation parameters (including the choice of suitable injection technique, temperature, and volume; liner size and its design; solvent expansion volume; column flow rate; column dimensions) can reduce in some extent the number of active sites available for interaction (lower surface area) and the time of its duration [8]. However complete elimination of the matrix effects is hardly obtained. In Figures 6 the application of a pressure pulse during the injection illustrates outcomes of effort to reduce residence time or thermal degradation in the injection port [11]. More details on the function of these injection techniques are provided in the next Section.

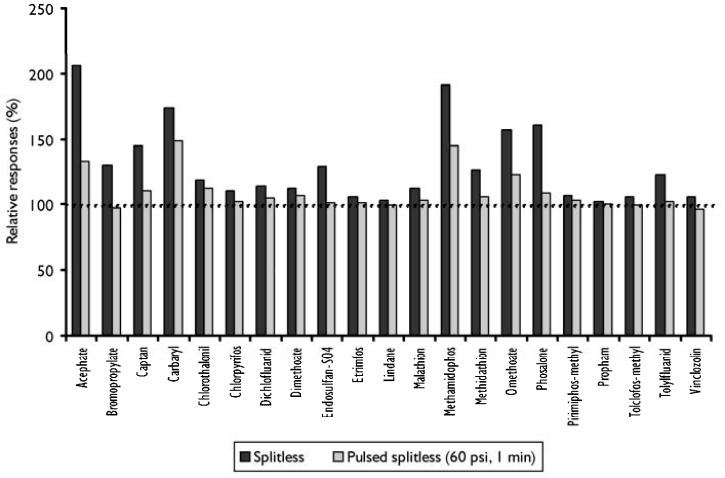


Figure 6. Matrix effects expressed as relative response of analytes in GC systems employing different injection techniques. Based on data published in [11].

3. Sample introduction

A number of options exists for GC inlet systems the most common being split/splitless, programmed temperature vaporiser, and cold on-column injector. The choice of optimum sample introduction strategy depends besides of other aspects on the concentration range of target analytes (with special requirements in (ultra)trace analysis), their physico-chemical properties and on the occurrence of matrix co-extracts present in the sample. In any case, to generate accurate and reproducible data in the GC analysis of analytes occurring in foods at trace levels (like most of food toxicants), the cause of following adverse effects due to the injection process should be avoided in maximum feasible extent:

(*i*) Discrimination, *i.e.* changed composition of sample that enters the column as compared to that of injected sample

(*ii*) Poor reproducibility of the amount of sample entering the column

(*iii*) Poor reproducibility of retention times

(*iv*) Thermal degradation, adsorption, rearrangement, and/or other changes of analyte

(*v*) Impairment of separation column performance

3.1. Split/splitless injection

Due to an easy operation, split/splitless injection remains the dominating sample introduction technique in the analysis of GC amenable food toxicants.

In a *split injection* mode, small volume of sample extract (typically in a range of 0.1–2 μL) is rapidly delivered into a heated glass liner. Under these conditions, thanks narrow input band of analyte, even microbore capillary can be used supposing fast separation is an option. However, considering the lost of most of injected sample (depends on the setting of split ratio) this technique is obviously not suitable for trace analysis where very low detection limits are required [12]. Another problem associated with split injection is potential discrimination due to the heating of the syringe resulting in a change of relative abundances of sample components when the mixture of analytes largely differing in boiling points is analysed. Another adverse phenomenon related to this technique is non-linear splitting due to adsorption of sample components on liner surfaces or deposited matrix "dirt".

Nowadays in trace quantitative analysis, hot *splitless injection* is the most commonly used injection technique since entire injected sample is introduced onto the GC capillary. As already discussed earlier, the major limitation of this inlet is that it suffers from the potential thermal degradation and/or adsorption of susceptible analytes what may result either in matrix-induced response enhancement or its diminishment. The extent of these effects depends on many factors like the inlet temperature, sample residence time (splitless period), the nature of sample matrix and/or liner material. To overcome, or at least partly compensate these problems, *pulsed splitless injection* should be applied whenever possible. Increased column head pressure for a short time period during the sample injection (usually 1–2 min) leads to a higher carrier gas flow rate through the injector (8–9 mL/min compared to 0.5–1 mL/min during classical splitless injection), *i.e.* faster transport of sample vapours onto the GC column. In this way, the residence time of analytes and, consequently, their interaction with active site in the GC inlet is fairly reduced [11]. The detection limits of troublesome compounds (mentioned above) obtained with pulsed splitless injection are thus significantly lower and their further improvement can be obtained by injection of higher sample volumes (for most liners up to 5 μL) without the risk of backflash [13, 14].

It should be noticed that this splitless technique does not permit a real large volume injection (10–100 μL) and some compounds may still thermally degrade in the injector even when the injector temperature is lowered. In addition, for injections >1–2 μL, a retention gap

prior to the analytical column is generally required to avoid excessive contamination of separation column and peak distortion (Figure 7).

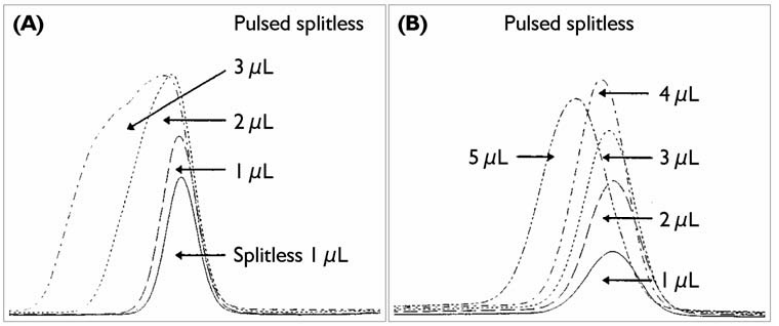


Figure 7. Peak shapes obtained by pulsed splitless injections of different volumes of standard solution onto the GC column (A) without a retention gap; (B) with an installed retention gap (Reproduced from [11] with permission from Wiley© 1999).

3.2. Cold on-column (COC) injection

In COC injection, sample aliquots are directly introduced by a special syringe onto the analytical column or a retention gap at temperatures lower (*e.g.* 60–80°C) as those used in hot split/splitless mode (commonly 200–300°C). On this account, COC is expected to cause less thermal stress on analytes during injection process (some thermal degradation can still occur on the GC column during the temperature ramp but this would occur regardless of the inlet employed). Generally, some improvements in COC performance can be achieved by modifying the polarity of column stationary phase, or reducing its diameter and thickness to allow lower temperatures of separation and faster analysis times [15]. In any case, this low-temperature injection eliminates both syringe needle and inlet discrimination and might be suitable namely for high-boiling analytes, on the other hand, the introduction of entire sample, both analytes and interferences, into the GC system is associated with increased demands for cleaning and maintenance when such complex samples as food is analysed [16]. Due to high sensitivity to nonvolatile matrix co-extracts, the use of COC in routine residue analysis is rather limited, it is more often employed as a control when optimising other techniques.

There are two alternative approaches available to perform on-column injection; details relevant to food toxicants analysis are summarised below.

- *Small volume on-column injection*

In this "classical" approach, small volumes of sample (up to 1–2 μL) are injected onto the separation column, or preferably, in the case of dirty samples, onto a retention gap to mitigate band broadening in space [17].

- *Large volume on-column injection* (*LVI*)

In this mode, large volume of sample (up to 1000 μL) can be introduced into the GC system [18]. The bulk of solvent is eliminated via special solvent vapour exit so that it does not overload the analytical column and detector. After the most of solvent is vented, solvent vapour exit is closed and analytes, together with remaining traces of solvent, are transferred onto the analytical column where they are focused like in the splitless injection. A modification of the chromatographic system is required in this case to include a large diameter retention gap (10–15 m × 0.53 mm), connected to a retaining pre-column (3–5 m × 0.32 mm) assisting in retention of volatile analytes. There are several technical reasons to use retention gap: (*i*) to perform injections into the column with wider bore is easier than into the narrow bore columns, although this option is also possible; (*ii*) analytical column is protected against the damage to transfer of non-volatile coextracts; (*iii*) refocusing of analytes zone is assisted. Another parameter that to be changed is the sample injection speed, which has to be slowed down in LVI-COC to prevent flooding of the system during large volume injections with injection speeds in LVI-COC of 20–300 μL/min as compared to LVI-PTV at 50–1500 μL/min [19].

3.3. Programmable temperature vaporisation injection (PTV)

PTV injector represents the most versatile GC inlet offering mitigation of most problems encountered when using hot vaporising device such as splitless and/or cool on-column injection in trace analysis. Regardless PTV is operated in split or splitless mode, the important fact is that injector chamber at the moment of injection is cool. A rapid temperature increase, following withdrawal of the syringe from the inlet, allows efficient transfer of the volatile analytes onto the front part of separation column while leaving behind non-volatiles in the injection liner. With regard to these operational features, PTV is ideally suited for thermally labile analytes and samples with a wide boiling range (when needed, PTV temperature can be programmed higher than the usual column temperature allowing injection of analytes that would not pass through classic split/splitless inlet). In addition to elimination of discrimination phenomenon and diminishing adverse affects of non-volatile matrix deposits on the recovery of injected analytes (*i.e.* improved tolerance of dirty samples), PTV enables to introduce, all at once or over period of time, large sample volumes, up to hundreds μL, into the GC system. No retention gaps or pre-columns are needed for this purpose, instead of that the liner size is increased. This feature makes PTV particularly suitable for trace analysis and also enables its on-line coupling with various enrichment and/or clean-up techniques such as automated SPE approaches. From practical point of view, another important PTV characteristic is its compatibility with any capillary column diameter including narrow bore columns. However, to attain optimal PTV performance in particular application, many parameters have to be optimised. Due to the inherent complexity of this inlet, method development might become on some occasions a rather demanding task. In spite of that, the

use of PTV in toxicants analysis is rapidly growing, in paragraphs below two in routine most commonly used ways of this injector operation are described. The other alternative approaches such as PTV vapour overflow injection and/or PTV Solid Phase Extraction-Thermal Desorption are used only exceptionally.

- *PTV splitless injection*

The sample is introduced at temperature below or close to the boiling point of solvent. Split exit is closed during sample evaporation and solvent vapours are vented via analytical column. PTV splitless injection has been employed for both large volume injections of 20 μL of sample and for conventional small volume injections [20]. The advantage of this technique is that no losses of volatile analytes occur (contrary to the solvent vent technique, see below). Operating parameters must be carefully optimised to avoid inlet overflow by sample vapours (and consequent losses of volatile compounds) as well as column flooding by excessive solvent (causing poor peak shapes of more volatile analytes). It has been already suggested that for some analytes the PTV splitless injection may produce better stability of responses and less matrix influence [21].

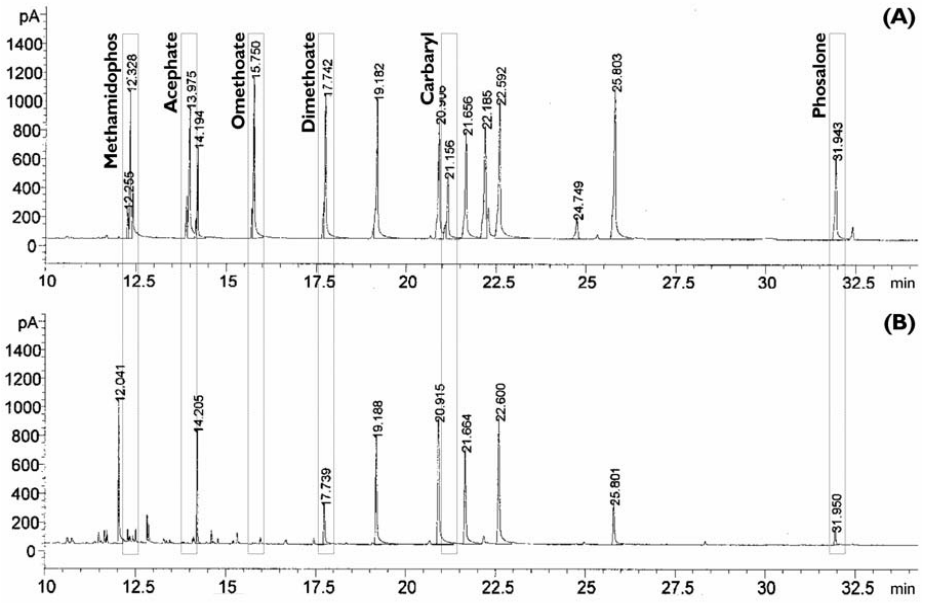


Figure 8. Chromatograms obtained by PTV injection into (A) empty liner and (B) liner packed with glass wool plug (Reproduced from [24] with permission from Wiley© 2001).

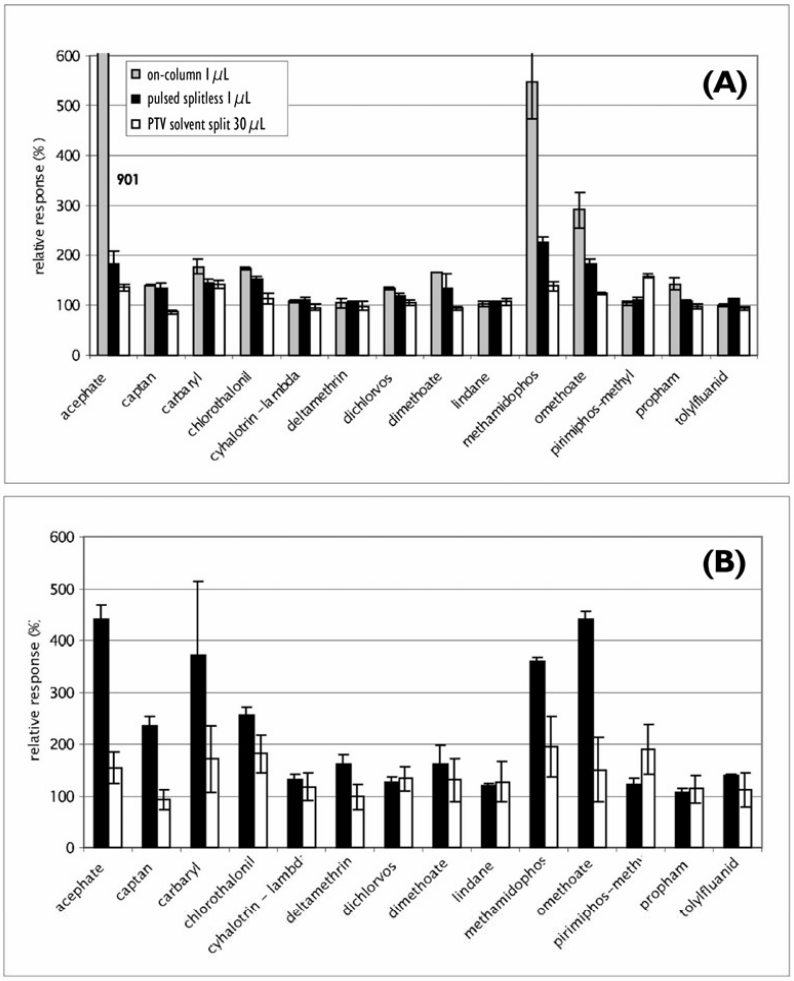


Figure 9. Relative responses (average of three consequent matrix-matched standards divided by average response of two surrounding solvent standards×100%) of analytes in GC systems employing different injection techniques. (A) Injection into "clean" GC system (after 10 injections of wheat samples) shows strong matrix effects using on-column injection. (B) Injection into "dirty" GC system (after 66 injections of wheat samples); considerable drop of analyte responses after a few injections of matrix-matched standards observed when using on-column injection. In both cases, the application of PTV resulted in suppression of matrix effects compared to COC and pulsed splitless injections (Reproduced from [16] with permission from Elsevier© 2001).

- *PTV solvent vent injection*

When employing this technique, sample is injected at temperatures well below a boiling point of solvent, holding the temperature of inlet port at low value thus enables elimination of solvent vapours via split exit. After venting step, inlet is rapidly heated and analytes are transferred onto the front part of analytical column. In that way, sample volumes up to hundreds μL can be injected [22, 23]. For injection of large volumes injector liner is often packed with various sorbents (Tennax, polyimide, Chromosorb, glass wool, glass beads, PTFE and Dexsil) in order to protect solvent from reaching bottom of injector what may lead to column flooding with liquid sample [24]. However, some labile compounds can be prone to degradation / rearrangement due to catalytic effects of the sorbent, alternatively, strong bounding onto the packing material causes poor desorption. As far as selection of suitable sorbent fails to eliminate these adverse impacts then the only conceivable solution is the use of empty or open liner; under this conditions rather lower volumes (in maximum ca. 50 μL) of sample are injected, typically employing multiple injections concept to get the total injection volume. Chromatograms shown in Figure 8 clearly document the damaging effect of glass wool inserted into PTV liner on the analysis of modern pesticides mixture.

In any case, obtaining good performance of PTV injector in solvent vent mode requires thorough experimental optimisation of all relevant parameters such inlet and column temperature, PTV heating rate, vent flow, vent time *etc.* To make method development more convenient advanced programmable PTV injectors such as Optic 3 (ATAS) are equipped with a built-in thermal conductivity sensor allowing determination of the time when the solvent is removed.

Figure 9 presents results obtained within examination of large series of food samples for multiple pesticide residues. PTV with solvent split technique shows the highest tolerance for matrix components among other injection techniques (COC, splitless) discussed until now.

3.4. Direct Sample Introduction (DSI) / Difficult matrix introduction (DMI)

DSI/DMI is a novel large volume injection technique employing automated liner exchange system with disposable sample microvial placed inside it. DMI strategy is based on a direct sample introduction (DSI) concept developed earlier by Amirav *et al.* [25] Contrary to any other sample introduction technique mentioned above, only volatile and semivolatile compounds are selectively excluded into the GC system during thermal desorption process while non-volatile sample matrix is retained on the wall of microvial. The individual phases of one of the most common commercial DMI represented by ATAS are shown in Figure 10.

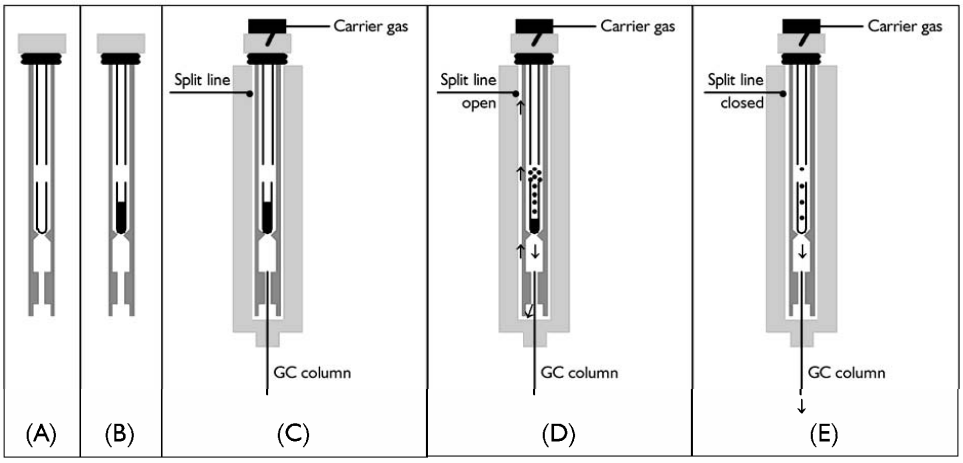


Figure 10. DMI injection. (A) Empty liner containing a microvial; (B) transfer of an aliquot of the sample extract to a microvial placed in the liner by an autosampler; (C) transfer of the liner to the special adapted injector; (D) venting of the solvent via split line; (D) transfer of (semi)volatile compounds onto the GC column.

As documented in studies concerned with pesticide residues analysis, in this way, time-consuming and expensive purification step can be omitted / significantly reduced for some matrices [10, 27, 28]. However, without cleanup, bulk (semi-)volatile matrix components introduced from the sample into the injector may still influence the quantitative aspects of the injection process and/or interfere in analytes detection. On this account, instruments with tandem (in space or in time) MS analysers (MS/MS) enabling reduction of chemical noise hence providing more accurate and unbiased results should be preferably used [29]. In Figure 11 the distinct improvement obtained by sample clean-up is illustrated. As far as minimal purification is still the option, then improved chromatographic and spectral resolution of sample components is the must for achieving reliable data. Under these conditions comprehensive two-dimensional gas chromatography (GC×GC) coupled to high-speed time-of-flight mass analyser is an ideal solution. In any case, regardless sample preparation strategy, reduced demands for the GC system maintenance represents another positive feature of this challenging technique.

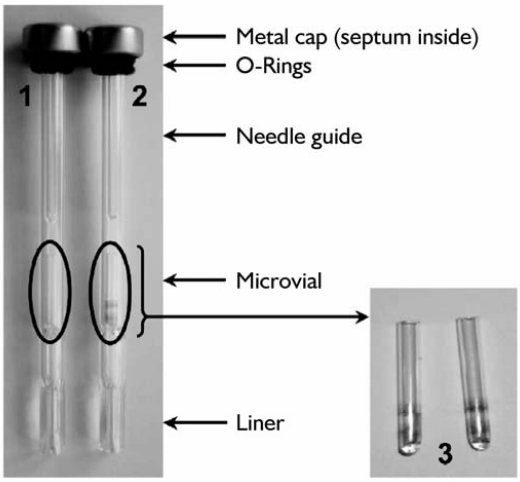


Figure 11. DMI liner after injection of: (1) purified baby-food extract; (2) crude extract; and (3) detail of microvials used for introduction of crude extracts (Reproduced from [10] with permission from Wiley© 2005).

3.5. Solid-phase microextraction (SPME)

SPME represents solvent-free sample introduction technique using a fused-silica fibre that is coated on the outside with an appropriate stationary phase. Volatile analytes present in the sample are directly extracted (from the headspace or by direct immersion) and concentrated to the fibre coating followed by their thermal desorption in the hot GC injection port [30]. The main features of SPME include unattended operation via robotics (if fully automated option available) and the elimination of maintenance of the liner and column (contamination by non-volatiles does not occur). It must be noticed, however, that this sample introduction technique relates to strong matrix effects, thus complications in quantification. In addition, variability of limit of detections for different analytes depends on the equilibrium between the coating material and the matrix [31].

4. Gas chromatographic separation

To be suitable for the GC an analyte should possess not only appreciable volatility at temperatures below 350–400°C (as a general rule, the greater the molecular weight or polarity of a particular analyte, the lower is its volatility), but also must be able to withstand high temperatures without degradation or reaction with other compounds. This requirement is met by many food toxicants, examples are given in the last Section.

With regard to (typically) complex mixture of matrix components occurring in food extracts (often even after purification) in fairly higher amounts as compared to concentrations of isolated toxicants, the optimisation of GC separation requires careful attention to a number of important variables and their interaction. Both physical (length, internal diameter, and stationary phase), and parametric (temperature and flow velocity) column variables affect separation process. To illustrate a wide range combinations to be considered when selecting GC capillary column, the overview of available internal diameters are shown in Table 1.

Table 1. Classification of capillary column (Reproduced from [31] with permission from Elsevier© 2003).

Category	Column diameter range (mm)	Standard commercial column diameters (mm)	Max flow-rate (mL/min) *)
Megabore	≥0.5	0.53	≥660
Wide bore	≥0.3 to <0.5	0.32, 0.45	≥85 to <660
Narrow bore	≥0.2 to <0.3	0.20, 0.25, 0.28	≥17 to <86
Microbore	≥0.1 to <0.2	0.10, 0.15, 0.18	≥1 to <17
Sub-microbore	<0.1	Various	<1

*) Flow rate calculated using helium carrier gas at 690 kPa, 200°C oven, vacuum outlet conditions and 10-m column length.

Figure 12 shows relative polarities of commercially available stationary phases. In the latter case not only the range of stationary phases including those dedicated for specific applications (*e.g.* dioxins) is growing but also their quality represented reduced bleed and increased temperature limit is improving (in case of most popular silicon columns this is achieved by addition of phenyl group into the backbone of a standard polysiloxane). The detailed discussion of the impact of manipulation of all conceivable settings on column efficiency, resolution and analysis speed is very complex and beyond the scope of this book. In any case, successful optimisation requires some level of expertise, recently computer software helping in prediction of optimal separation conditions have been developed. The limits of inlet pressure, sampling system and MS detector parameters have to be involved into consideration. In paragraphs below recent challenges in chromatographic set-up that may improve performance characteristic attainable in GC–MS analysis of food toxicants are presented.

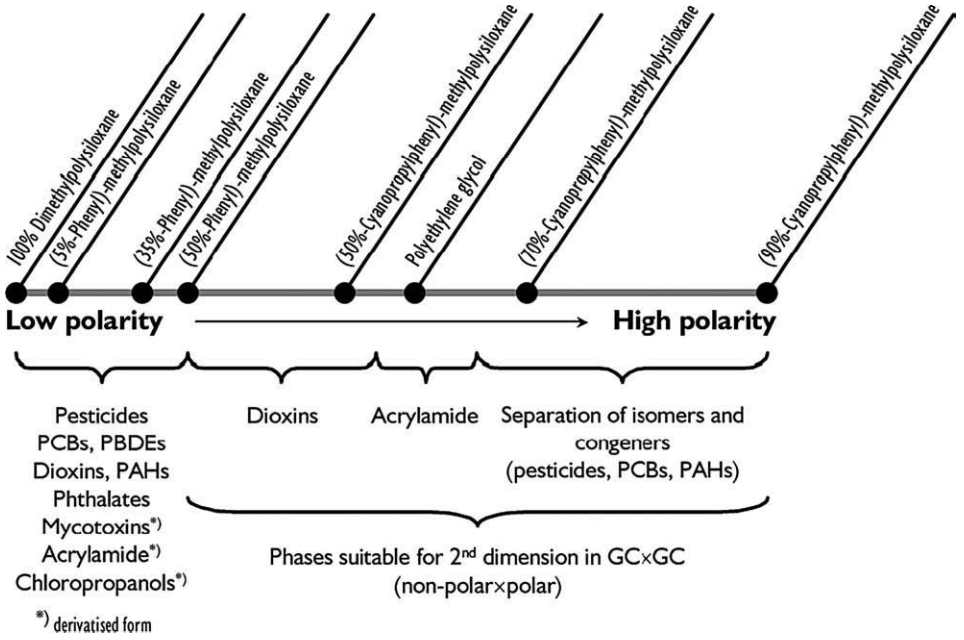


Figure 12. Classification of GC stationary phases with regard to polarity, typical uses in food toxicants analysis indicated.

4.1. Fast GC

Increasing demands for higher sample throughput together with the need to reduce laboratory operating costs has began focus attention of many residue laboratories on the implementation of high-speed GC (HSGC) systems. The basic principles and theory of this technique were formulated as soon as in 1960, nevertheless its development resulted at the end of last century from introduction of novel technologies such as new methods of fast column heating, inlet devices capable to inject very narrow samples plugs and, last but not least, MS detectors capable of high speed characterization of sample components. It should be noted, that full exploitation of potential of this challenging technique in routine practice is conditioned by reduction of sample preparation time and other operations limiting laboratory throughput. Using approximate terms, classification of GC analyses on the basis of speed is shown in Table 2.

Table 2. Classification of GC analyses

Type of GC analysis	Typical separation time	Full width at half-maximum
Conventional	>10 min	>1 s
Fast	1–10 min	200–1000 ms
Very fast	0.1–1 min	30–200 ms
Ultra-fast	<0.1 min	5–30 ms

Alike in conventional GC, the separation time this is defined as the retention time (t_R) for the last target component peak eluting from the column:

$$t_R = \frac{L}{\bar{u}}(k+1) \qquad (1)$$

where k is the solute capacity ratio (capacity factor, retention factor) for the last compound, L is the column length and $\bar{u}$ is the average linear carrier gas velocity.

The value of retention factor is related to column temperature (T_C):

$$\ln k = A/T_C + B \qquad (2)$$

where constants A and B have unique values for every compound and every stationary phase type and phase volume ratio.

4.1.1. Practical approaches to fast GC analysis

As indicated in Table 3 the increase of separation speed is compromised reduction of resolution (R) or sample capacity (Q_c). The acceptability of these losses has to be considered for each particular case separately. Availability of compatible sample introduction technique and MS detection parameters play an important role in these considerations.

Reduction of column length (*use of a short column*) represents one of the simplest approaches to decrease time of GC analysis. In practice, almost all fast GC analyses are performed with short columns (usually ≤10 m) in combination with other approaches. Reduction of the length of a given column results in reduced resolution ($R \sim \sqrt{L}$). Spectral resolution of co-eluting peaks supposing suitable mass selective detector is employed may compensate to some extent sacrificed separation efficiency.

Use of column with small internal diameter (microbore column) is another attractive way towards faster GC analysis. However, the instrumental requirements, especially the difficulties with the sample introduction and also the lower sample capacity limit their application in many real-world analyses.

Table 3. Approaches to faster GC separation

(A) Reduced column length ($\downarrow L$)
(B) Decreased retention factor ($\downarrow k$)
(*i*) Increased isothermal temperature
(*ii*) Faster temperature programming
(*iii*) Altered stationary phase to improve selectivity
(*iv*) Thinner film of the stationary phase
(*v*) Larger diameter capillary column (for fixed length)
(C) Increase carrier gas velocity ($\uparrow \bar{u}$)
(*i*) Higher than optimum carrier gas velocity
(*ii*) Increased optimum carrier gas velocity
(a) Hydrogen as a carrier gas
(b) Vacuum outlet operation (low-pressure GC)

Use of column with thin film of stationary results in the decrease of the capacity (retention) factor and thus in the faster GC analysis. In addition, due to decreased contribution of mass transfer in the stationary phase, separation efficiency is increased. On the other hand, reduced ruggedness and sample capacity, the latter limiting attainable sensitivity in (ultra)trace analysis are the fee for faster separation.

Fast temperature programming is the most popular approach in application of fast GC for food toxicants analysis (several studies concerned with pesticide residues and PBDEs were published [32, 33]). Either convection heating facilitated by conventional GC oven or resistive heating (described in next paragraph) can be employed. As far as "fast" separation in terms of classification shown in Table 2 (*i.e.* analysis time in the range 1–10 min) is required, conventional GC oven can be used. At faster programming rates heat losses from the oven to the surrounding that occur namely at higher temperatures may cause poor reproducibility of analytes elution. Figure 13 well illustrates the superiority of resistive heating in terms of retention times stability of analytes.

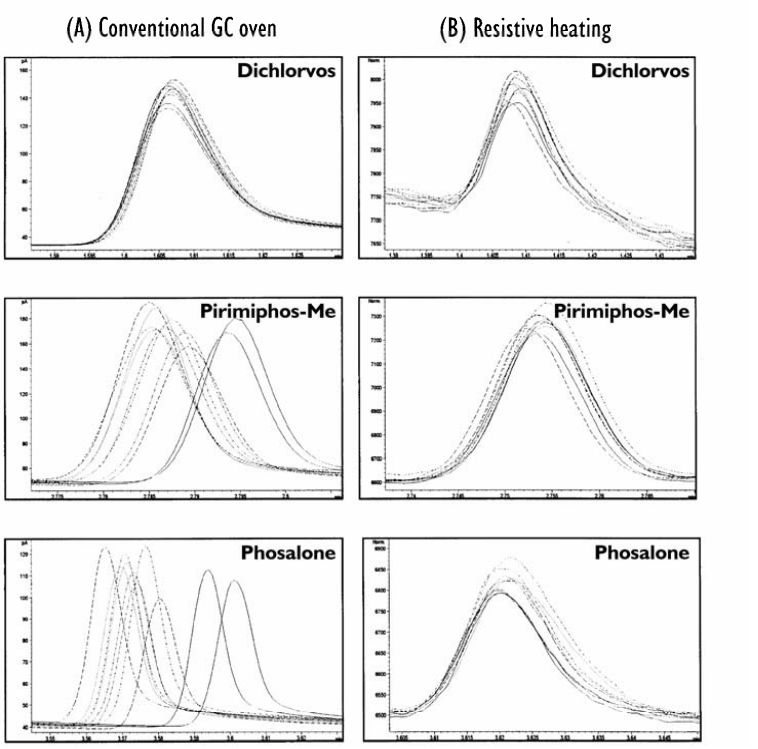


Figure 13. Overlay of 10 repeated injections obtained by analyses of the standard solution, fast temperature programming realised by: (A) a conventional GC oven, (B) resistive heating (flash GC); – comparison of retention time repeatability of three selected analytes (Reproduced from [32] with permission from Elsevier© 2001).

Operation of column outlet at low pressure (*low-pressure GC*) is another fast GC alternative that may find a wide use in routine laboratories concerned with food toxicants analysis. Due to operating of (megabore) separation column at low pressure, optimum carrier gas linear carrier gas velocity is attained at higher value because of higher diffusivity of the solute in the gas phase. Consequently, faster GC separations can be achieved under reduced pressure at the same column dimensions as atmospheric column outlet pressure operation with a disproportionately smaller loss of separation power [34]. The main attractive features of LP-GC–MS involve: (*i*) reduced peak tailing (Figure 14) thus their improved detection limits; (*ii*) increased sample capacity of megabore column allowing injection of higher sample volume resulting in lower detection limits for compounds not limited by matrix interferences; (*iii*) reduced thermal degradation of thermally labile analytes (*e.g.* carbamates) [35].

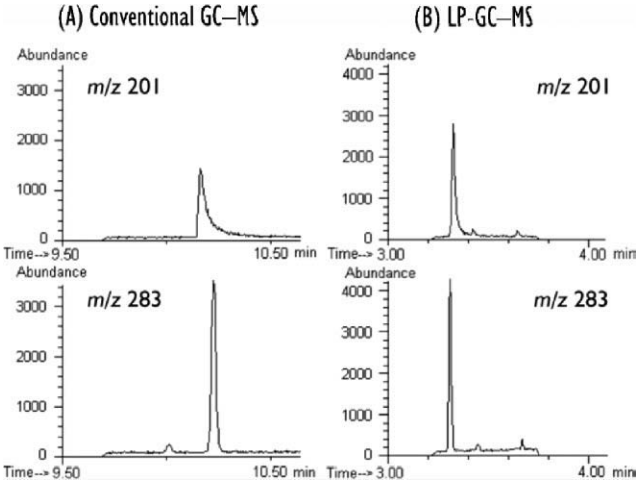


Figure 14. Comparison of peak shapes of thiabendazole (*m/z* 201) and procymidone (*m/z* 283) obtained by (A) conventional GC–MS and (B) LP-GC–MS (Reproduced from [35] with permission from Elsevier© 2003).

Use of hydrogen as a carrier gas. Hydrogen with the highest diffusion coefficient is obviously the best carrier gas for fast GC. Moreover, its low viscosity results in lower inlet pressure requirements. In practice, however, helium is usually preferred as a carrier gas flow for safety and inertness reasons.

4.1.2. Instrumental requirements in fast GC

Unavoidably, fast GC analysis puts special requirements on the instrumentation used and these demands are growing with increasing separation speed. Although under real-world conditions HSGC analysis of food toxicants can be performed using modern conventional GC instruments, attention has to be paid to minimisation of extra-column band broadening that may become very significant and thus limiting the optimum column performance. Both injector and detector may contribute to this undesirable phenomenon.

Inlet systems should allow as short as possible injection time in order to generate sufficiently narrow sample vapour plugs. Mechanical computer controlled micro valves or cryofocusing inlets have been proposed as effective tools to deliver narrow injection band widths and minimise uncertainty of shot-to-shot injection times. As far as the concentration of toxicant is not a limiting factor *split injection* is a favoured sample introduction mode in HSGC due to its speed at high split ratio. However, the sample loss resulting in reduced

sensitivity might be a problem in trace analysis. Under these circumstances, *splitless injection*, during which the injected sample band is refocused at the front part of analytical column is the only conceivable strategy to attain low detection limits. As far as solvent effect takes part in trapping process, too long flooding zone formed typically when narrow bore capillaries are used may cause severe discriminations and peak distortions. Either the use of short retention gap with larger internal diameter or, preferably, employing *PTV injector* operated in solvent vent may eliminate these problems. Much faster and therefore more suitable for narrow-bore columns than classical hot splitless injection seems to be "cold" splitless injection using a PTV injector, where sample is transferred to a cold injector and then vaporised by a very rapid heating. Additional benefit of this technique is the possibility of solvent elimination, if PTV in solvent vent mode is employed.

Regarding the *detectors* applicable in fast GC, attainable data collection rate must be high enough to give enough points across (narrow) peaks to enable acceptable precision of measurements of their areas and heights. Worth to mention that large discrepancy exists in the literature as regards the minimum points needed to define chromatographic peak. While some authors consider as little as 3–4 points sufficient for quantitative needs (peak recovery is degraded by ±1.44% when considering Gaussian curve), the requirement for higher numbers was documented in several other studies with 10–20 points as maximum in one of them [31]. Figure 15 illustrates the experimental evidence in the case of a quadrupole MS instrument on the repeatability of peak area/height with respect to spectral sampling rate. This plot indicates that 5–6 points across a chromatographic peak essentially achieve the minimum relative standard deviation of peak area/height.

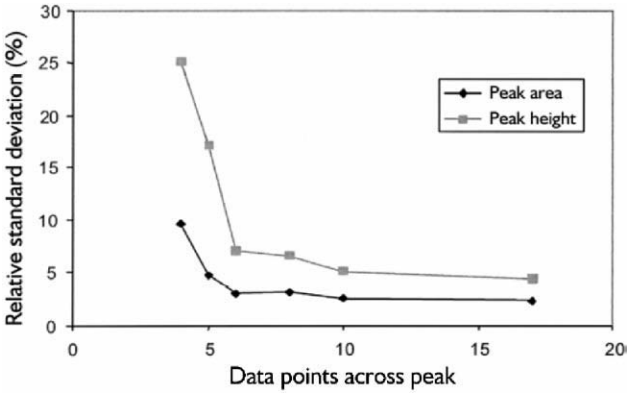


Figure 15**.** Influence of scan rate on RSDs of peak area and peak height. Test analyte: C14 *n*-alkane with C15 as internal standard (1 ng injected, n = 7 per data point) (Reproduced from [36] with permission from Wiley© 2002).

Most of the modern conventional GC detectors (FID, NPD, FPD, μ-TCD, μ-PID, μ-ECD) usually have maximum sampling frequencies in the range 20–200 Hz, which is adequate for fast GC analysis. Typical acquisition rates of modern scanning mass spectrometers, such as quadrupole, ion traps and magnetic sector instruments, are in the range of 5–15 spectra/s, hence capable of offering the speed for MS detection in the case of fast GC separation (analysis in the minutes range). Alternatively, time-of-flight analysers allowing acquisition rates up to 500 spectra/s can be used for detection of eluted peaks under the conditions of fast GC.

Figure 16 lists practical ways to achieve the most prioritised feature (speed, sensitivity, or selectivity) using different types of GC–MS instruments.

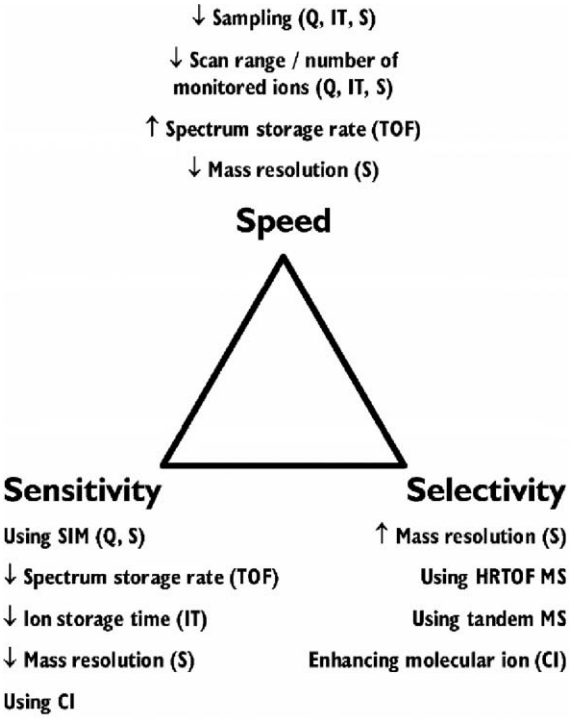


Figure 16. Approaches in MS to achieve speed, sensitivity, or selectivity. (Q–quadrupole, IT–ion trap; TOF–time-of-flight, S–sector, CI–chemical ionisation)

Relevant parameters of *temperature and pressure programming* functions of GC system are important for performing fast GC. Although modern instruments use sophisticated algorithms and microprocessor-controlled oven heaters programming rates of 1 to 2°C/s seem to be

practical achievable limit. A number of techniques have been used in the past to obtain faster temperature ramping than that obtained with a standard convection GC oven. These include the oven inserts to decrease the volume of the GC oven, oven inserts which can add extra heating capability to the standard oven *etc.* However, in sequential approach like GC, not only fast temperature programming rates during separation, but also minimal cooling-down period between two subsequent runs (*i.e.* rapid equilibration to initial conditions) can significantly contribute to the increased sample throughput. Slow cool-down process is a common drawback of all the above approaches. *Resistive heating* of separation column represents an alternative technique eliminating the conventional GC ovens. Electrical current is employed to heat a conductive material (a metal) located in its close proximity. Its temperature can be determined by controlled by resistance measurements. Since the thermal mass of the heater is minimised, the heating-up and cooling-down rates can be very fast. Using this approach temperature programming rates up to 30°C/s are attainable [37].

High carrier gas velocities typically employed in fast GC analyses controlled by electronic pressure control (EPC) systems that are available in all current instruments. In addition to possibility to make fine tuning of carrier gas flow rates, sufficiently high inlet pressure (up to 150 psi), especially when narrow bore capillary columns are used can be set. A special column set up (Figure 17) is needed for performance of low-pressure GC–MS.

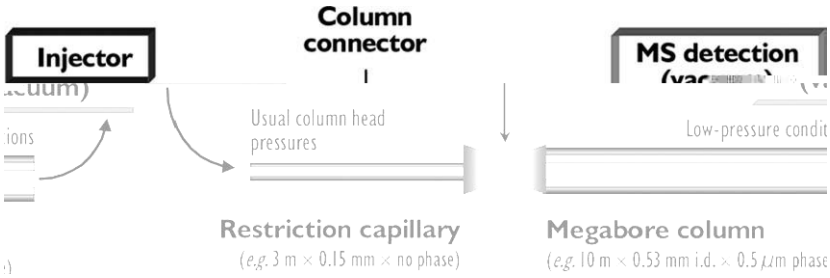


Figure 17. Column set-up for LP-GC–MS.

The narrow restriction capillary connected to the front part of wide bore analytical column allows (thanks to common head pressure) performing of sample introduction by conventional GC injector, while the separation occurs under vacuum delivered by MS detector. The comparison of pressure drop across the megabore and narrow bore column is illustrated in Figure 18.

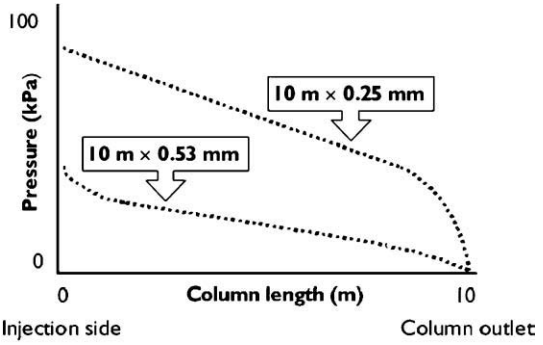


Figure 18. Local gas-pressure inside the capillary (Reproduced (adapted) from [34] with permission from Wiley© 2000).

4.2. Multidimensional high-resolution GC

Using the peak capacity (n_c) for characterisation of resolving power (R) of chromatographic system, then its value for column generating N theoretical plates and for retention window from t_1 to t_2 is given by equation:

$$n_c = 1 + \frac{\sqrt{N}}{4R} \ln \frac{t_2}{t_1} \qquad (3)$$

Considering modern high-resolution GC, peak capacity for a 50 m capillary developing 2×10^5 theoretical plates is 260 (calculated from Equation 3 using $R = 1$). In the analysis of complex mixtures such food extracts (hundreds of randomly distributed peaks can be contained) by one-dimensional chromatography, overlap of some sample components inevitably occurs. To achieve considerable increase in peaks capacity, two independent displacement processes with axis x and y oriented at right angles (peak capacities along them are, respectively, n_1 and n_2) have to be employed in analysis of sample. Supposing the orthogonality criterion is met, *i.e.* coupled separations are based on two different mechanisms, the maximum peak capacity calculated as $n_1 \times n_2$ is typically enhanced by at least one order of magnitude.

Large variety of modes how the columns differing in stationary phase selectivity can be used in two-dimensional (2D) manner exists. While the common feature of all these processes is that segment or band of effluent from first separation is directed onto the secondary column, the key difference in these methods lies in the mechanism by which the overflow from the primary column is interfaced to the secondary column. Typical set-up is schematically shown in Figure 19.

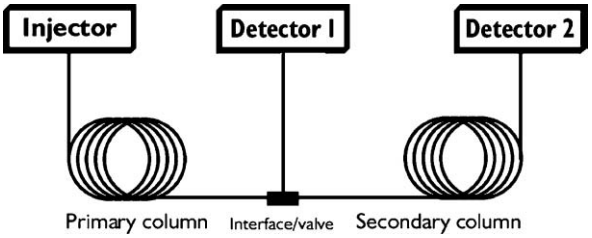


Figure 19. Schematic diagram of a coupled column system.

Most of successful applications reported in food toxicants analysis such as persistent organic pollutants (besides resolution of co-eluting congeners, separation of enantiometric species was often carried out in this way) since 1960 up to 1990 employed so called "heart cut" mode, in which only narrow fraction(s) containing analytes of interest is (are) transported for further separation onto the second column, see example in Figure 20.

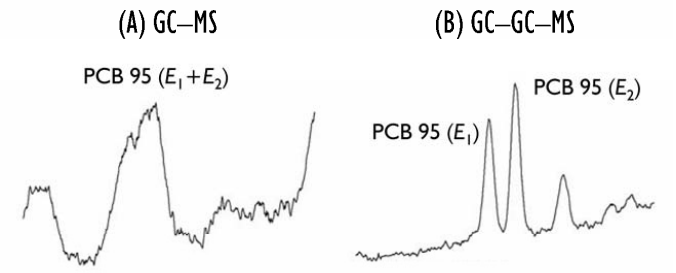


Figure 20. Separation of PCB 95 enantiomers (E_1+E_2) under the conditions of (A) conventional GC–MS, and (B) heart cut GC–GC–MS (HP-8–Chirasil Dex columns) (Reproduced (adapted) from [38] with permission from Elsevier© 2005)

However, this approach has limitations since increasing the width of the first column fraction or isolation of too many parts of 1D analysis subject them to 2D separation, becomes troublesome, time-demanding reconstruction of generated chromatograms may become a serious problem. The introduction of systems allowing (thanks to technical achievements in interface technology between the two dimensions) all of the sample from the first column to be analysed on the second column has enabled both target and non-target screening of food toxicant in a wide range of matrices. With regard to exponentially growing number of applications of this challenging technique, in residue analysis, comprehensive two-dimensional gas chromatography (GC×GC) is introduced in following section.

4.3. Comprehensive two-dimensional gas chromatography (GC×GC)

4.3.1. GC×GC set-up

The heart of the GC×GC system is a modulator connecting the first dimension conventional-size column with a short microbore column in the second dimension (**Figure 21**).

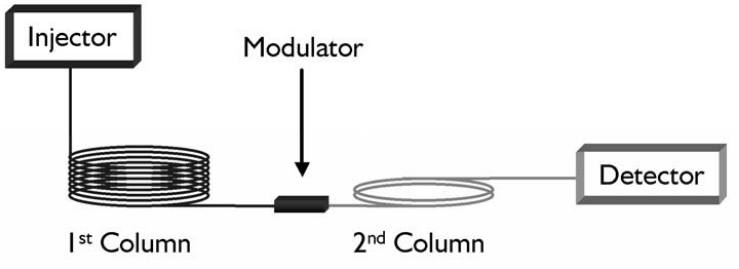


Figure 21. GC×GC instrument configuration.

The function of this interface between the two dimensions is: (*i*) to trap small adjacent fractions (typically 2–10 s) of the effluent from the first separation column; (*ii*) refocusing these fractions (either in time or in space); (*iii*) injection of the refocused fractions as narrow pulses into the second-dimension column. The separation on the latter column is extremely fast and takes only 3–10 s *vs.* 45–120 min for the first dimension and is therefore performed under essentially isothermal conditions. Under these conditions separation according to boiling points practically does not take place hence the separation is governed only by specific interactions of sample components with stationary phase [39].

A large series of high-speed chromatograms as the outcome of the transfer of chromatographic band from the first to second dimension is generated during the GC×GC run. As shown in Figure 22 these adjacent pulses are usually stacked side-by-side by a special computer program to a form a two-dimensional chromatogram with one dimension representing the retention time on the first column (t_{R1}) and the other, the retention time on the second column (t_{R2}). The most convenient way to visualise GC×GC data is as contour plots representing the bird's eye view, where peaks are displayed as spots on a plane using colours and shading to indicate the signal intensity, see Figure 22.

4.3.2. Optimisation of operation conditions and instrumental requirements in GC×GC

Compared to conventional 1D-GC, the optimisation of GC×GC analysis requires different and definitely more complex approach. For instance, the changes in operational parameters such oven temperature or carrier gas flow rate have different impacts on the performance of

separation columns since these differ both in their geometry and separation mechanism. Furthermore, new parameters such as modulation frequency and modulator temperature have to be optimised [40].

Conventional columns, typically 15–30 m × 0.25–0.32 mm I.D. × 0.1–1 μm film thickness, are used in the first dimension. This allows application virtually all conventional sample introduction techniques (splitless, on columns, LVI-PTV, and/or SPME). Stationary phases commonly used in first-dimension columns are 100% dimethylpolysiloxane or (5% phenylene)-dimethylpolysiloxane. The separation on these relatively non-polar columns is governed by volatility. The size of columns for second dimension is commonly in the range 0.5–2 m × 0.1 mm I.D. × 0.1 μm film thickness. More polar stationary phases such as 35–50% phenylene–65–50% dimethylpolysiloxane, polyethyleneglycol (Carbowax), carborane (HT-8) and/or cyanopropyl–phenyl–dimethylpolysiloxane are employed. Analytes interact with these medium-polar/polar phases via various mechanisms like π-π interaction, hydrogen bonding *etc*., hence the requirement for different, independent separation principle is met. In most applications orthogonality is achieved by non-polar×polar separation.

To obtain acceptable separation in both dimensions, compromise has to be adopted with regard to both columns; due to their connection in series, they are not independent. The linear velocity of the carrier gas in the (narrow bore) first dimension column is usually rather lower than optimal (ca. 30 cm/s) while at the same time, the linear velocity in the (microbore) second dimension capillary is relatively high, typically exceeds 100 cm/s. Also when setting the temperature programming rate the requirement for obtaining at least three or four modulations over each first-dimension peak has to be taken into account. In most analyses programming rates as low as 0.5–5°C/min what is less than in conventional 1D-GC. For better tuning of the GC×GC set-up, systems with programmable second oven are preferred [39].

As already emphasised, effective and robust modulation is a key process in the GC×GC analysis. Theoretically, thermal modulation in a capillary GC can be performed by both heating and cooling. Heated modulators use a thick-film modulation capillary to trap subsequent fractions sample components eluting from the first column by means of stationary phase focusing. Subsequently these compounds are released by the application of heat. Contrary to heated modulators, cryogenic cooled modulators do not use a modulation capillary. Instead, they trap and focus the analyte-containing fractions eluting from the first column at the front end of the secondary column itself. Cooled modulators allow the maximum operation temperature 100°C higher when compared to heated modulators. The common disadvantage of moving heated/cooled modulators is their low robustness (fragile capillary was often broken). These shortcomings of moving modulators have been overcome by two-stage jet modulators, using a stream of nitrogen or carbon dioxide for cooling a short section of the second column for trapping/focusing of the analytes eluting from the first column (Figure 23) [41, 42]. Their introduction has lead to increasing use of GC×GC separations in routine laboratories.

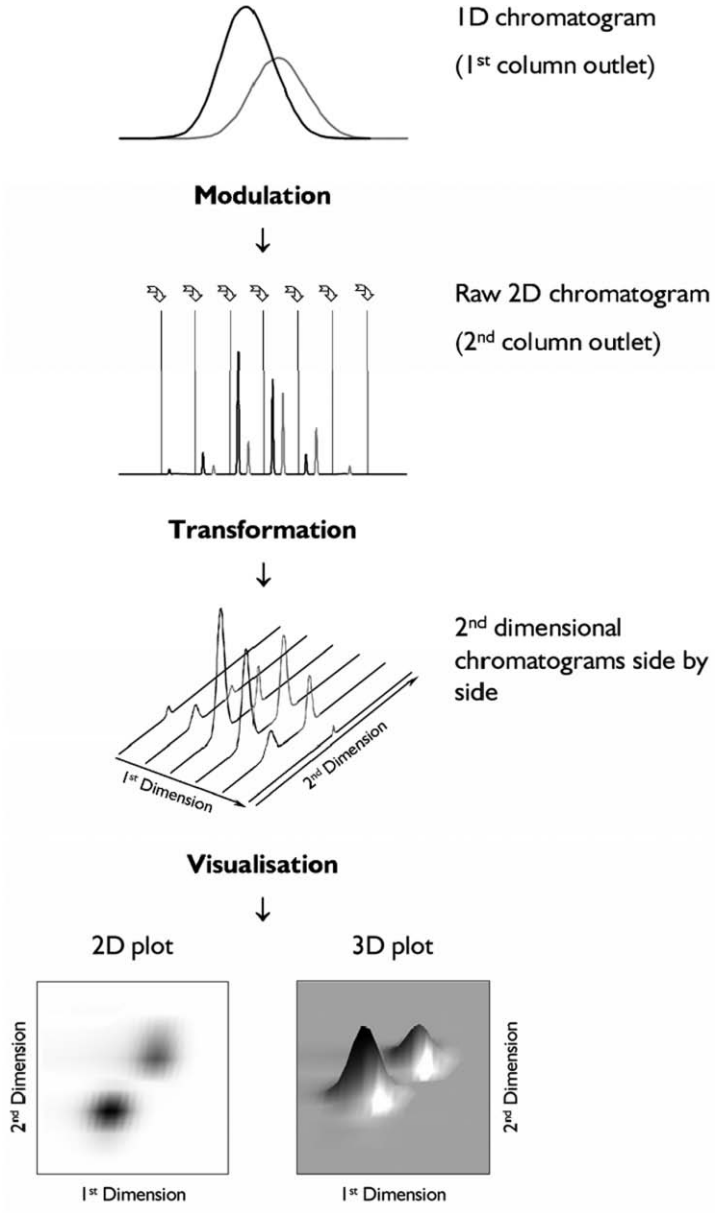


Figure 22. Generation and visualisation of a GC×GC chromatogram.

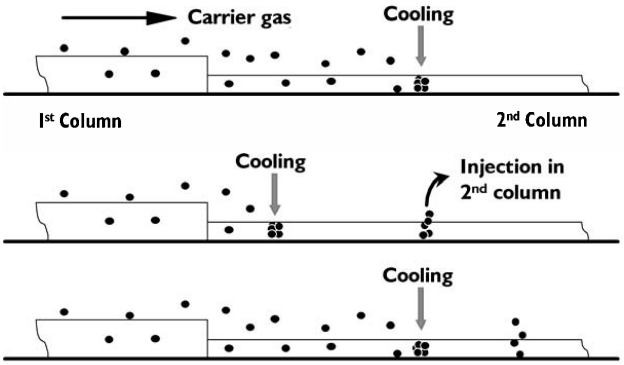


Figure 23. Schematic of a two-jet cryogenic modulator. (1) The modulator (right-hand jet) retains part of a peak eluting from the first column. (2) The right-hand jet is switched off, the cold spot heats up very quickly and the analytes are released and launched for separation in the second column. Meanwhile, the left-hand jet is switched on to prevent material eluting from the first column to interfere with the focused fraction. (3) The right-hand jet is switched on again and the next modulation cycle is started (Reproduced (adapted) from [39] with permission from Elsevier© 2002).

In practice, fixed modulation frequency, typically in the range 0.1–10 Hz is employed during the analysis. Under ideal experimental conditions the retention time of the most retained compound in the second dimension is shorter than modulation time. As long as this is not the case, *i.e.* analytes do not elute in their modulation cycle, so called "wrap-around" phenomenon that may cause coelutions occurs. Its avoiding can be achieved *e.g.* by increase of second dimension column temperature (supposing independent oven is available). In any case, optimal function of modulator is essential for the quality of separation and detection process.

The fast separation on short and microbore second dimension column results in very narrow peaks with widths of 100–600 ms at the baseline. As discussed earlier, fast analogue detectors such as an FID or ECD are fully compatible with fast chromatography and provide reliable peak recognition but do not provide structural information. Coupling GC×GC separation with MS detector results in fact to three dimensional system that may contribute to the identification of 2D separated peaks and brings a deeper understanding of structured chromatograms (see below). However, conventional scanning MS detectors are too slow and do not provide reliable spectra and peaks reproduction. As illustrated in Figure 24 under extreme conditions, very narrow peak may remain "invisible" for detector with low acquisition speed. At present, only time-of-flight mass spectrometers can acquire the fifty or more mass spectra per second, which are required for the proper reconstruction of the chromatogram and for quantification [39].

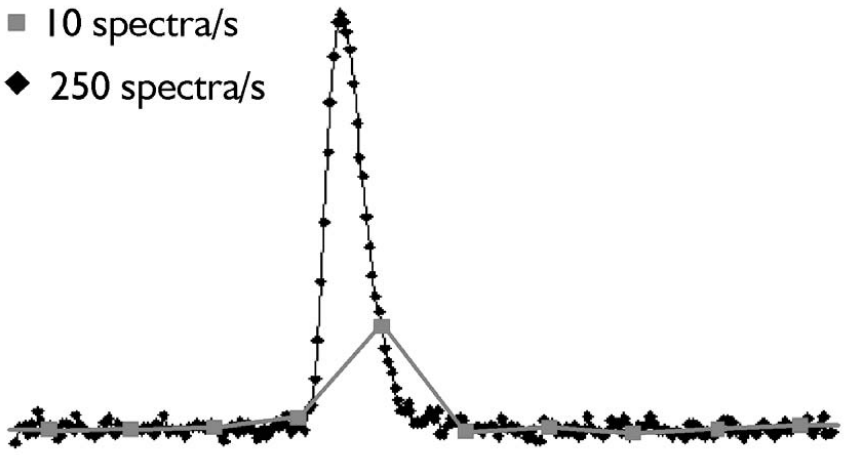


Figure 24. Detection of narrow chromatographic peak (150 ms) using instrument with different acquisition rates.

4.3.3. Application of GC×GC, examples of merits

A number of characteristics of GC×GC have been reported that documents superiority of this technique over conventional 1D-GC. Few examples are given below.

High peak capacity. The peak capacity, characterised as a maximal number of chromatographic peaks that can be placed side by side into the available separation space (chromatogram), is significantly enhanced. Separation of several thousands of compounds by means of GC×GC, in reasonable time period has been reported. Under real-world conditions, the total peak capacity in GC×GC is rather lower than calculated value due to imperfections in sample transfer between the two columns, however it still greatly exceeds the limits of conventional GC. The merit in pesticide residue analysis resulting from the separation power is illustrated in Figure 25. While due to analyte overlap with abundant matrix co-extract yielding the same low, unspecific ions it was not possible to determine traces dichlorvos by conventional 1D-GC–TOF MS in apple extract (deconvolution failed), efficient resolution of dichlorvos from interference (identified on the basis of its mass spectrum as 5-(hydroxymethyl)-2-furancarboxaldehyde), the use of GC×GC separation enabled its reliable identification and quantification [43].

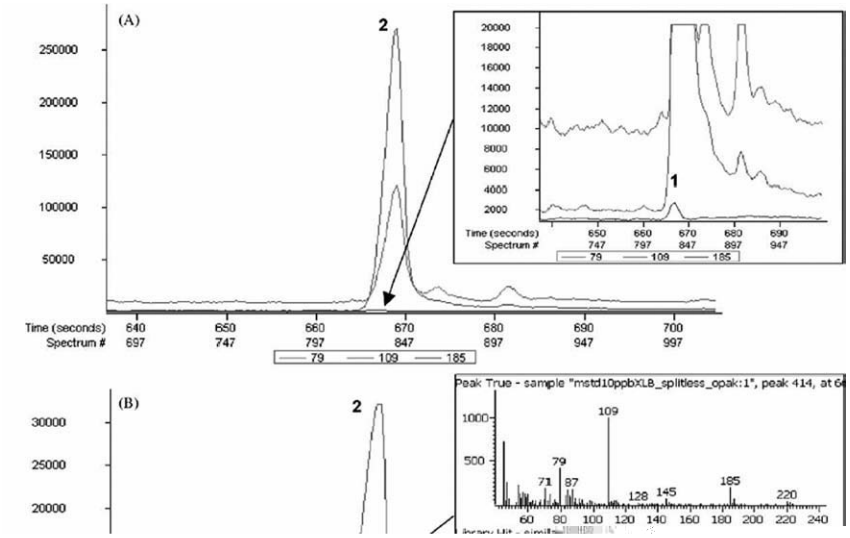


Figure 25. Separation of: (1) dichlorvos from matrix co-extract, (2) 5-(hydroxymethyl)-5-furancarboxaldehyde, spiked apple sample at 10 ng/g. Plotted are three most abundand ions in the mass spectrum of dichlorvos (79, 109, and 185). Chromatogram from: (A) 1D-GC–TOF MS analysis of zoomed section shows the peak of dichlorvos at mas 185, at masses 79 and 109 matrix interference is recorded; (B) GC×GC–TOF MS analysis, base modulation, matrix inteference has been resolved on medium polar DB-17 column (Reproduced from [43] with permission from Elsevier© 2003).

Enhanced sensitivity. Compared to 1D-GC separation pronounced improvement of detection limits in GC×GC system is obtained thanks to compressing the peak in the modulation capillary and/or front part of second column (following fast chromatography avoids band broadening of focused peaks). Further, thanks to improved separation of chemical noise in GC×GC system, the signal to noise ratio is also improved. Example is given in Figure 26 that illustrates 1D-GC *vs.* GC×GC analysis of hexachlorobenzene (2 pg injected). The enhancement in detectability (1.5–50-fold) for selected pesticides determined in real-world samples (peach extracts) when compared to 1D-GC is also documented in Table 4 [43].

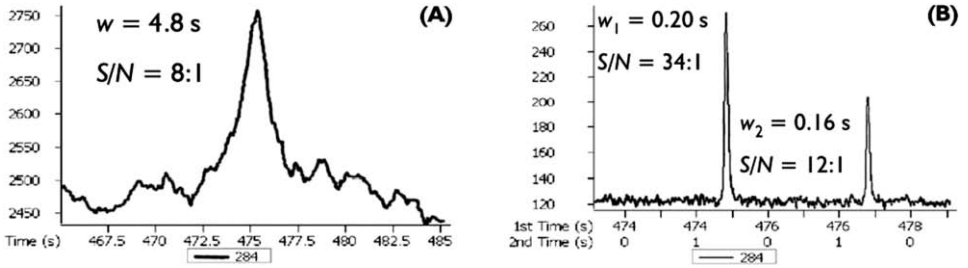


Figure 26. Improvement of detectability of HCB (2 pg injected, *m/z* 284). (A) 1D-GC, (B) GC×GC (DB-5×BPX-50 columns). Data acquired by TOF MS at acquisition rates of 10 spectra/s and 200 spectra/s, respectively.

Table 4. Limits of detection (ng/g) of pesticides in peach extracts [43]

Pesticide	1D-GC	GC×GC	Detection enhancement factor*)
Dichlorvos	10	0.2	50
Acephate	10	1.5	6
Pirimiphos-Me	10	2	5
Carbaryl	7	0.6	11
Chlorpyrifos	5	1.7	3
Procymidone	15	0.7	20
Endosulfan I	19	4.5	4
Phosalone	14	6	2
Deltamethrin	140	100	1.5

*) Ratio of LOD for 1D-GC analysis to GC×GC.

Structured chromatograms. Thanks to complementary separation mechanisms occurring on both columns, the chromatograms resulting from GC×GC are ordered, *i.e.* molecules have their definite locations in the retention space based on their structure. In the reconstructed 2D contour plots characteristic patterns are obtained, in which the members of homological series differing in their volatility are ordered along the first dimension axis, whereas the compounds differing by polarity are spread along the second dimension axis (see Figure 27). The formation of clusters of the various subgroups of compounds in a GC×GC contour plot may be useful for the group type analysis and provisional identification of unknown compounds.

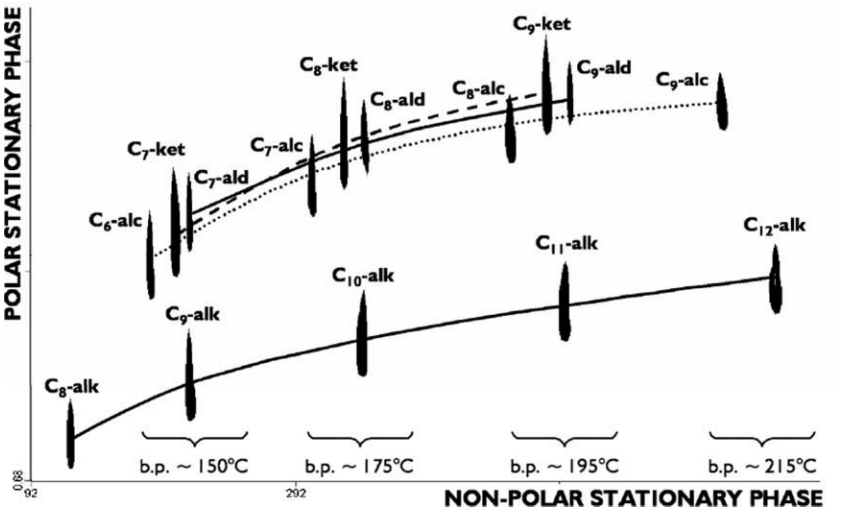


Figure 27. Separation of selected alkanes (alk), alcohols (alc), aldehydes (ald), and ketones (ket) using GC×GC–TOF MS with a combination of non-polar×polar stationary phases (DB-5×BPX-50).

Improved identification of unknown. Non-target screening allows obtaining of overview of the sample constituents. This approach consists from: (*i*) peak finding and deconvolution (algorithm for recognising of partly coeluting peaks in the GC–MS chromatogram and obtaining of their "pure" mass spectra); (*ii*) library searching, and (*iii*) further post-processing. Since a large amount of data has to be processed, automated data processing is employed. Figure 28 shows an example of non-target screening.

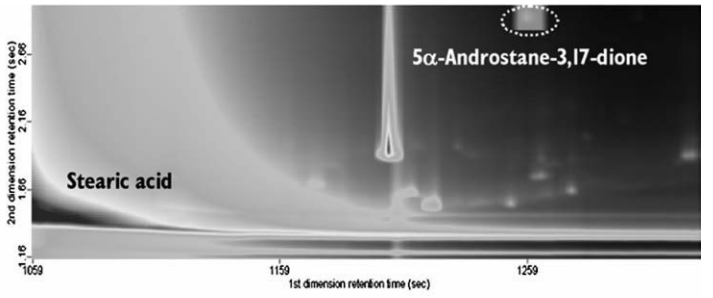


Figure 28. Illustration of non-target screening using GC×GC–TOF MS. The food supplement (creatine pyruvate) routinely controlled for the presence of several banned anabolic steroids (nandrolone, testosterone, 17α-methyl-testosterone, progesterone). While none of these compounds were detected, the result of data processing reported presence of generic compound 5α-androstane-3,17-dione with a similarity match of 841.

5. Mass spectrometric detection

Most of organic food toxicants are xenobiotics containing elements / groups other than those commonly occurring in natural components. On this account, GC coupled with various element / group selective and/or specific detectors used to be a traditional tool for screening residues occurrence. However, due to an inherent lack of structural information, GC coupled with mass selective detector was needed for confirmation to avoid biased results. In spite of some unique features of conventional (*e.g.* extreme sensitivity of ECD in detection of polychlorinated analytes), GC–MS has become in recent years the preferred primary tool for determination of a large number of food contaminants.

Advantages of GC–MS over traditional approaches include:

(*i*) Simultaneous quantification and confirmation of target analytes

(*ii*) Detection and identification of non-target sample components

(*iii*) Possibility to spectrometrically resolve co-eluting peaks

However, under certain circumstances simple MS detectors such as single quadrupole may due to high chemical noise fail to detect residues overlapped by abundant matrix interferences (this may be the case when low, unspecific ions *m/z* are yielded from an analyte). Either GC×GC separation or the use of tandem mass spectrometry may provide improved performance. To optimise MS detection/identification strategies and fully exploit potential of this technique, analyst should be familiar with all potential options in this area. The overview of fundamental information relevant to food toxicants analysis is summarised below.

5.1. Ionisation techniques

Depending on the purpose of analysis, ionisation potentially resulting in extensive fragmentation of particular molecule yielding characteristic ions pattern or soft ionisation providing information on molecular ion are the basic options.

5.1.1. Electron ionisation (*EI*)

In GC, the most widely used ionisation technique is electron ionisation (formerly called electron impact ionisation), in which sample molecules are in the first phase bombarded by high-energy electrons (70 eV), creating high-energy, single charged molecular ions followed by the lose excess energy via fragmentation, producing a collection of fragment ions characteristic of the compound [44]. EI is employed for generating spectra with controlled and reproducible fragmentation patterns. Virtually identical spectra can be obtained employing different types of EI mass spectrometer as long as the electron energy is the same, which has led to the compilation of extensive libraries for 70 eV EI spectra (*e.g.* the NIST—National Institute of Standard and Technology library and the Wiley library). EI is therefore preferred for identification of unknowns, determination of molecular structure and confirmation of target component identity through consistent ion abundance ratios.

5.1.2. Chemical ionisation (*CI*)

In some cases, however, EI fragmentation is too extensive, leaving little or no trace of a molecular ion, which makes the determination of the molecular weight (and, consequently identification of particular compound) somewhat difficult. This problem can be solved by applying of low energy or "soft" techniques such as *positive chemical ionisation* (PCI) [45]. In PCI, the ion source is charged with a reagent gas (*e.g.* methane), at a relatively high pressure (0.1–100 Pa), which undergoes EI ionisation, producing an excess of reagent ions. Sample molecules are subsequently ionised by the reagent gas ions via proton transfer, producing pseudomolecular ions and, depending on the choice of a reagent gas, adduct ions may be formed. The pseudomolecular ions generally have low internal energy and, consequently, they are less prone to the fragmentation than the molecular ions generated under EI conditions, providing unambiguous molecular-weight information. Due to little or no fragmentation, PCI is less suitable for confirmation, what can be, however, highly appreciate in some analysis, since the pseudomolecular ion is more intensive and specific than any lower-mass fragment ions [46]. In other words, PCI can offer both increased sensitivity and improved detectability (reduced chemical ionise from background or coeluting analytes results in increased S/N ratio).

The production of a large population of low-energy electrons during CI operation provides an opportunity for another ionisation technique: *negative chemical ionisation* (NCI), alternatively called *electron capture negative ionisation* (ECNI) or *negative ion chemical ionisation* (NICI). The basic mechanism of this technique is the same as that of an ECD: a low-energy electron is captured by an electronegative sample molecule, forming the molecular anion, which may undergo fragmentation, depending on its structure [44]. ENCI has two important advantages over EI and CI: (*i*) allowing a 100-fold or greater improvement in sensitivity, and (*ii*) it is highly selective, since only a limited number of analytes are prone to efficient electron capture (*e.g.* analytes containing a halogen atom, a nitro group, or an extended aromatic ring system). An illustration of the influence of ionisation techniques (EI *vs.* NCI) for the fragmentation of an analyte is shown in Figure 29.

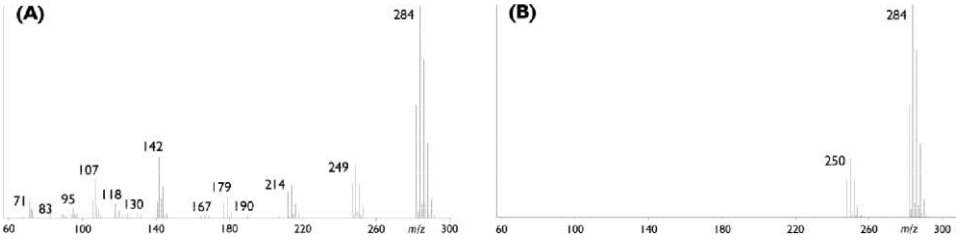


Figure 29. The (A) EI and (B) NCI (methane as a reagent gas) mass spectra of hexachlorobenzene. The ionisation techniques (EI *vs.* NCI) influence the amount of fragmentation.

5.2. Mass analysers

Wide range of MS instruments, both scanning and non-scanning are nowadays available for residue analysis The choice of optimal mass analyser depends on many aspects such as physico-chemical properties of analytes, complexity of matrix they are contained in, target performance characteristics (low detection limits are often prioritised criterion), instrument cost, demands on personnel competence, throughput demands *etc*. have to be considered. Listed below there are the key parameters characterizing MS systems that are closely associated with the type of mass analyser.

- *Mass range*. The limit of *m/z* over which a mass spectrometer can detect ions (*m/z*). The high mass range is less necessary in combination with GC since volatility/thermolability effectively dictates the upper mass limit.
- *Mass accuracy.* The absolute deviation between measured mass from true mass of an ion, typically expressed as an error value (ppm, mDa). This parameter is important for structural interpretation.
- *Spectral acquisition speed.* The time it takes to record the mass spectrum. For scanning instruments terms scans/s, s/scan, or s/decade are used; for non-scanning instruments term spectra/s is preferred. *Maximal spectral acquisition speed* is a critical parameter in detection of very narrow peaks generated in fast GC and/or GC×GC.
- *Acquisition mode.* The alternative modes of mass spectra collection in scanning instruments are full scan/spectra and selected ion monitoring (SIM). In the latter case structural information is sacrificed in favour of detection sensitivity.
- *Sensitivity.* Instrument characteristic often specified by the instrument producers as minimal signal-to-noise ratio at given concentration of a reference. For this purpose, compounds such as octafluoronaphthalene or hexachlorobenzene are widely used.
- *Linear dynamic range.* Range of analyte concentration over which the detector continues to proportionally respond to changes in its concentration. Narrow dynamic range makes quantification complicated.
- *Versatility.* Possibility of use different ionisation techniques (EI, PCI, NCI) hence extend the scope of applications.
- *Mass resolution.* Ability of the instrument to separate two ions of similar mass. Two approaches are generally used for classification of this parameter: (*i*) 10% valley—the difference between two peak masses when they are separated by a 10% valley; or (*ii*) full width at half maximum (FWHM)—value obtained by dividing the peak mass by the width of a peak at a point 50% above the baseline. This usually gives a number twice the magnitude of the 10% valley definition. Possibility of exact mass setting enables removing/reduce chemical noise and decrease detection limits. Ions with close masses can be resolved.
- *MS/MS.* Ability to provide tandem MS (either *in space* or *in time*). This function provides more selectivity and thanks to elimination of chemical noise, improved detectability.

- *Performance/cost*

Although there are many different types of MS analysers, the basic principles are the same in all cases: molecules introduced into ion source are ionised, ions are separated according to their mass-to-charge ratio (*m/z*), and accelerated towards a detector where they are counted. The data system compiles a spectrum showing the mass distribution of the ions produced from the sample – a snapshot of ion intensities plotted against their *m/z*. Basically, mass analysers can be divided into two groups: (i) scanning, and (ii) non-scanning. Non-scanning mass analysers, such as *time-of-flight* (TOF) or *Fourier transform ion cyclotron resonance instrument*, are characterised by the extremely fast acquisition rates and are therefore highly suitable as detectors for fast GC applications. Unfortunately, their cost is still substantially higher than the cost of common scanning mass spectrometers. The only considerably expensive scanning mass spectrometer is *magnetic sector* (*double-focusing*) *instrument*, offering a relatively wide mass range and high mass resolution. Nevertheless, in routine practise, less expensive and demanding scanning instruments – with *quadrupole* and/or *ion trap* analyser – are usually used. The general specifications and features of the most frequently used mass analysers are summarised in Table 5.

5.2.1. Quadrupole instruments

Four parallel metal rods of circular cross-section are electronically connected in pairs and a combination of DC and RF voltages are applied. By varying these voltages (scanning), at a fixed frequency, the mass spectrum is produced from low energy ions [47].

A quadrupole can be operated in two modes: (*i*) full scan (of a selected mass range, usually *m/z* 50–500), and (*ii*) selected ion monitoring (SIM). In SIM mode, sensitivity is enhanced by monitoring only few selected *m/z* ions, thus proportionally increasing the acquisition time of the ions of interest, but spectral information has to be sacrificed.

5.2.2. Ion-trap instruments

An ion trap is a device that stores ions for a period of time by the use of electric and/or magnetic fields [47].

In addition of full scan and SIM modes an ion trap can also be operated in MS/MS mode (potentially in MS^n, however, for relatively small molecules orders $n>3$ are not practical). The application of MS/MS mode results in increased specificity and improved detectability (increased S/N ratio) compared to both the SIM and full scan modes, however it requires careful (and relatively time-consuming) optimisation of several MS/MS parameters for all involved analytes, which is a certain drawback of this mode. In MS/MS, sample molecules are ionised in the ion source, specific (precursor, parent) ions are isolated and then fragmented into product (daughter) ions. An ion trap instrument is referred to as a *tandem-in-time* instrument, because the same ion region is used for all MS/MS processes.

Table 5

General specifications and features of currently available mass analysers

Criteria	Quadrupole	Triple quadrupole	Ion trap	DF magnetic sector	High-speed TOF	High-resolution TOF
Mass range	<1050 Da	<1500 Da	<1000 Da	<4000 Da	<1000 Da	<1500 Da
Mass accuracy			0.1–0.2 Da	<5 ppm		<5 ppm
Maximal spectral acquisition speed (*m/z* 50–500 Da)	15 Hz		5 Hz	7 Hz	500 Hz	20 Hz
Acquisition mode	Full scan, SIM, simultaneous full scan/SIM	Full scan, SIM, full scan of product ions	Full scan, SIM, full scan of product ions	Full scan, SIM	Full spectra	Full spectra
Sensitivity	pg in full scan fg in SIM	pg in full scan pg in MS/MS	pg in full scan	fg in SIM	pg	fg–pg
Linear dynamic range	>5 Orders of magnitude	>5 Orders of magnitude	4–5 Orders of magnitude	>5 Orders of magnitude	4 Orders of magnitude	4 Orders of magnitude
Versatility	EI, PCI, NCI	EI, PCI, NCI	EI, PCI, NCI	EI, PCI, NCI	EI	EI, PCI, NCI
Mass resolution	Unit mass	Unit mass	Unit mass	>10000 (10% valley definition)	Unit mass	>7000 (FWHM)
Performance/cost	Low	High	Low	Very high	High	High
MS/MS	None	MS^2 (*in space*)	MS^n, n = 2–10 (*in time*)	Only with special configuration	None	None

5.2.3. Time-of-flight instruments

Time-of-flight mass analyser identifies sample molecules by measuring their flight time in a field free tube. The gaseous ions generated in an ion source from neutral molecules of analyte are in the first phase accelerated to get constant kinetic energy and then ejected into a mass analyser using pulsed electric-field gradient oriented orthogonally to the ion beam (orthogonal acceleration TOF), which has a positive influence on mass resolution of the instrument. Further enhancement of mass resolution is obtained by using reflectron (ion mirror). This device consists of a series of ring electrodes with increasing voltage creating retarding fields. After reaching the reflectron area, ions with higher energy penetrate more deeply inside, what extents the time until they are reflected. As a consequence of this phenomenon, the ions of the same *m/z* value with different initial energies hit the detector at almost the same time. In addition, the mass resolution is substantially improved by making the ions to pass twice along a TOF flight tube before reaching the detector. The flight times of the ions separated in a field free region are proportional to the square root of respective m/z value. Compared to scanning instruments those used electron multipliers for detection of ions, the TOF MS systems employ mostly a multichannel plate (MPC) detector. The outgoing pulses are registered either by time-to-digital converter (TDC), or by analogue-to-digital (ADC)-based continuous averager, also called digital signal averager (DSA) or integrating transient recorder (ITR) [48, 49].

The effort to exploit unique features of rapidly developing TOF MS technique resulted in introduction of two types of spectrometers differing in their basic characteristics: (*i*) instruments employing high-resolution analysers characteristic with only moderate acquisition speed, and (*ii*) unit-resolution instruments that feature a high acquisition speed. The application potential of these approaches is obviously complementary.

5.2.4. Double-focusing magnetic sector instruments

A *single sector magnetic mass analyser* uses only a magnetic field to separate ions with different *m/z* ratios. The ions entering the mass analyser are initially accelerated using an electric field and only ions with a certain charge are passed through. These ions are then passed through a magnetic field. Charged ions tend to move in a circular trajectory in a magnetic field depending on their mass and thus reach the ion detector at different locations. The *double sector mass analyser* uses, on the contrary, an additional electric field to filter ions such that only ions with a certain kinetic energy are passed through to the magnetic sector. The ions are then separated according to their mass in the magnetic sector as before.

Due to its high specificity and sensitivity, GC coupled to high-resolution MS has been employed for many years and is still being used to solve some specific problems in different application areas. There are two main types of applications of HRMS. The first is based on a very high capacity to remove the contribution of matrix interfering compounds in the

determination of the analytes (*e.g.* analysis of polychlorinated dibenzo-*p*-dioxins and -furans). Using SIM mode at a mass resolution of 10000 (10% valley definition), the presence of abundant matrix components in the extracts does not interfere with the detection process and high level of mass accuracy can be obtained. The second type of application represents the determination of complex mixtures of contaminants (*e.g.* polychlorinated terphenyls), where, in addition to the matrix interferences, some problems arise due to interferences between fragment ions of congeners with different degrees of chlorination. In order to eliminate these specific interferences, HRMS at a resolving power higher than 10000 is needed [50].

5.3. Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) is a method involving at least two stages of mass analysis, either in conjunction with a dissociation process or a chemical reaction that causes a change in the mass or charge of an ion. Basically there are two different approaches in MS/MS: *in space* by coupling of two physically distinct parts of instrument (*e.g.* triple quadrupole, QqQ); or *in time* by performing an sequence of events in an ion storage device (*e.g.* ion trap, IT) [51].

The main tandem MS/MS scan modes are product ion, precursor ion, neutral loss, selected reaction monitoring, multiple reaction monitoring, and MS^n scans (Figure 30). MS/MS methods generally involve activation of selected ions, typically by collision with an inert gas, sufficient to induce fragmentation (collision induced dissociation, CID) [51].

- *The precursor ion scan* involves selection of the ion of interest, its activation and mass analysis of the product ions.
- *The product ion scan* represents opposite process compared to the precursor ion scan.
- *The neutral loss scan* involves scanning for a fragmentation (neutral loss of fixed, predetermined mass). It is useful for rapid screening in metabolic studies.
- MS^n is commonly applied on ion trap analysers. A precursor ion is selected and isolated by ejecting all other masses from the mass spectrometer. CID of the precursor ion yields ions that may have different masses (MS/MS). A product mass of an analyte is selected and other fragment ions are ejected from the cell. This product ion can be, again, subjected to CID, generating more product ions that are mass analysed (MS/MS/MS). This process can be repeated several times. However, as already mentioned, for small molecules such as those of food toxicants only MS/MS or MS/MS/MS is mainly used in practice.
- *Selected reaction monitoring* (*SRM*) is a special case of SIM in which a tandem instrument is used to enhance the selectivity of SIM.
- The term *multiple reaction monitoring* (*MRM*) is used if several different reactions are monitored.

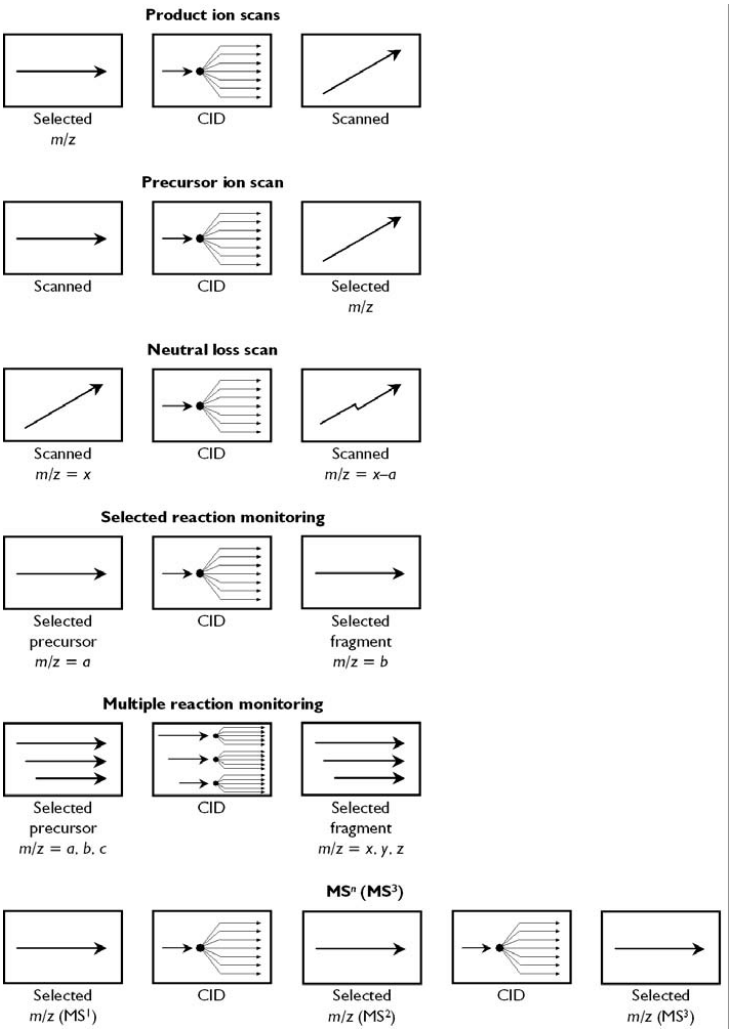


Figure 30 . Main processes in tandem MS; CID stands for collision-induced dissociation, as occurs when an inert gas is present in the collision cell

The main advantage of using MS/MS is the discrimination against the chemical noise, which can originate from different sources (*e.g.* matrix compounds, column bleed, contamination from an ion source). Figure 31 illustrates the effect of multiple MS/MS steps on S/N ratios. Each MS-step leads to some loss in intensity of the analyte signal, but a greater

loss in the noise signal and, consequently, S/N increases [52]. Practical example demonstrating improvement of detection of heptachlor present at trace level in fish oil extract is shown in Figure 32; in this case the use of tandem MS led to unambiguous identification/quantification of this analyte compared to single MS (SIM mode), or by using conventional detection (ECD).

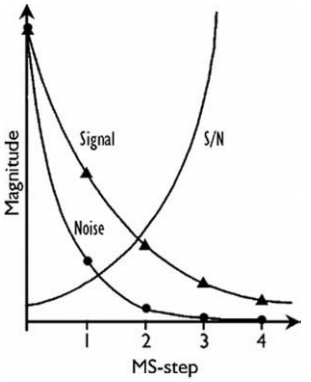


Figure 31. Influence of MS-step *vs.* signal, noise, and S/N (Reproduced (adapted) from [52] with permission from American Chemical Society© 1982).

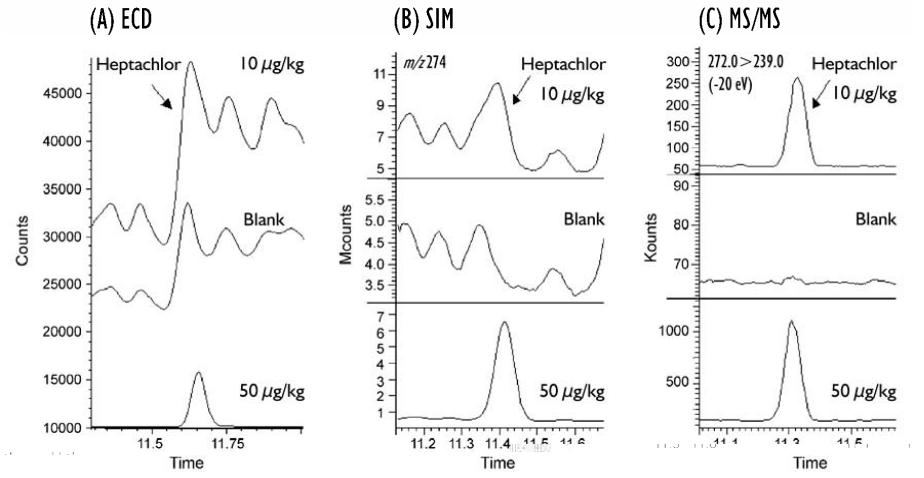


Figure 32. Comparison of selectivity between (A) ECD, (B) SIM, and (C) MS/MS for heptachlor at 10 µg/kg in fish oil. Chromatograms of the corresponding blank extract and a higher matrix-matched standard are provided for contrast and identification (Reproduced from [53] with permission from Elsevier© 2005).

6. Applications of GC–MS to food toxicants analysis

Since a large range of food toxicants is (semi)volatile compounds, the GC–MS is widely used for their control. The choice of an optimal GC–MS set-up depends on the requirements for the performance characteristics of methods used, cost, speed, and several other factors. In paragraphs below the current state-of-the-art of GC–MS methods for several groups of food toxicants is discussed with special attention paid to applicability of recent advantages in the field of this technique for their analysis.

6.1. Pesticides

Modern pesticides represent a group of food contaminants possessing a wind range of physico-chemical properties. With regards to this fact and considering a number of registered compounds (almost 1000), the analysis of multiple residues in various food matrices is obviously a difficult task. Although multiresidue methods based on LC–MS/MS are of a growing popularity in this fields, GS–MS methods still play a very important role in controlling residues and in some cases (poor ionisation under LC–MS conditions) remains the only methods of choice.

A conventional approach to GC analysis in multiresidue pesticide analysis employs capillary columns with low-bleed stationary phases mostly consisting of (5%-phenyl)-methylpolysiloxane (or cyanopropyl, cyanopropylphenyl or increased phenyl content up to 50%). Relatively long analytical column (30–60 m) of 0.25–0.32 mm inner diameters are commonly used in routine practice, with GC analysis time approaching or even exceeding 1 h. However, the growing number of required analyses leads to afford to decrease analysis time using fast GC techniques (mostly in combination with MS detection), thus increasing sample throughput and reducing laboratory operating costs. The types of MS instruments used for pesticide analysis include quadrupole, ion trap, and TOF systems. In the case of SIM and MS/MS modes time segments are needed, which limits the number of targeted analytes that can be detected in a given time. The selectivity of detection is increased, especially in MS/MS, but full-scan operation generally provides sufficient information for confirmation (depending on the pesticide) and can monitor hundreds of analytes, whereas SIM and MS/MS is typically limited to about 100 pesticides in a single chromatogram. This represents a critical point especially in fast-GC analysis. Other trade-offs with SIM relate to the difficulty of identifying analytes due to fewer ions monitored and higher chance of matrix interferences than in MS/MS [54, 55].

A recent progress in instrumentation design as well as the use of fast recording electronics together with improvements of signal processing techniques has led to “re-discovery” of TOF mass analysers (both types, high-resolution and high-speed) for determination of a wide range of pesticide residues. The advantage of using HSTOF MS is illustrated in Section 4.3.3 (Figure 25) on example of separation of dichlorvos from the abundant matrix co-extracts [43].

On the other hand, the main advantage of using the HRTOF MS instrument in GC analysis of phosalone illustrates Figure 33. Thanks to the high mass resolution and monitoring of exact mass of target analyte chemical noise originating from various sources (*e.g.* matrix co-extracts, column bleed) can be significantly reduced hence limit of quantification (LOQ) improved. In addition, the identification of analyte can be based not only on retention time and mass spectrum (as traditionally used in GC–MS) but also employing elemental composition determination, which adds further dimension [49].

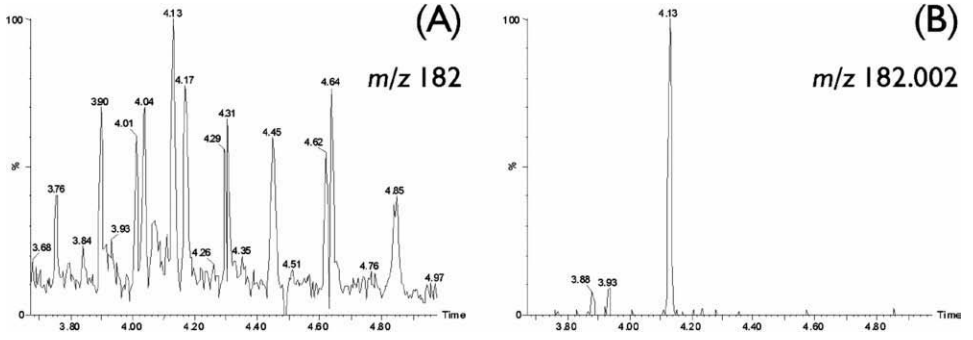


Figure 33. LP-GC–HRTOF MS chromatograms of phosalone (t_R = 4.13 min) at concentration 0.01 mg/kg in apple baby food extract. Different mass window setting (A) 1 Da, and (B) 0.02 Da used for extraction of quantification ion.

Various ionisation techniques are also possible in GC–MS to provide in particular case higher degree of information and lower LODs, nevertheless in the case of multiresidue analysis by GC–MS, EI is commonly preferred. CI (both PCI and NCI) as a softer ionisation technique tends to give lower LODs depending on the pesticide, but it is not as widely applicable in multiclass pesticide methods and does not provide as much structural information about the analyte as EI.

6.2. Polychlorinated biphenyls (PCBs)

PCBs are highly persistent, lipophilic, and bioaccumulative toxic industrial contaminants formed as a result of human activities. Their occurrence in food chain is therefore monitored in many routine laboratories. Capillary columns with high-resolution GC combined with specific detectors allow for good resolution of PCB congeners. GC separation and detection with ECD or MS is the method of choice for routine analysis. Typically, non-polar columns such as 100%-methylpolysiloxane or (5%-phenyl)-methylpolysiloxane are employed for their separation. However, because of coelution of a number of congeners (critical pairs), alternative phases such as (50%-phenyl)-methylpolysiloxane or (8%-phenyl)-polycarborane-siloxane have to be used. Since separation of all PCB isomers in real-world samples is hardly

feasible, the application of two columns in parallel using a single injection with splitting to the two columns that end in two ECD detectors is preferred.

The numerous operation modes that enhance the specificity of MS make it a flexible tool for PCB analysis. Although EI mode can be used, the NCI allows obtaining lower LODs of these analytes. GC–MS is a reliable technique for planar PCBs quantification because of its improved selectivity, particularly given by the availability of ^{13}C-labelled PCB standards. Either single quadrupole (SIM) or ion trap (MS/MS) is frequently applied in the routine analysis of PCBs. The use of HRMS permits quantification of lower PCBs differ by two chlorines because of the high mass resolution allows unbiased measurement of ions. In addition, the application of high-resolution TOF MS or high-speed TOF MS (the latter with GC×GC) represents a tool successfully applied in the analysis of PCBs [56, 57].

6.3. Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/PCDFs), dioxine-like polychlorinated biphenyls, and dioxine-like PCBs

PCDDs/PCDFs and dioxine-like PCBs are highly toxic substances. The analytical methods for these compounds require laborious and time-consuming sample cleanup processes. Additionally, analytical methods of high selectivity and high sensitivity are required since samples often contain matrix components (potential chemical interferences) at concentrations several orders of magnitude higher than those of target analytes. One of the key factor, which makes the analysis of dioxins so difficult, is the existence of many isomers (*i.e.* 75 PCDDs, 135 PCDFs). Since differences in toxicities of several orders of magnitude exist between various isomers, the separation and positive identification/quantification of each is a crucial task.

Since the monitoring of all PCDDs/PCDFs is hardly attainable, the legislation reduces the monitoring only of those compounds with the highest toxicological potential.

For GC separation long narrow bore capillary columns are often used (60–30 m × 0.32–0.25 mm × 0.25–0.15 μm) with different stationary phases: (5%-phenyl)-methylpolysiloxane (*e.g.* DB-5ms, HP-5ms, RTX-5ms, BPX5, CP-Sil 8 CB), (50%-cyanopropylphenyl)-dimethylpolysiloxane (*e.g.* DB-225ms, BP225), or 44%-methyl–28%-phenyl–20%-cyanopropylpolysiloxane–8% Carbowax 20M (DB-Dioxin).

Quantitative determination of PCDDs/PCDFs is often performed using low-resolution (unit mass) quadrupole or ion trap analysers or sector instruments, which are operated under low-resolution conditions. Although PCI/NCI techniques can be used, a majority of laboratories employ EI. High-resolution systems (mass resolution of 10000) have greater sensitivity than quadrupole systems, even if the high-resolution system is operated at low-resolution conditions. In addition, the HRMS allows higher selectivity compared to unit mass resolution instruments if levels of potentially interfering compounds are too great [58].

According the EU legislation (2002/69/EC), several specific requirements for GC–MS methods should be applied where foodstuffs are analysed for the official control of the levels of dioxins and the determination of dioxine-like PCBs:

- Addition of ^{13}C-labelled 2,3,7,8-chlorine substituted internal PCDD/F standards and ^{13}C-labelled internal dioxine-like PCB standards must be carried out at the very beginning or start of the analytical method in order to validate the analytical procedure.
- At least one congener for each of the tetra to octa-chlorinated homologous groups for PCDD/F and at least one congener for each of the homologous groups for dioxine-like PCBs must be added.
- The use of all 17 ^{13}C-labelled 2,3,7,8-substituted internal PCDD/F standards and all 12 ^{13}C-labelled internal dioxine-like PCB standards is preferred for a confirmatory method.
- Prior to GC–MS analysis, 1 or 2 recovery (surrogate) standard(s) must be added.
- Separation of dioxins from interfering chlorinated compounds (*e.g.* PCBs, chlorinated diphenyl ethers) should be carried out by suitable chromatographic technique (*e.g.* florisil, alumina and/or carbon column).
- Gas chromatographic separation of isomers should be sufficient (<25% peak-to-peak between 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF).
- Positive results have always to be confirmed by a confirmatory method (GC–HRMS).

In addition, a relatively lower accent on method performance characteristics (mainly for precision and recovery of internal standards) is required for screening methods compared to the confirmatory methods (Table 6).

6.4. Polybrominated diphenyl ethers (PBDEs)

PBDEs belong to a group of brominated flame retardants, chemicals widely used in various products (*e.g.* plastics, textiles, furnishing foams) to prevent a fire hazard. Currently, there is a growing interest in PBDE analysis in environmental and food samples due to the continual increase in the levels of these compounds in the general environment and human tissues during the last decade also related health concerns.

Although 209 BDE congeners are theoretically possible, only a small number of these contaminants can be found in technical PBDE mixtures (*e.g.* BDE 28, 47, 99,100, 153, 154, 183, 209), what allows to use a single-capillary GC column that offers sufficient resolution for a congener-specific PBDE determination. A non-polar or medium-polar column, *e.g.* 100% methyl-polysiloxane, (5%-phenyl)-dimethyl polysiloxane, 14% cyanopropylphenyl 86% dimethyl-polysiloxane, with a length of 25–60 m and small diameters (<0.25 mm) are most frequently employed. The use of sufficiently long columns is important for achieving enough separation between BDE congeners and possible interferences.

Table 6. Characterisation of screening and confirmatory methods for the analysis of dioxins.

	Screening method	Confirmatory method
Objectives	• Detection of presence of dioxins and dioxin-like PCBs • Capacity for a high sample throughput • Used to sift large numbers of samples for potential positives • Designed to avoid false negatives	• Full or complementary information enabling those compounds to be identified/quantified unequivocally at the level of interest
Performance characteristics		
• False positive rate	<1%	
• Trueness		–20% to +20%
• Precision	<30%	<15%
• Recovery of internal standards	30%–140%	60%–120%

Special attention is usually paid to BDE 209 because of its sensitivity for higher temperatures and the higher susceptibility for degradation in the GC system. For that reason, the analysis of BDE 209 is often carried out separately from the analysis of the other PBDEs. Typically, non-polar columns (100% methyl-polysiloxane) with a length of 10–15 m and 0.1–0.2 μm film thickness. The relatively short column reduces the residence time of this congener.

The most widely used detectors for determination of PBDEs are mass spectrometers operated either in EI or NCI. The LRMS is more routinely applied compared to the HRMS that requires more experienced users and is much more costly and labour intensive. However, HRMS has a number of advantages over LRMS (increased sensitivity and selectivity), but is almost exclusively operated in EI mode. For LRMS, NCI, in addition of EI, can be applied to obtain an increased sensitivity for higher brominated BDE congeners. Although the EI is not routinely used for determination of PBDEs, this technique is preferred for the identification of mixed organohalogenated compounds. Another advantage of EI mode is possibility to use ^{13}C-labelled internal standards for a more precise determination of the recovery of the analysis. This is not possible in NCI, since generally only the Br^- ions (*m/z* 79 and 81) are monitored. The benefits of NCI are efficient ionisation, higher sensitivity, and less fragmentation than EI. Worth to notice that the spectra obtained using NCI are strongly

dependent on the experimental conditions (type of reagent gas, reagent gas pressure, the instrument used, and the ion source temperature) [59].

The potential of GC coupled to high-resolution TOF MS under NCI conditions in the simultaneous analysis of PBDEs and PCBs is documented in Figure 34. In addition, quantitative results for high-resolution TOF MS and quadrupole (both operated in NCI) are compared demonstrating comparable quality of generated data (Table 7).

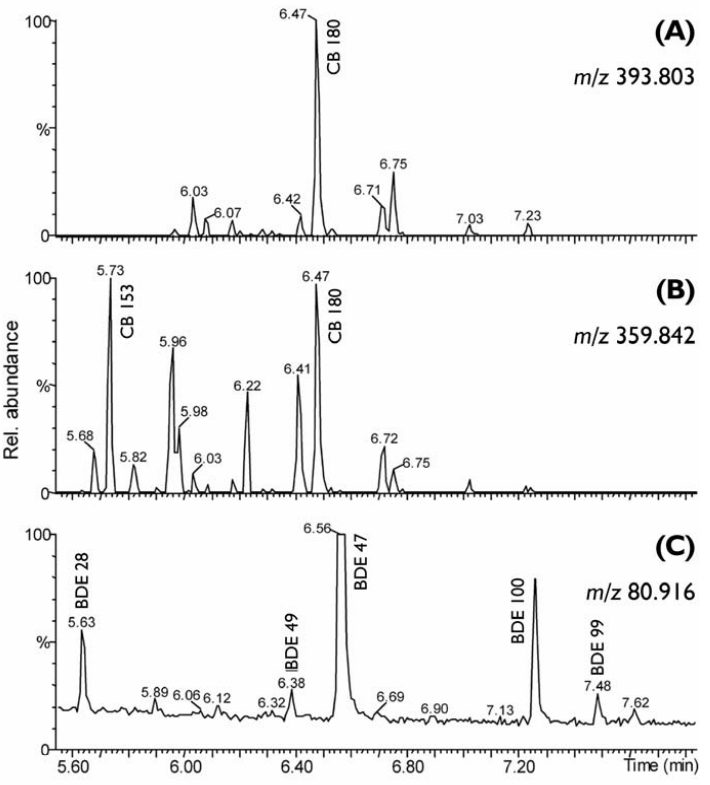


Figure 34. GC–HRTOF MS chromatograms of selected PCBs and PBDEs in fish extract in NCI mode. Ions extracted using a mass window of 0.02 Da. (A) Chromatogram of ion *m/z* 393.803 corresponding to hepta-CBs. (B) Chromatogram of ion *m/z* 359.842 corresponding to hexa-CBs. (C) Chromatogram of ion *m/z* 80.016 corresponding to PBDEs (Reproduced from [60] with permission from Wiley© 2005).

Table 7. Comparison of levels (mean±SD) of PBDEs (ng/g muscle) in fish extract employing alternative detection.

Congener	HRTOF MS *)	Quadrupole **)
BDE 28	0.10±0.01	0.10±0.01
BDE 47	2.23±0.20	2.30±0.22
BDE 49	0.05±0.00	0.05±0.00
BDE 99	0.09±0.01	0.12±0.01
BDE 100	0.40±0.03	0.30±0.03
BDE 153	0.12±0.01	0.16±0.01
BDE 154	0.18±0.02	0.24±0.02

*) 0.32 mg sample equivalent injected.
**) 6.4 mg sample equivalent injected.

6.5. Polycyclic aromatic hydrocarbons (PAHs)

Due to the higher concentrations of PAHs in the environmental samples GC–MS operated in SIM mode represents appropriate technique for their determination. In the case of food sample, where PAHs can be present at ultratrace levels, the use of HPLC–UV is generally preferred. However, due to the improvement in detection, the MS can also be used as a tool for determination of their residues. As an example, the occurrence of selected PAHs in olive oil extract is shown in Figure 35, which combines the advantages of coupling GC×GC to TOF MS.

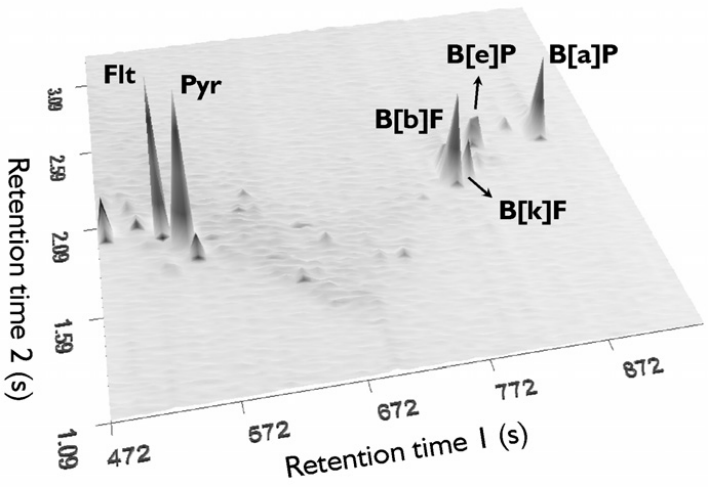


Figure 35. Separation of PAHs present in olive oil extract using GC×GC–TOF MS (LC50×BPX-50 columns).

6.6. Mycotoxins

The GC–MS methods for determination of mycotoxins are performed either by direct analysis or (mostly) employing various chemical derivatisations. Two groups of derivates can be applied, the trimethylsilyl ethers and the perfluoro-esters. In the case of GC separation of derivate mycotoxins, non-polar columns fit to the purpose. The use of MS detection of pentafluoropropionyl or heptafluorobuturyl esters allows to achieve better sensitivity if the MS can be operated with a mass range >*m/z* 800. The fluorinated derivates can be detected selectively to very low levels using NCI but also EI or PCI. Although quadrupole MS (SIM) is mostly used, the high molecular weight of derivatised analytes predetermines their molecules for MS^n-measurements, especially in complex matrices like foodstuff and beverages [61, 62].

6.7. Veterinary drug residues

The use of any pharmacologically active chemical to a food-producing animal inevitably leads to the occurrence of residues in food. Due to the widely use of drugs in veterinary medicine as therapeutic or grown-promoting agents, the development of analytical methods for the determination of drug residues in meat and other animal by-products (milk, egg) represents an important task of toxicological and regulatory concern. Although in resent years, LC–MS-based strategies have been widely applied for confirmation of veterinary drug residues, also some GC–MS-based methods exist although their main drawback is the need of extensive clean-up and time-consuming derivatisation procedures prior to GC analysis. As examples can serve the determination of sulphonamide residues in egg and milk [63].

6.8. Acrylamide

Discovery of acrylamide occurrence in various heat-processed starch-rich foodstuffs in 2002 resulted in an urgent requirement for the development of a reliable analytical procedure allowing to control the levels of this, potentially carcinogenic, contaminant in a wide range of various food matrices.

The majority of current GC–MS methods employ bromination to obtain a less polar, more volatile target analytical form yielding more specific ions (higher *m/z*). Instead of 2,3-dibromopropionamide, 2-bromopropenamide, generated under experimental conditions via dehydrobromination, is employed due to its better stability. Some laboratories, however, find using bromine uncomfortable, pointing at certain workplace hazards; moreover, the sample preparation procedure involving the derivatisation step is relatively laborious and time consuming. For GC separation of derivatised acrylamide non-polar and medium polar column are frequently used.

Alternative approach to the analysis of derivatised acrylamide represents its direct GC–MS determinations. In this case, however, special attention has to be paid to sample preparation strategy to avoid co-isolation of acrylamide precursors (sugars, asparagine) those increase the

risk of additional analyte formation in the hot GC injector, generating acrylamide overestimation. Due to a higher polarity compared to its derivatised form, separation is mainly carried out on polar columns.

In both cases, isotopically labelled internal standards (*e.g.* d_3-acrylamide, ^{13}C-acrylamide) are employed for compensation of potential target analyte losses and/or matrix effects.

For the final determinative step, mass spectrometric detection employing either quadrupole or ion trap/triple quadrapole (tandem MS) can be used in EI or CI mode. Alternatively, HRMS (sector or TOF) instruments (if available) are applied in the acrylamide analysis, since due to the mass resolution interfering compounds (chemical noise) with ions close to those of acrylamide can be eliminated. In this way improvement of S/N ratio can be effectively achieved [64, 65].

6.9. Chloropropanols

1,3-Dichloropropan-2-ol (1,3-DCP), 3-monochloropropane-1,2-diol (3-MCPD), 2-monochloropropane-1,2-diol (2-MCPD) are the most toxic chloropropanols found as contaminants in acid-hydrolysed vegetable protein. All resent methods for the determination of these toxicants are based on GC–MS after their previous derivatisation using phenylboronic acid, butaneborinic acid, heptafluorobutyric acid anhydride, heptaluorobutyrylimidazole, or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide. Narrow bore non-polar columns ((5%-phenyl)-methylpolysiloxane) are used for separation of chloropropanols with quadrupole (SIM) or ion trap analysers (MS/MS) for their detection. As in the case of acrylamide, the use of isotopically labelled internal standards (d_5-3-MCPD, d_7-3-MCPD, d_5-1,3-DCP) is employed to improve accuracy of the method [66, 67].

References

[1] J. Hajšlová and J. Zrostlíková, J. Chromatogr. A, 1000 (2003) 181.
[2] D.R. Erney, A.M. Gillespie, D.M. Gilvydis, J. Chromatogr. 638 (1993) 57.
[3] B. Dušek, J. Hajšlová, V. Kocourek, J. Chromatogr. A, 982 (2002) 127.
[4] A. Fajgelj, Á. Ambrus (eds.), Principles and Practices of Method Validation, Royal Society of Chemistry, Cambridge, UK, 2000.
[5] K. Maštovská, S.J. Lehotay, J. Hajšlová, J. Chromatogr. A, 1054 (2004) 335.
[6] D.R. Erney, T.M. Pawlowski, C.F. Poole, J. High Resolut. Chromatogr. 20 (1997) 375.
[7] Quality control procedures for pesticide residues analysis – guidelines for residues monitoring in the European Union, 3rd ed., European Commission document SANCO/10476/2003, Brussels, 2004.
[8] M. Anastassiades, K. Maštovská, S.J. Lehotay, J. Chromatogr. A, 1015 (2003) 163.
[9] K. Maštovská, S.J. Lehotay, M. Anastassiades, Anal. Chem., 77 (2005) 8129.
[10] T. Čajka, K. Maštovská, S.J. Lehotay, J. Hajšlová, J. Sep. Sci., 28 (2005) 1048.
[11] M. Godula, J. Hajšlová, K. Alterová, J. High Resolut. Chromatogr. 22 (1999) 395.
[12] E. Matisová, M. Dömötörová, J. Chromatogr. A, 1000 (2003) 199.
[13] Y. Ito, Y. Ikai, H. Oka, J. Hayakawa, T. Kagami, J. Chromatogr. A, 810 (1998) 81.

[14] H.B. Wan, M.K. Wong, P.Y. Lim, C.Y. Mok, J. Chromatogr., 662 (1994) 147.
[15] A. Covaci, P. Schepens, J. Chromatogr. A, 923 (2001) 287.
[16] J. Zrostlíková, J. Hajšlová, M. Godula, K. Maštovská, J. Chromatogr. A, 937 (2001) 73.
[17] H.J. Stan, H.M. Müller, J. High Res. Chromatogr., 11 (1988) 140.
[18] B. Grolimund, E. Boselli, K. Grob, J. High Res. Chromatogr., 21 (1998) 378.
[19] R. Bailey, J. Environ. Monit., 7 (2005) 1054.
[20] K. Grob, Z. Li, J. High Res. Chromatogr., 11 (1988) 626.
[21] K. Grob, Th. Läubli, J. High Res. Chromatogr., 11 (1988) 462.
[22] M. Termonia, B. Lacombiez, F. Munari, J. High Res. Chromatogr., 11 (1988) 890.
[23] J. Staniewski, J.A. Rijks, J. High Res. Chromatogr., 16 (1993) 182.
[24] M. Godula, J. Hajšlová, K. Maštovská, J. Křivánková, J. Sep. Sci, 24 (2001) 355.
[25] H. Jing, A. Amirav, Anal. Chem., 69 (1997) 1426.
[27] K. Patel, R.J. Fussell, D.M. Goodall, B.J. Keely, Food Addit. Contam., 21 (2004) 658.
[28] S. de Koning, G. Lach, M. Linkerhägner, R. Löscher, P.H. Tablack, U.A.Th. Brinkman, J. Chromatogr. A, 1008 (2003) 247.
[29] S.J. Lehotay, J. AOAC Int., 83 (2000) 680.
[30] H. Kataoka, H.L. Lord, J. Pawliszyn, J. Chromatogr. A, 880 (2000) 35
[31] K. Maštovská, S.J. Lehotay, J. Chromatogr. A, 1000 (2003) 153.
[32] K. Maštovská, J. Hajšlová, M. Godula, J. Křivánková, V. Kocourek, J. Chromatogr. A, 907 (2001) 235.
[33] P. Tollbäck, J. Björklund, C. Östman, J. Chromatogr. A, 991 (2003) 241.
[34] J. de Zeeuw, J. Peene, H.G. Jansen, X. Lou, J. High Resol. Chromatogr. 23 (2000) 677.
[35] K. Maštovská, S.J. Lehotay, J. Hajšlová, J. Chromatogr. A, 926 (2001) 291.
[36] J. Dallüge, R.J.J. Vreuls, D.J. van Iperen, M. van Rijn, U.A.Th. Brinkman, J. Sep. Sci, 25 (2002) 608.
[37] H.M. McNair, G.L. Reed, J. Microcol. Sep., 12 (2000) 352.
[38] T.D. Bucheli, R.C. Brändli, J. Chromatogr. A, 1110 (2006) 156.
[39] J. Dallüge, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A, 1000 (2003) 69.
[40] J. Dallüge, R.J.J. Vreuls, J. Beens, U.A.Th. Brinkman, J. Sep. Sci., 25 (2002) 201.
[41] M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, TrAC-Trend Anal Chem, 25 (2006) 438.
[42] M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, TrAC-Trend Anal Chem, 25 (2006) 540.
[43] J. Zrostlíková, J. Hajšlová, T. Čajka, J. Chromatogr. A, 1019 (2003) 173.
[44] W.G. Fong, H.A. Moye, J.N. Seiber, J.P. Toth (eds), Pesticide residues in food: methods, techniques, and regulations, John Wiley & Sons, UK, 1999.
[45] A.G. Harrison, Chemical ionization mass spectrometry, CRC Press, USA, 1983.
[46] G.C. Mattern, G.M. Singer, J. Louis, M. Robson, J.D. Rosen, J. Agric. Food Chem., 38 (1990) 402.
[47] J. Barker, Mass spectrometry, John Wiley & Sons, UK, 1999.
[48] M. Guilhaus, D. Selby, V. Mlynski, Mass Spec. Rev., 19 (2000) 65.
[49] T. Čajka, J. Hajšlová, J. Chromatogr. A, 1058 (2004) 251.
[50] F.J. Santos, M.T. Galceran, J. Chromatogr. A, 1000 (2003) 125.
[51] E. de Hoffmann, V. Stroobant, Mass spectrometry, principles and applications, John Wiley & Sons, UK, 2002.
[52] R.G. Cooks and K.L. Busch, J. Chem. Educ., 59 (1982) 926.

[53] K. Patel, R.J. Fussell, M. Hetmanski, D.M. Goodall, B.J. Keely, J. Chromatogr. A, 1068 (2005) 289.
[54] P. Worsfold, A. Townshend and C. Poole (eds.), Encyclopedia of Analytical Science, Elsevier, UK, 2005.
[55] S. Otles (ed.), Methods of Analysis of Food Components and Additives, CRC Press, 2005
[56] F.E. Ahmed, TrAC-Trend Anal Chem, 22 (2003) 170.
[57] J. de Boer, J. Chromatogr. A, 843 (1999) 179.
[58] R.E. Clement, H.M. Tosine, Mass Spec. Rev., 7 (1988) 593.
[59] A. Covaci, S. Voorspoels, J. de Boer, Environ. Int., 29 (2003) 735.
[60] T. Čajka, J. Hajšlová, R. Kazda, J. Poustka, J. Sep. Sci, 28 (2005) 251.
[61] K.F. Nielsen, U. Thrane, J. Chromatogr. A, 929 (2001) 75.
[62] Y. Onji, Y. Aoki, N. Tani, K. Umebayashi, Y. Kitada, Y. Dohi, J. Chromatogr. A, 815 (1998) 59.
[63] M. Careri, F. Bianchi, C. Corradini, J. Chromatogr. A, 970 (2002) 3.
[64] Y. Zhang, G. Zhang, Y. Zhang, J. Chromatogr. A, 1075 (2005) 1.
[65] L. Dunovská, T. Čajka, J. Hajšlová, K. Holadová, Anal. Chim. Acta (2006), in press, doi:10.1016/j.aca.2006.07.001
[66] S. Abu-El-Haj, M.J. Bogusz, Z. Ibrahim, H. Hassan, M.A. Tufail, Food Control, 18 (2005) 81.
[67] W. Chung, K. Hui, S. Cheng, J. Chromatogr. A, 952 (2002) 185.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 13

Liquid chromatography with conventional detection

Mervi Rokka, Marika Jestoi, Susanna Eerola

Finnish Food Safety Authority (Evira), Department of Animal Diseases and Food Safety Research, Unit of Chemistry and Toxicology, Mustialankatu 3, FIN-00790 Helsinki, Finland

1. Introduction

High performance liquid chromatography (HPLC) is the most commonly used separation technique in the chemical analyses of food toxicants. Although it lacks the high resolution power of gas chromatography (GC), it is capable of separating compounds with a wide range of chemical and physical properties. HPLC is the preferred choice for the determination of thermally labile, polar and non-volatile compounds. In addition, it allows simultaneous determination of several compounds - even with variable chemical properties.

As compared to spectrophotometric methods, which also have been applied to food toxicant analyses, chromatographic methods offer special advantages, such as the confirmation of positive detection by the retention time. In addition, in case the toxicant appears as enantiomers, proper chromatographic systems are able to separate these optically-active isomers. Chromatographic systems moreover able to diminish the effect of detection-interfering compounds often present in sample extracts.

Thin layer chromatography was gradually replaced by HPLC in food toxicant analysis, which offered advantages such as the ability to handle larger samples, higher resolution and reliable quantitative detection [1,2]. Compared to GC, HPLC presents numerous advantages when applied to the study of organic contaminants in foods: a complex clean-up is unnecessary, reducing the likely sources of error and increasing absolute recoveries; and there is no need to use “appropriate” internal standards to correct for poor recovery and/or instrumental errors due to injection discrimination [3]. In addition, most often GC requires derivatization prior analysis, which can reduce reproducibility and therefore add uncertainty.

HPLC is suitable to determine some GC-amenable compounds. In some cases, however, the sensitivity of the method is lower using GC, as is the case for malondialdehyde in milk powder [4]. Also, polycyclic aromatic hydrocarbons (PAH’s) can be separated using either GC or HPLC. With connection to proper detectors (mass spectrometry or fluorescence detector, respectively) both methods provide selective and sensitive analyses. Reversed phase-HPLC is, anyhow, suitable in particular for PAH’s with high molecular weight [5]. HPLC is somewhat faster than GC and offers lower resolution efficiency in separating low-molecular

mass PAH's, but instead it can readily separate a number of PAH-isomers that are difficult to separate with GC [6].

To date, hundreds of food toxicants have been recognized, and the chemical diversity of these compounds is truly enormous. These toxic compounds can be found, usually at low concentrations, in a range of matrices with differential chemical compositions, e.g. grains, cheese and wine. Therefore the background effect may complicate the analytical techniques used for the determination of food toxicants [7].

2. Separation modes for food toxicant analysis

Several modes of separation have been used in the HPLC analyses of chemical food toxicants. Although normal phase (NP)-separations (with polar stationary phase and apolar mobile phase) were earlier successful in many cases, nowadays reversed phase (RP)-separations have been used more often, as for most compounds both techniques are applicable. The reason for the generalization of RP-HPLC is mainly due to the better performance obtainable and the flexibility in the selection of (modified) sorbents. In fact, there are no applications employing NP-separations (Tables 1.-4).

Ion-pairing - and ion-exchange -chromatography are also utilized in food toxicant analyses [e.g. 8–13]. These techniques are well suitable for the separation of polar compounds, the retention of which may otherwise be cumbersome. The formation of an ion-pair or the use of ion-exchange sorbents enables the retention of these compounds, allowing the successful separation, for instance for cationic biogenic amines [14]. The same applies for acidic compounds such as mycotoxin moniliformin [15].

Latest technological innovations are originating from the development of new LC column packing materials. These include e.g. narrow-bore columns and new stationary phase sorbents [16]. For instance, the availability of special column materials for PAH-analysis eliminated common disadvantage - poor resolution [17]. Figure 1 represents a chromatogram of perfectly separated PAH-isomers using RP-PAH stationary phase. Hydrophilic interaction chromatography (HILIC), instead, can replace ion-pairing chromatography in case desired separation can not be achieved [18].

3. Detection systems

3.1. UV/VIS-detection

The most common detection technique in the analysis of food toxicants is spectrophotometry with UV/VIS-detectors. It is based on the measurement of the intensity of absorption of ultraviolet (UV) or visible light (VIS) at the wavelengths of 190-800 nm by

sample chromophores, that is molecules that absorb light (i.e. double-bond, carbonyl- or aromatic-group). The concentration of the analyte in sample can be determined by measuring the absorbance at specified wavelength and applying the Lambert-Beer law (1):

$$A = \varepsilon \cdot c \cdot d \qquad (1)$$

A = absorbance
ε = molar absorbtion coefficient
c = concentration
d = light path

The light source of an UV/VIS-detector is usually a deuterium (UV) or a tungsten lamp (VIS). The wavelengths of these continuous light sources are selected with a wavelength separator i.e. monochromator (prism, grating or filter) before the light beam passes the sample cell.

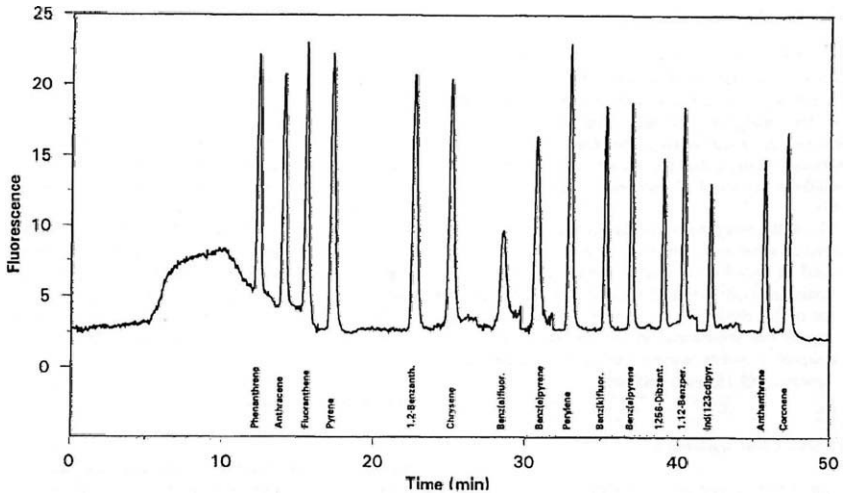


Figure 1. Chromatogram of fully separated PAH-isomers in spiked olive oil, separated using RP-PAH stationary phase (Reproduced from [128] with permission from Elsevier© 1996).

The advantages of UV/VIS-detectors include their simplicity of use, broad linear range, moderate sensitivity and robustness. Problems may arise regarding selectivity as an adequate separation of sample components or the need of more selective but less sensitive detection wavelength. Generally, the use of UV/VIS-detection for confident sample identification is

limited but it is very useful for quantitative measurements, especially in cases when a generic idea of the structure of the analyte is known. The lowest detection capability is at ppb-levels. It must be taken into account that low wavelengths applied will decrease the selectivity and sensitivity of detection, as UV-detection is not a specific technique. The lower the detection wavelength used the more interferences will occur in the analysis. This necessitates intense sample preparation, especially when complex sample matrices are concerned, as was reported for N-methylcarbamate insecticides in vegetables [19] and heterocyclic amines in different foods [20]. The choice of the analysis method will therefore always depend on sample nature and on the number of compounds that are present in it [19].

Recently, post-column reaction systems in HPLC have attracted increasing interest as a mean of converting compounds with unfavourable detection properties into derivatives which show enhanced sensitivity and often a higher selectivity towards such specific detectors as fluorescence and electrochemical detectors. Unfortunately, this technique is not simple because it needs prior laborious optimization of the derivatization reactions that can reduce the final recoveries of some compounds. Also more complicated, expensive and non-universal equipment is also needed. Cardoso et al. [21] instead, used 2,4-dinitrophenolhydrazone to derivatize ketones and aldehydes in spirits to achieve a simultaneous analysis of these compounds using UV-detection with high reproducibility and sensitivity. In addition, as the absorption maximum of the derivatives was higher than those of the parent molecules, higher detection wavelength could be used for the detection, the interferences resulting from the background were reduced and higher selectivity was obtained.

Conventional UV-detection has been applied for several food toxicants on various kinds of food matrices. Detailed information of the methods is presented in Table 1.

Biogenic amines represent a group of harmful compounds that can be analysed using UV-detection. UV allows the detection of tyramine, tryptamine, phenylethylamine and histamine. However, putrescine and cadaverine do not absorb light without derivatisation and therefore either pre- or post-column derivatisation are needed for their successful determination. Another possibility for the detection of biogenic amines is to use ion-pair chromatography without derivatisation [38]. The limits of detection for biogenic amines using UV-detection are at the ppm-level depending on the analyte. Also some of the toxic secondary metabolites produced by filamentous fungi, mycotoxins, can be determined using UV. Mycotoxins having chromophores include type-B trichothecenes (e.g. deoxynivalenol), beauvericin, moniliformin, fusaproliferin and some *Penicillium*-toxins (e.g. cyclopiazonic acid, mycophenolic acid). Type-A trichothecenes, instead, lack the carbonyl-group at the C8-position, and therefore UV-detection can not be applied. In addition to biogenic amines and mycotoxins, other food toxicants analysed using simple UV include e.g. drug residues, aldehydes, shellfish toxins and glycoalkaloids (Table 1).

Table 1. Ultraviolet detection applied in food toxicants analysis

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
PAH's					
	edible oils			RP-C_{18}	[22]
	smoked foods		0.02-0.56 µg/kg	RP-C_{18}	[23]
	smoked foods		0.02-0.39 ppb	RP-C_{18}	[24]
	fish		2-20 ng/ml	RP-C_{18}	[1]
Toxic carbonyl compounds					
hydroxymethylfurfural	honey		1.4 mg/kg	RP-C_{18}	[25]
Malondialdehyde	milk powder	TBA, DNPH	1 ng	RP-C_{18}	[4]
2-furaldehyde, 5-hydroxymethyl-2-furaldehyde	beer	DNPH		RP-C_{18}	[26]
Malondialdehyde	broiler meat	DNPH		RP-C_{18}	[27]
Aldehydes	alcoholic beverages	DNPH	10-50 µg/l	RP-C_{18}	[28]
Hexanal	potato crisps	DNPH	9 ng/ml	RP-C_{18}	[29]
Mycotoxins					
Patulin	apple, baby-food, apple juices			RP-C_{18}	[30]
DON, NIV, FX	wheat		20-40 ppb	RP-C_{18}	[31]
NIV, DON	maize		0.02 mg/kg	RP-C_{18}	[32]
MON	corn		0.025 µg/g	RP-C_{18}	[11]
FUS, BEA	maize			RP-C_{18}	[33]
MON, ZEN, DON	corn			RP-C_{18}	[34]
DON	cow's milk		5 ng/ml	RP-C_{18}	[35]
Biogenic amines					
	dry sausage	dansyl chloride	1-5 mg/kg	RP-C_{18}	[36]
	cheese	dansyl chloride		RP-C_{18}	[37]
	cheese, chocolate,wine,		0.2-0.8 mg/kg	RP-IP	[38]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
	fish, sauerkraut				
	cheese, meat, sausage, fish	dabsyl chloride	0.12-0.52 pmol	RP-C_{18}	[39]
	salmon, tuna, salami, cheese	dansyl chloride		RP-C_{18}	[6]
	cheese	dabsyl chloride	1.5 mg/l	RP-C_{18}	[40]
	canned mackerel	Benzoyl chloride		RP-C_{18}	[41]
tyramine	cheese	NBD-Cl	8 µg/g	RP-C_{18}	[42]
Heterocyclic amines					
	meat, pan residue		0.01-0.03 ng/g	RP-C_{18}	[43]
	meat			RP-TSK gel	[44]
Drug residues					
Malachite green	fish		1 µg/kg	RP-C_{18}	[45]
Fenbendazole	pig tissue			RP-C_{18}	[46]
3-amino-2-oxazolidone	liver			RP-C_{18}	[47]
Phenylbutazone	bovine milk			RP-C_{18}	[48]
Tylosin	honey		<0.2 mg/kg	RP-C_{18}	[49]
Rofecoxib	breast milk			RP-C_{18}	[50]
PSP toxins					
Ciguatoxin	red bass, red emperor			RP-C_{18}	[51]
Neurotoxins PbTX-2, PbTX-3, brevetoxin	shellfish			RP-C_{18}	[52]
Mushroom toxins					
Agaritine	mushroom		3 mg/kg	RP-C_{18}	[53]
Psilocin, psilocybin	mushroom			RP-C_{18}	[54]
Psilocin, psilocybin	mushroom			RP-C_{18}	[55]
Furanocoumarins					
Bergamottin	grapefruit			RP-C_{18}	[56]
	celeriacs			RP-C_{18}	[57]
	fruits			RP-C_{18}	[58]
Glycoalkaloids					

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
	potato tuber			RP-C_{18}	[59]
	potato tuber, top		0.2 ppm	RP-NH_2	[60]
	potato tuber, sprout			RP-C_{18}	[61]
Pesticides	drinking water			RP-C_{18}	[62]
Carboxylic acids	foods, beverages	2-nitrophenyl-hydrazine hydrochloride, EDC		RP-C_8 RP-C_{18}	[63]
Glucosinolates	vegetables			RP-C_{18}, RP-HILIC	[18]
N-methylcarbamate	vegetable		0.5-7.5 ng	RP-C_{18}	[19]

Abbreviations:
TBA: thiobarbituric acid
DON: deoxynivalenol
FX: fusarenon X
FUS: fusaproliferin
ZEN: zearalenone
PbTX-3: Ptychodiscus brevis toxin 3
DNPH: 2,4-dinitrophenylhydrazine
NIV: nivalenol
MON: moniliformin
BEA: beauvericin
PbTX-2: Ptychodiscus brevis toxin 2
NBD-Cl: 4-chloro-7-nitrobenzofurazan
EDC: 1-ethyl-3-(dimethylaminopropyl)carbodi-imide hydrochloride

Diode array –detectors (DAD) supplement additional features to spectrophotometric detection compared with the conventional UV/VIS-detectors. In addition to the identification by the retention time, qualitative information of the analyte can be obtained with DAD. DAD allows the selection of the wavelength(s) with the highest absorbance for separate analytes. Moreover, DAD allows the peak purity testing by absorbance rationing when the peak shape in itself does not reveal the possible co-elution of (an)other compound(s). In absorbance rationing, the absorbance is measured at two wavelengths and the ratio is calculated. In addition to the peak purity testing, the use of absorbance measurement at two different wavelengths allows the removal of the impurity from the chromatogram as well as the qualitative identification of the analytes using spectral libraries. In Figure 2. is represented chromatograms of corn samples analysed with HPLC-DAD for mycotoxin zearalenone and the scanned UV-spectrums to confirm the identification. UV/VIS spectral libraries are useful tools for confirmation of peak identity. Second order derivatives reveal more specific details of the spectra [64].

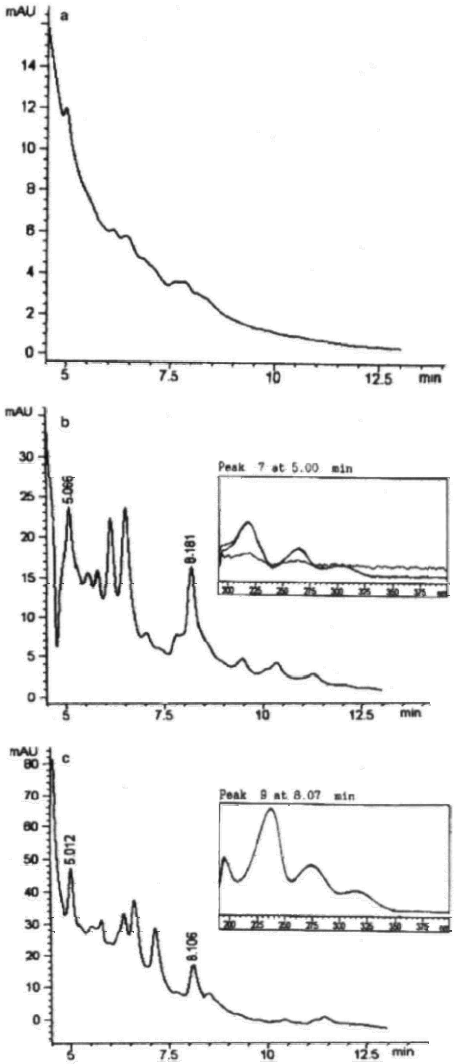


Figure 2. HPLC-DAD chromatograms (λ = 274 nm) for a blank corn sample (a), blank corn sample spiked with 100 ppb zearalenone (RT 8.1 min) (b) and a blank corn sample spiked with 200 ppb of zearalenone. Zearalanol (RT 5.0 min) was used as an internal standard. UV spectrums of zearalanol and zearalenol were used to confirm the identification (Reproduced from Ref. [192] with permission from Elsevier© 2006)

Table 2. Diode array detection applied in food toxicants analysis.

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
PAH's					
	water			RP-C_{18}	[68]
Toxic carbonyl compounds					
Ketones	spirits, rum	DNPH	10-20 µg/l	RP-C_{18}	[21]
5-hydroxymethyl-furfuraldehyde	apple juice		< 0.01 mg/l	RP-C_{18}	[70]
Furaldehydes	apple juice		0.025 mg/l	RP-C_{18}	[71]
Aldehydes	honey		0.01-0.08 mg/l	RP-C_{18}	[72]
5-hydroxymethyl-furfuraldehyde	honey			RP-C_{18}	[73]
Mycotoxins					
Patulin	apple juice		< 0.01 mg/l	RP-C_{18}	[70]
MON	maize		0.12 mg/kg	ion-exchange	[9]
Penicillium-toxins	cheese, bread			RP-C_{18}	[74]
BEA	corn		50 µg/kg	RP-C_{18}	[75]
Penicillium- toxins	chestnuts			RP-C_{18}	[76]
Ochratoxin A	wine, beer		0.05 µg/l	RP-C_{18}	[77]
Mycotoxins					
Mycophenolic acid	blue cheese		3 ppb	RP-NH_2	[78]
Biogenic amines					
	sea bream	benzoyl chloride		RP-C_{18}	[79]
	beef		1.0 µg/g	RP-C_{18}	[80]
	beef		0.5-1.0 µg/g	RP-C_{18}	[81]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
Heterocyclic amines					
	meat extract		0.1-14 ng/ml	RP-TSK-Gel, RP-C_{18}	[82]
	fried meat		2.0-5.0 mg	RP-C_8	[83]
	fast-food, meat products			RP-TSK-Gel, RP-C_{18}	[65]
	pork, juice, egg, bean cake		0.03-0.3 ng	RP-C_{18}	[84]
	meat			RP-TSK-Gel, RP-C_{18}	[85]
	meat dishes	PFPA	0.4-2.0 ng	RP-TSK-Gel, RP-C_{18}	[86]
Drug residues					
Thiouracil, tapazole, methylthiouracil, propylthiouracil, phenyltiouracil	bovine matrices		2.45-4.52 ng/g	RP-C_{18}	[87]
Enrofloxacin, ciprofloxacin	goat milk		15 µg/kg	RP-C_8	[88]
Nitrofurazone, furazolidone, furaltadone	egg		2.5-5.0 µg/kg	RP-C_{18}	[89]
PSP toxins					
Domoic acid	sand crabs and mussels		2.5 µg	RP-C_{18}	[90]
Domoic acid	blue whale		0.2 µg/ml	RP-C_{18}	[66]
Okadaic acid, dinophysistoxin-1	shellfish	ADAM	0.26 µg/g	RP-C_{18}	[91]
Domoic acid	shellfish			RP-C_{18}	[92]
Mushroom toxins					

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
Agaritine	mushrooms		0.02 µg/ml	RP-C_{18}	[93]
Furanocoumarins					
6'7'-Dihydroxybergamottin	grapefruit juice			RP-C_{18}	[94]
Furanocoumarins	fruit			RP-C_{18}	[95]
Glycoalkaloids					
	S. brevidens' leaves			RP-C_{18}	[96]
Glucosinolates	plant *A. thaliana*			RP-C_{18}	[97]
Glucosinolates	plants			RP-C_{18}	[13]
Carbofuran	water		0.06-8.9 µg/l	RP-C_{18}	[98]
Glycyrrhizin	roots of *Glycyrrhiza* plants			RP-C_{18}	[99]
Glycyrrhizin	roots of *Glycyrrhiza* plants			RP-C_{18}	[100]
Glycyrrhizin	root of liquorice			RP-C_{18}	[101]

Abbreviations:
MON: moniliformin
NIV: nivalenol
3-AcDON: 3-acetyl deoxynivalenol
PFPA: pentafluoro-propionic acid
BEA: beauvericin
DON: deoxynivalenol
15-AcDON: 15-acetyl deoxynivalenol
ADAM: 9-anthryldiazomethane

DAD is preferred for complex chromatograms including matrix impurities and several analytes with different absorption maxims, due to its above mentioned broader features compared with simple UV (Table 2.). One example of food toxicants commonly analysed using DAD is heterocyclic amines. In fact, the presence of the heterocyclic amines is difficult to confirm by simple UV spectra in many cases [65].

Several studies have used the peak purity option of DAD to confirm the absence of co-eluting compounds [e.g. 25, 66] sometimes in on-line connection with other analytical detectors [43, 67, 68]. Pascale et al. [69] also used this option to confirm the positive detection of mycotoxin fusaproliferin and observed that by using simple UV-detection, matrix impurities could interfere the analysis.

3.2. Fluorescence detection

Electromagnetic spectroscopy is based on a three-stage process - excitation, excited-state lifetime and emission. This phenomenon occurs in certain types of molecules, fluorophores, and it is called fluorescence. It is accomplished with a beam of (UV)-light, that excites the electrons of fluorophores to vibrational states. Usually, the fluorescence excitation spectrum of a fluorophore is identical to its absorption spectrum. After the excited electron returns to the electronic ground state, it loses the excess energy. This causes the emission of light, the intensity of which can be measured.

Fluorescence detectors (FL) consist of an excitation source (UV-lamp), wavelength separators, a sample cell and a detector that records emission photons. Overall, the only difference to UV/VIS-detectors is that two optical systems are needed (separators for excitation and emission photons). As the detection of a component depends on the chosen excitation and emission wavelengths, a selective detection of analytes is permitted. Modern fluorescence-detectors allow the switch of the excitation and emission wavelengths, which enables the simultaneous detection of several components in a mixture.

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance (Lambert-Beer –law), fluorescence quantum yield of the analyte, excitation source intensity and fluorescence collection efficiency of the instrument. In addition, several physico-chemical parameters affect the intensity of fluorescence: pH, solvent, temperature and concentration of the analyte.

Only about 15% of compounds have a natural fluorescence. The presence of conjugated π-electrons especially in the aromatic components gives the most intense fluorescent activity. Other fluorophores are carbonyl- and amino-groups as well as conjugated double bonds. Typical compounds with natural fluorescence include PAH's and mycotoxins ochratoxin A and zearalenone. In case of deficiency or weak natural fluorescence, a derivatisation to achieve a fluorescent molecule is commonly carried out. Both pre- and post-column derivatisation techniques have been used with several different derivatisation reagents [102] (Table 3.), such as most often used reagents *o*-phthalaldehyde (OPA) [e.g. 103, 104], dansyl chloride [e.g. 105], 9-anthryldiazomethane [e.g. 106, 107] and 1-bromoacetylpyrene [e.g.108, 109]. In Table 3., if the derivatization reagent is not presented, the compound analysed is naturally fluorescent.

Fluorescence detectors are by far among the most sensitive HPLC-detectors. Fluorescence sensitivity may be even 10-1000 times higher than that of the UV/VIS-detectors [20, 110, 111] or electrochemical detectors [112]. In some cases fluorescence detection also has been more sensitive than gas chromatography connected to mass spectrometry [111]. The reason for the high sensitivity of fluorescence techniques is that the emission signal is measured above a low background level. In addition to sensitivity, fluorescence detectors are very specific and selective. The excellent selectivity of fluorescence detection is shown in Figure 3., which represents a chromatogram of a corn sample spiked 200 ppb zearalenone and α-zearalenol. Please refer to Figure 2. to observe the dramatic difference between UV/VIS-detection as compared to FL. It must e, however, noticed that before FL-detection more intense sample purification (immunoaffinity clean-up) was used, as compared to UV/VIS-detection. Table 3 presents the LC fluorescence applications for determining food toxicants.

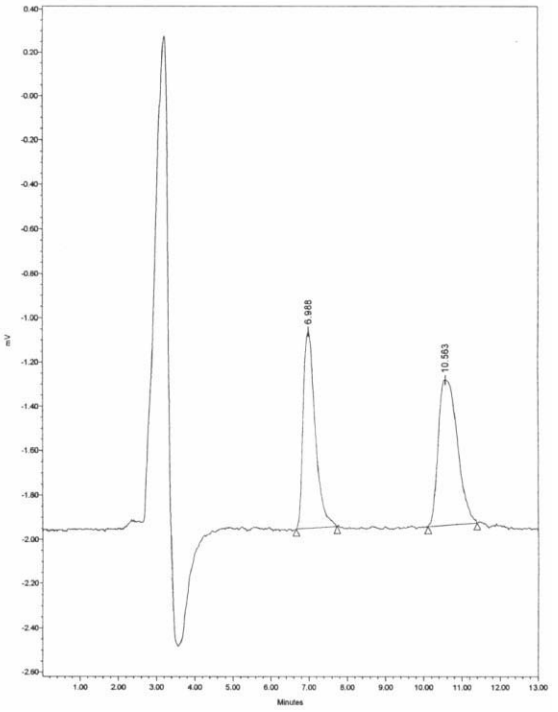


Figure 3. A chromatogram of a corn sample spiked with 200 ppb zearalenone (RT 10.6 min) and α-zearalenol (RT 7.0 min) (Reproduced from [193] with permission from Elsevier© 2003).

Table 3. Fluorescence detection applied in food toxicants analysis

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
PAH's					
Benzo[a]pyrene	Mozzarella		0.0058 µg/kg	RP-C_{18}	[113]
	edible oils			RP-C_{18}	[22]
	edible oils, mayonnaises, margarines, oils from canned fishes		0.05-1.5 ng/g	RP-C_{18}	[114]
nitro-PAH's	smoked food (fish, meat, cheese)	zinc		RP-C_{18}	[115]
	drinking water		0.007-1.3 µg/l	RP-C_{18}	[116]
Benzo[a]pyrene	smoked food		0.0019-0.0067 µg/kg	RP-C_{18}	[117]
Benzo[a]pyrene	smoked foods		0.049 µg/l	RP-C_{18}	[118]
	instant coffee		0.01-0.05 µg/kg	RP-C_{18}	[3]
	chorizo sausage		0.05-4.0 µg/kg	RP-C_{18}	[119]
	smoked foods		0.01-0.4 µg/kg	RP-C_{18}	[23]
	Meat			RP-C_{18}	[120]
	Meat			RP-C_{18}	[121]
	smoked foods		0.03-0.44 ppb	RP-C_{18}	[24]
	Milks		1.3-76 ng/kg	RP-C_{18}	[111]
	vegetable oil		0.08-10.1 µg/l	RP-C_{18}	[122]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
	fish, shellfish, meat products		2-27 pg/inj.	RP-C_{18}	[123]
	spinach, malt, cooking oils, dairy products, milled wheat, cereals			RP-C_{18}	[124]
	Italian diet			RP-C_{18}	[2]
	Shellfish		0.25-10 ng/g	RP-C_{18}	[125]
	Water			RP-C_{18}	[68]
	Seafood		0.12-5.96 ng/ml	RP-C_{18}	[5]
Benzo(a)pyrene	olive oils		0.5 µg/kg	RP-C_{18}	[67]
	grilled food			RP-C_{18}	[126]
Nitro-PAH's	meat, sausage, fish, cheese	$NaBH_4$	0.5-150 pg	RP-C_{18}	[127]
	edible oils, fats		< 0.1 µg/kg	RP-C_{18}	[128]
	fish, shellfish			RP-C_{18}	[129]
Benzo[a]pyrene	vegetable oils		0.23 µg/kg	RP-C_{18}	[130]
	different meals			RP-C_{18}	[131]
	fish bile		20-480 ng/g	RP-C_{18}	[132]
	vegetable			RP-C_{18}	[17]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
	oils				
	smoked foods		0.11-0.80 μg/kg	RP-C_{18}	[133]
Toxic carbonyl compound					
Malondialdehyde	dry sausage	TBA		RP-C_{18}	[134]
Aldehyde-bisulfites	beer	OPA	5-25 nM	RP-C_{18}	[112]
1,3-diethyl-2-thiobarbituric acid-malonaldehyde adduct	fish	BHT	5 pmol	RP-C_{18}	[135]
Mycotoxins					
Ochratoxin A	wheat, kidney			RP-C_{18}	[136]
Type-A trichothecenes (T-2-, HT-2-, DAS), type-B trichothecenes (NIV, DON, FX, 3-AcDON, 15-AcDON)	cereal	coumarin-3-carbonyl chloride	0.2-1 ng/g	RP-C_{18}	[137]
FB_1, FB_2	corn	OPA	0.03-0.05 μg/g	RP-C_{18}	[104]
Aflatoxin M1	milk			RP-C_{18}	[138]
Moniliformin	cereals	DDB	0.02 mg/kg	RP-C_{18}	[139]
Zearalenone	maize		0.1 mg/kg	RP-C_8	[32]
Ochratoxin A	roasted and instant coffee		0.2 ng/g	RP-C_{18}	[140]
Mycotoxins					
Ochratoxin A	wheat			RP-C_{18}	[141]
Ochratoxin A	beer		3 ng/l	RP-C_{18}	[142]
Aflatoxin M1	milk		50 ng/kg	RP-C_{18}	[143]
Ochratoxin A	wine, beer			RP-C_{18}	[144]
Biogenic amines					
Putrescine, cadaverine	beef	OPA	1.0 μg/g	RP-C_{18}	[80]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
	hake	OPA		ion-exchange	[145]
	cheese	OPA	0.066-0.149mg/100g	RP-C_{18}	[146]
	tulum cheese			RP-C_{18}	[147]
Heterocyclic amines					
	beef extracts			RP-TSK Gel, RP-C_{18}	[148]
	meat, pan residue		0.01-0.03 ng/g	RP-TSK-Gel, RP-C_{18}	[43]
	fast-food meat products			RP-TSK-Gel, RP-C_{18}	[65]
	meat			RP-TSK-Gel, RP-C_{18}	[44]
	beef extracts		0.02 ng/g	RP-C_{18}	[149]
Drug residues					
Ivermectin	sheep milk	N-methyl-imidazole TFA (anhydride)		RP-C_8	[150]
Ivermectin, moxidectin	bovine milk	N-methyl-imidazole		RP-C_{18}	[151]
Ivermectin, doramectin	pig liver	1-methyl-imidazole, TFA (anhydride)	0.8 ng/g	RP-C_{18}	[152]
Abamectin, doramectin, eprinomectin, ivermectin, milbemectin, moxidectin	bovine muscle, liver	N,N-dimethylformamide-acetic anhydride-1-methylimidazole		RP-C_{18}	[153]
Oxolinic acid, flumequine	salmon		3.2 ng/g	RP-C_{18}	[154]
Emamectin,	salmon	1-methyl-imidazole,	0.5 ng/g	RP-C_{18}	[155]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
ivermectin		TFA (anhydride)			
PSP toxins					
dinophysistoxin-1, okadaic acid	mussel	ADAM		RP-C_{18}	[156]
saxitoxin, neosaxitoxin, gonyautoxins	mussel	H_5IO_6	0.03-0.20 nmol/g	RP-C_{18}	[157]
Okadaic acid	mussel	ADAM		RP-C_{18}	[158]
	shellfish	H_5IO_6, H_2O_2		RP-C_{18}	[159]
Okadaic acid, dinophysistoxin-1, dinophysistoxin-2, dinophysistoxin-3	smoked mussel	ADAM		RP-C_{18}	[106]
Okadaic acid, dinophysistoxin-2	mussel	BAP	0.1 µg OA/g	RP-C_{18}	[108]
Okadaic acid, dinophysistoxin-1, dinophysistoxin-2, dinophysistoxin-3	mussel, clam, scallop	BAP	0.5 ng OA on-colum	RP-C_{18}	[109]
Okadaic acid, dinophysistoxin-1	mussel, mussel products	ADAM	0.08 mg DSP/kg	RP-C_8, RP-C_{18}	[160]
Domoic acid	seafood	NBD-F	< 1 ng/ml	RP-C_{18}	[161]
Okadaic acid, dinophysistoxin-1, dinophysistoxin-2	mussel	BAP, ADAM	0.4 ng on-column	RP-C_{18}	[162]
Saxitoxin, gonyautoxins, tetrodotoxin	puffer fish			RP-C_{18}	[163]
Gonyautoxins, saxitoxin	shellfish	O_2, IO_4		RP-C_{18}	[164]
Okadaic acid, dinophysistoxin-1	shellfish	CA	12-20 ng/g	RP-C_{18}	[165]
Saxitoxin, neotoxin,	shellfish	MnO_2	0.1-2.0 ng/inj.	polymer RP	[166]

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref
gonyautoxins					
Saxitoxin, gonyautoxins	mussel	KIO_4		RP-C_8	[167]
Gonyautoxins, saxitoxin	shellfish	H_5IO_6	20-120 fmol	RP-C_{18}	[168]
Okadaic acid, dinophysistoxins	shellfish	ADAM		RP-C_{18}	[107]
Saxitoxin, gonyautoxins	shellfish	H_5IO_6, HCO_2NH_4, NaOH		RP-C_{18}	[169]
Saxitoxin, gonyautoxins	green mussels	IO_4, O_2		RP-C_{18}	[170]
Glycoalkaloids					
	potato tuber			RP-C_{18}	[171]
Carbamates					
carbanyl, carbofuran, methiocarb	cucumber, strawberry	OPA		RP-C_{18}	[172]
N-methylcarbamates	apple, pear, cucumber	NaOH, OPA	3-12 ng/g	RP-C_{18}	[173]
N-Methylcarbamate	apple, broccoli, cabbage, cauliflower, potato	OPA, 2-mercaptoethanol	5-10 ppb	RP-C_{18}	[174]
N-methylcarbamates	pear, apple, cucumber	OPA	< 0.5 mg/kg	RP-C_{18}	[103]
N-Methyl carbamate	fruit, vegetable	OPA	0.8-1.9 ng/ml	RP-C_8	[110]
***N*-Nitrosamine**	beer	HBr, dansyl chloride	0.08-0.75 µg/l	RP-C_{18}	[105]
PCBs, PCDFs	mussel, clam, cockles		5.3-104 ng/ml	RP-C_{18}	[175]

Abbreviations:
$NaBH_4$: sodium borohydride
OPA: *o*-phthalaldehyde
DAS: diacetoxyscirpenol
DON: deoxynivalenol
3-AcDON: 3-acetyl deoxynivalenol
FB_x: fumonisin BX
$PHFB_1$: partially hydrolyzed fumonisin B1
DDB: 1,2-diamino-4,5-dichloro-benzene
TBA: thiobarbituric acid
BHT butyl hydroxyl-toluene
NIV: nivalenol
FX: fusarenone X
15-AcDON: 15-acetyl deoxynivalenol
HFB_1: hydrolysed fumonisin B 1
NH_3: ammonia
HBO_2: boric acid

KOH: potassium hydroxide
ADAM: : 9-anthryldiazomethane
H_2O_2: hydrogen peroxide
NBD-F: nitrobenzofurazan
IO_4:periodate
NaOH: sodium hydroxide
CA: 9-chloromethylanthracene
HBr: hydrobromic acid
PCDF: polychlorinated dibenzofurans
TFA: trifluoroacetic
H_5IO_6: periodic acid
BAP: 1-bromoacetylpyrene
O_2: peroxide
HCO_2NH_4: ammonium formate
KIO_4: potassium periodate
MnO_2: manganese dioxide
PCB: polychlorinated biphenyl
$HCONH_2$: formamide

Polyaromatic hydrocarbons (PAH´s) represent an excellent example of fluorophores, the molecular properties of which can be selectively and sensitively analysed using fluorescence detection. Their different optimal emission and excitation wavelengths means that programmed fluorescence detection can be gainfully used for their simultaneous determination [176]. Detection parameter settings previously reported in the literature, or measured on other equipments, might not give the best results for compounds under investigation. It might also be necessary to further modify the conditions according to the relative concentrations and sensitivities of the components in the samples. When complete resolution of components is not achieved, a compromise set conditions must be used for these components [176]. For the fluorescence analysis of nitro-PAH´s, a reduction to amino-PAH´s is needed to achieve enough sensitivity [127]. Although, PAH´s can be detected more specifically using fluorescence (Table 3.), also UV has been used for their determination [22] but some limitations e.g. the four lightest PAH´s (naphthalene, acenaphthylene, acenaphthene, fluorene) coeluted with fat fraction and could not be analysed. FL permits the determination of PAH´s at ppb-level, the same as in UV-detection.

The major impurities in the PAH´s fractions appear to be alkylated PAH´s, which have very similar responses in optical detection systems to their unsubstituted analogues [177]. Confirmation of peak purity and identification is possible by using DAD, but due to the broad absorption bands in UV spectra it is highly probable that there will be some interference, if one particular wavelength is chosen for quantification. Fluorescence detection provides very high selectivity and sensitivity [178].

Paralytic Shellfish Poisoning (PSP) toxins are non-volatile and have no useful UV-absorbing chromophores, so analysis by GC or HPLC-UV/VIS detection is not possible [166]. Therefore, fluorescence detection is commonly applied for the determination of shellfish poisons. The current reference method in the European Union is the mouse bioassay, but alternative methods including the HPLC methodology are preferred for ethical reasons [179]. Shellfish poisons can easily be derivatised into their fluorescent derivatives. 9-fluorenylmethylchloroformate (FMOC) has been used for toxin analysis in phytoplankton and in sea water samples using fluorometric detection [161]. Although improved detection limits were reported, this method was not successful for the analysis of shellfish tissue [180]. The main problems with shellfish tissue analysis are associated with chromatographic

interferences due to reagent products and other compounds in the matrix, which is common problem in the case of food contaminant analyses. Fluorometric analysis [181] of the 9-anthrylmethyl derivatives of the acidic diarrhetic toxins has become an established method and this protocol was recently subjected to critical examination [182]. The derivatising reagent, 9-anthrylmethyldiazomethane (ADAM), is somewhat unstable and this may give rise to interferences from artefact peaks in HPLC [162]. Post-column derivatization for paralytic shellfish toxin has a great advantage over other method in its ability to quantitate each toxin in a crude sample of small size. It is a powerful tool in the research field, especially for analysis of toxin production by dinoflagellates [162]. However, unstable reagents such as ADAM require special storage conditions and constant tests to ensure stability of reagent. The use of 9-chloromethylanthracene for derivatisation of okadaic acid and dinophysistoxin-1 should significantly reduce these requirements while still maintaining good reproducibility and similar detection limits as ADAM reaction [165].

Other examples for common fluorescence analysis are determination of mycotoxins. Fluorescence detection coupled to HPLC is an official technique for analysis of ochratoxin A in cereals as well as for aflatoxins in milk, cereals and nuts [183 – 186]. Method performances were demonstrated in interlaboratory trials, showing good sensitivity and reproducibility. Whereas, ochratoxin A and aflatoxins show natural fluorescence and can be detected without previous derivatization step, common *Fusarium*-mycotoxins trichothecenes lack this property. Recently, Jiménez et al. [187] and Dall'Asta et al. [137] reported a novel method for determination of both type-A and type-B trichothecenes with fluorescence detection after derivatisation using coumarin-3-carbonyl chloride. Comparable limits of detections (at ppb-levels) with GC-MS were achieved. Different sources of variation in the GC-analysis of trichothenes have been observed [188], contributing to the uncertainty of analyses, and therefore HPLC with fluorescence detection could be interesting choice providing more precise quantification with less sample preparation.

Other food toxicants determined with fluorescence include e.g. carbamate-pesticides, biogenic and heterocyclic amines and drug residues (Table 3).

3.3. Electrochemical detection

The electrochemical detector (ED) gives a response to molecules that are able to either oxidize (lose an electron) or reduce (gain an electron). In electrochemical detection, the signal is a response to a chemical reaction occurring at the electrodes of the detector, in contrast to physical measurements as is the case e.g. in UV/VIS or fluorescence detectors. The concentration of an analyte can be determined by measuring the produced current and applying the Faraday's law. The compounds that are capable to be measured using electrochemical detectors include phenols, aromatic amines, thiols and nitroso-compounds but

also some aliphatic molecules. Electrochemical detection is particularly preferred to ionic compounds.

Electrochemical detector consists of three electrodes: working (surface for oxidation/reduction reactions), auxiliary (conduction of electricity from the signal source into the solution) and reference (to compensate changes in the background conductivity) electrodes. The nature of the working electrode is critical to the detector performance. Commonly used electrode materials include carbon, gold, nickel, silver, mercury or platinum. Electrochemical reactivity is highly dependent on the electrode material. In addition, a prerequisite for the electrochemical reaction to occur is that the working electrode is chemically inert. Since electrochemical detection depends on the transfer of electrons between mobile phase and electrode surface, the mobile phase needs to be conducting. The conductance is achieved by having salt dissolved in the mobile phase. This may set restrictions to the mobile phase composition. Usually, aqueous mobile phases are used, though a totally nonaqueous mobile phase can also be used as long as an appropriate salt is dissolved. Moreover, the mobile phase must permit the ionization of the analyte and it must be chemically inert to minimize the background signal.

Pulsed electrochemical detectors extend the applicability of the use of electrochemical detection by eliminating the contamination of electrodes, which is a common problem using conventional electrochemical detectors with constant potential. In pulsed detectors, the potential of the working electrode is varied to prevent the accumulation of reaction products on the electrode surface. With pulsed detectors, higher sensitivity can be achieved.

Generally, electrochemical detectors are very sensitive and selective. Sometimes electrochemical detectors have a debatable reputation due to the difficulties with the contamination and subsequent short lifetime of electrodes as well as to the sensibility to the temperature variations. It is, however, important to understand the difference of the operational process between electrochemical detectors and optical detectors, and that is also responsible for the high sensitivity achievable by using electrochemical detectors. Table 4 contains information on the applications of food toxicants using ED. Overall, the ED-applications are very rare as compared to other detection techniques (Tables 1.-3).

Electrochemical detectors have frequently been used to quantify heterocyclic amines [e.g. 10, 190]. However, matrix interferences may occur. Electrochemical detection is especially applicable to analyse mixtures of polar and less polar amines in different matrices [190]. Aminoimidazoquinoline and –quinoxaline derivatives (heterocyclic amines) gave a clean chromatogram and good detection limits with electrochemical detection. Also indolpyridine and imidazopyridine derivatives could be successfully analysed [148]. Usually, the limits of detection obtained with electrochemical detection for heterocyclic amines are 2-4 times better than the ones obtained by UV or fluorescence [20].

Table 4. Electrochemical detection applied in food toxicants analysis.

Compound	Matrix	Derivatisation reagent	LOD	Column	Ref.
Toxic carbonyl compound					
Hydroxymethylfuraldehyde, furaldehyde	coffee powder, instant coffee powder, honey, dry fruits, breakfast cereals, sherry, refreshments, fruit juice	aldehyde hydrogenase, NADH oxidase		RP-C_8	[189]
Mycotoxins					
DON, NIV, FX	wheat		10-20 ppb	RP-C_{18}	[31]
Biogenic amines					
	kiwi, cheese, fish, dry fermented sausage		1.25-2.50 ng	IE	[89]
Heterocyclic amines					
	beef extracts			RP-C_{18}	[148]
	beef extracts		0.8-2.5 ng/g	RP-C_{18}	[190]
	soup cube		0.1-0.42 ng	phenyl-hexyl	[10]

Abbreviations:
NADH: β-nicotinamide adenine dinucleotide
DON: Deoxynivalenol
NIV:nivalenol
FX: fusarenon X

It must be noted that additives, such as triethylamine or diethylamine, frequently used for improving peak shapes of. heterocyclic amines with UV or fluorescence detection, can not be incorporated into the mobile phase when working with ED. These additives would increase the background noise and, therefore, detection limits. Tailed peaks can appear under these conditions thus reducing sensitivity. In addition to the limited mobile phases, ED is not necessary enough for confirmation of heterocyclic amines, and other systems such as DAD have to be employed for this purpose [e.g. [20, 148].

The use of electrochemical detection of biogenic amines enables also a simultaneous analysis of their amino acid precursors from different sample matrices with less sample preparation. Accoring to Draisci et al. [89] the method offers a simplified, sensitive, selective and rapid alternative to spectrophotometric and fluorimetric HPLC-methods, which require intense purification and laborious derivatisation. In Figure 4. is represented a chromatogram of biogenic amines in cheese detected using electrochemical detection.

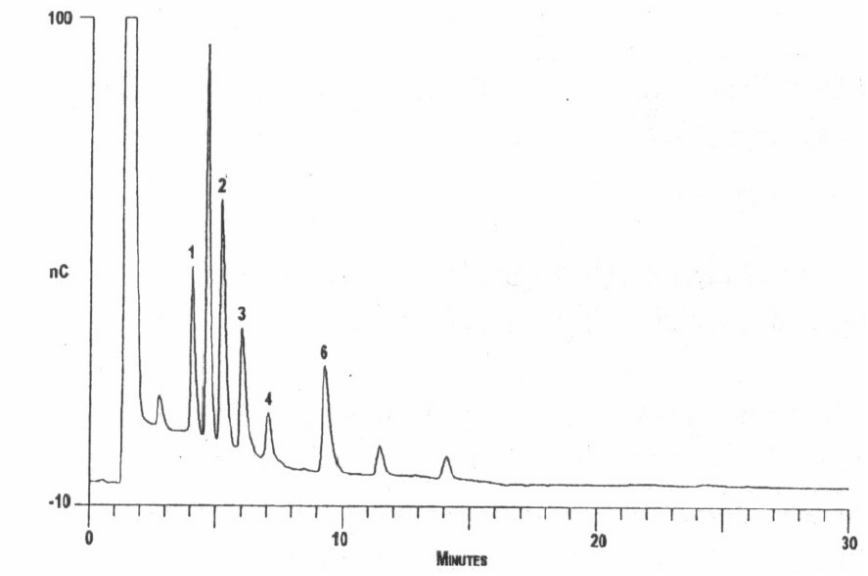


Figure 4. Chromatogram of a cheese sample analysed for biogenic amines using electrochemical detection. 1 = putrescine, 2 = histidine, 3 = tyrosine, 4 = cadaverine, 6 = tyramine (Reproduced from [8] with permission from Elsevier© 1998).

A rapid, simple, and inexpensive method for detection of individual trichothecene compound would be beneficial for routine screening of trichothecenes in food and animal feeds. Electrochemical methods could be ideal choice for such tasks since their selectivity often requires less intensive cleanup, allowing rapid and inexpensive detection. However, due to the difficulty in reducing or oxidizing trichothecenes, electrochemical methods do not currently play a major role in their detection. Only a few studies have used electrochemical detectors combined with HPLC separation for detection of trichothecenes [31].

3.4. On-line combination of multiple detectors

One possible approach to decrease the number of individual sample analyses and conserving sample is to have multiple detectors online [191]: UV detection with variable wavelengths, FL with certain assigned excitation and emission values and ED. By using multiple detectors, a chromatographic fingerprint of an unknown analyte in a sample can be obtained. In Figure 5 a is shows chromatograms of UV-, FL- and electrochemical detectors connected on line to obtain a chromatographic fingerprint of alkaloids in spiked orange juice. Please notice the ability of different techniques to detect different compounds with varying intensity. Although not all compounds can be detected by each detector or wavelength, it has been shown that each compound will be detected by at least one of the detectors [191]. In practice, effluent from the analytical column can be splitted to detectors, or detectors are consecutive connected, especially when non-destructive detectors are used.

Besides obtaining a chromatographic fingerprint of the sample, the connection of detectors online may also be used for simultaneous analysis of multiple analytes with variable properties of detection. This procedure demands, that the compounds can be separated with similar chromatographic conditions. This kind of determination could offer remarkably savings in time, money and the use of the instruments. An example of determination of several food toxicants with different properties of detection has been reported by Knize et al. [44] for heterocyclic amines in different foods using UV- and FL-detectors (Figure 6).

4. Conclusion

The method of choice for food toxicant analysis is naturally dependent on the facilities of the laboratory. As explained, most compounds can be detected with different modes and the choice of an optimal detection system is always a question of needed sensitivity and detection level, which is dependent on properties of the analyte. Also sample preparation procedures affect the applied technique, as method performance parameters are greatly dependent on sample matrix. Recently, mass spectrometry detection has replaced conventional modes in many food toxicant applications, especially when confirmation and increased specificity are needed. However, with conventional detection techniques comparable or even better sensitivity may be observed.

UV/VIS- and FL-detection are by far the most common detection techniques used in the food toxicant analyses, whereas ED has gained much less popularity. In the future, especially FL will remain its importance, in particular when a sensitive and specific detection is needed, as is the case e.g. for regulative purposes [17, 109, 117]. On the other hand, UV as an universal, fast, reliable and an economical detector will be suitable for routine analyses (quality control, basic research), but for any confirmatory purposes, at least photodiode array detection is needed to provide confidence to the determination, due to the lack of sensitivity

and specificity in most cases. For instance, according to the Directive of the European Commission [194] fluorescence and full-scan UV/VIS (DAD) with special remarks are suitable detection techniques for the determination of analytes for regulative purposes, whereas UV/VIS with single wavelength (UV) is inappropriate for confirmatory purposes.

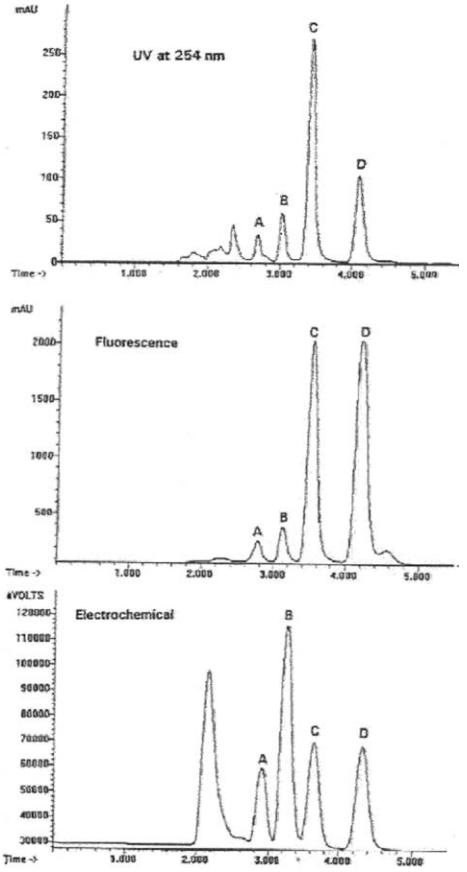


Figure 5. Chromatograms of orange juice spiked with alkaloids with connecting ultraviolet-, fluorescence- and electrochemical detections on line to obtain a chromatographic fingerprint. A = morphine, B = codeine, C = eserine, D = apomorphine (Reproduced from [191] with permission from Elsevier© 2006).

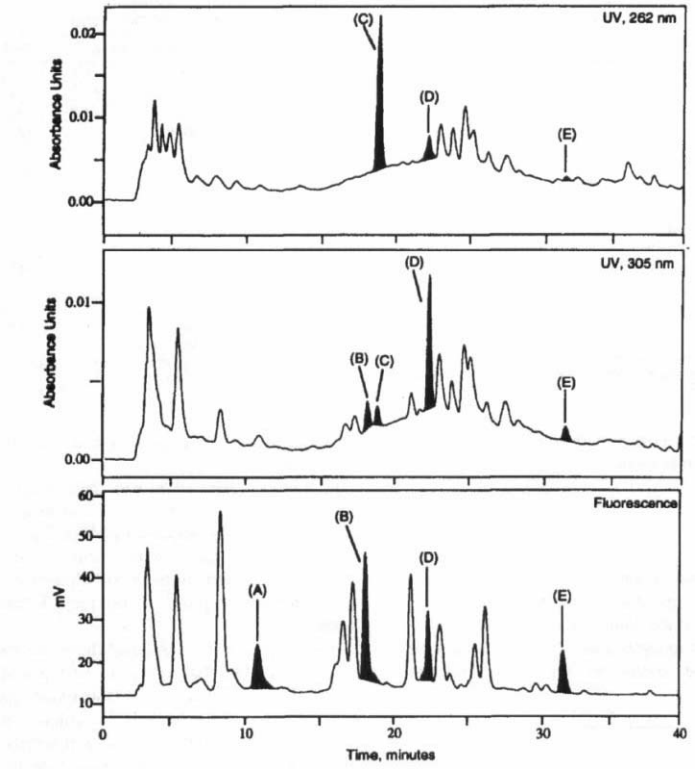


Figure 6. Chromatograms of grilled chicken sample spiked with heterocyclic amines connecting ultraviolet- and fluorescence detectors on line. A = 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine, B =, 1,5,6-trimethylimidazopyridine, C = 2-amino-1,6-dimethylimidazo[4,5-*f*]pyridine, D = 3,5,6-trimethylimidazopyridine (Reproduced from [44] with permission from Elsevier© 1997)

References

[1] E. Järvenpää, R. Huopalahti and P. Tapanainen,J. Liq. Chromatogr. Rel. Technol. 19(9) (1996) 1473.

[2] M. Lodovici, P. Dolara, C. Casalini, S. Ciappellano and G. Testolin, Food Add. Contam., 12(5) (1995) 703

[3] M.S. García-Falcon, B. Cancho-Grande and J. Simal-Gándara, Food Chem. 90 (2005) 643.

[4] F. Fenaille, P. Mottier, R.J. Turesky, S. Ali and P.A. Guy, J. Chromatogr. A 921 (2001) 237.

[5] G.A. Perfetti, P.J. Nyman, S. Fisher, F.L. Joe and G.W. Diachencko, J. AOAC Int. 75(5) (1992) 872.

[6] S. Moret and L.S. Conte, J. Chromatogr. A 729 (1996) 363.
[7] M. Jestoi, Emerging *Fusarium*-mycotoxins in Finland. EELA Publications 01/2005. Academic Dissertation. (2005) 120 p.
[8] R. Draisci, L. Giannetti, P. Boria, L. Lucentini, L. Palleschi and S. Cavalli, J. Chromatogr. A 798 (1998) 109.
[9] W. Kandler, M. Nadubinská, A.Parich and R. Krska, Anal. Bioanal. Chem. 374 (2002) 1086.
[10] C. Krach and G. Sontag, Anal. Chim. Acta 417 (2000) 77.
[11] C. Munimbazi, and L.B. Bullerman, J. AOAC Int. 81(5) (1998) 999.
[12] S. Novella-Rodríguez, M.T. Veciana and M.C. Vidal-Carou, J. Agric. Food Chem. 48 (2000) 5117.
[13] T. Prestera, J.W. Fahey, W.D. Holtzclaw, C. Abeygunawardana, J.L. Kachinski and P. Talalay, Anal. Biochem. 239 (1996) 168.
[14] M.T. Veciana-Nogues, T. Hernandez-Jover, A. Marine-Font and M.C. Vidal-Carou, J. Assoc. Off. Anal. Chem.78 (1995) 1045.
[15] G. Pineda-Valdes and L.G. Bullerman, J. Food Protec. 63(11) (2000) 1598.
[16] Anonymous, Abstract Book of 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Stockholm, Sweden, June 26-30, 2005.
[17] P. Welling and B. Kaandorp, Zeitschrift für Lebensmittel-Untersuchung und -Forschung A 183 (1986) 111.
[18] J.K.Troyer, K.K. Stephenson and J.W. Fahey, J. Chromatogr. A 919 (2001) 299.
[19] G.S. Nunes, M.L. Ribeiro, L. Polese and D. Barceló, J. Chromatogr. A 795 (1998) 43.
[20] P. Pais and M.G. Knize, (2000): Chromatographic and related techniques for the determination of aromatic heterocyclic amines in foods. J. Chromatogr. B 747 (2000) 139.
[21] D.R. Cardoso,S.M. Bettin, R.V. Reche, B.S. Lima-Neto and D.W. Franco, J. Food Comp. Anal. 16 (2003) 563.
[22] A. Barranco, R.M. Alonso-Salces, E. Corta, L.A. Berrueta, B. Gallo, F. Vicente and M. Sarobe, M, Food Chem. 86 (2004a) 465.
[23] E.A. Gomaa, J.I. Gray, S. Rabie, C. Lopez-Bote and A.-M. Booren, Food Add. Contam. 10(5) (1993) 503.
[24] F.L Joe, J. Salemme and T. Fazio, J. Assoc. Off. Anal. Chem.67(6) (1984) 1076.
[25] M. Driffield, D. Chan, R. Macarthur, S. MacDonald and P. Brereton, J. AOAC Int. 88(1) (2005) 121.
[26] F. Lo Coco, C. Valentini, V. Novelli and L. Ceccon, Anal. Chim. Acta 306 (1995) 57.
[27] S. Marcincák, J. Sokol, P. Bystrický, P. Popelka, P. Turek, M. Bhide and D. Máté, J. AOAC Int. 87(5) (2004) 1148.
[28] R. F. Nascimento, J.C. Marques, B.S. Lima Neto, D. De Keukeleire and D.W. Franco, J. Chromatogr. A, 782 (1997) 13.
[29] A. Sanches-Silva, A. Rodriguez-Bernaldo de Quirós, J. López.-Hernández and P. Paseiro-Losada, J. Chromatogr. A,1046 (2004) 75.
[30] B. Beretta, A. Gaiaschi, C.L. Galli and P. Restani, Food Add. Contam. 17(5) (2000) 399.
[31] W.L. Childress, I.R. Krull, C.M. Selavka, J. Chromatogr. Sci. 28 (1990) 76.
[32] D.R. Lauren and M.A. Ringrose, Food Add. Contam. 14(5) (1997) 435.
[33] A. Ritieni, A. Moretti, A. Logrieco, A. Bottalico, G. Randazzo, S.M. Monti, R. Ferracane and V. Fogliano, J. Agric. Food Chem. 45 (1997) 4011.
[34] P.G. Thiel, C.J. Meyer, C. and W.F.O. Marasas, J. Agric. Food Chem. 30 (1982) 308.
[35] D.K. Vudathala, D.B. Prelusky and H.L. Trenholm, J. Liq. Chromatogr. 17(3) (1994) 673.

[36] S. Eerola, R. Hinkkanen, E. Lindfors and T. Hirvi, J. AOAC Int. 76(3) (1993) 575.
[37] M.C. Gennaro, V. Gianotti, E. Marengo, D. Pattono and R.M. Turi, Food Chem. 82 (2003) 545.
[38] H.M.L.J. Joosten and C. Olieman, J. Chromatogr. 356 (1986) 311.
[39] I. Krause, A. Bockhardt, H. Neckermann, T. Henle and H. Klostermeyer, J. Chromatogr. A 715 (1995) 67.
[40] O. Pinho, I.M.P.L.V.O. Ferreira, E. Mendes, B.M. Oliveira and M. Ferreira, Food Chem. 75 (2001) 287.
[41] Y.H. Tsai, H.-F. Kung, T.-M. Lee, H.-C. Chen, S.-S. Chou, C.-I. Wei and D.-F. Hwang, Food Control 16 (2005) 579.
[42] M. Yigit and L. Ersoy, J. Pharm. Biomed. Anal. 31 (2003) 1223.
[43] M.A.E. Johansson, L. Fredholm, I. Bjerne and M. Jägerstad, Food Chem. Toxicol. 33(12) (1995) 993.
[44] M.G. Knize, C.P. Salmon, E.C. Hopmans and J.S. Felton, J. Chromatogr. A 763 (1997) 179.
[45] A.A. Bergwerff and P. Scherpenisse, J. Chromatogr. B 788 (2003) 351.
[46] B.P.S. Capece B. Pérez, E. Castelles, M. Arboix and C. Cristòfol, J. AOAC Int. 82(5) (1999) 1007.
[47] A. Conneely, A. Nugent, M. O'Keeffe, P.P.J. Mulder, J.A. van Rhijn, L. Kovacsisc, A.Fodor, R.J. McCracken and D.G. Kennedy, Anal. Chim. Acta 483 (2003) 91.
[48] E.J.I. De Veau, J. AOAC Int. 79 (1996) 1050.
[49] J . Kochansky, J. Api. Res. 43(2) (2004) 60.
[50] M. Zhang, G.A. Moore, S.J. Gardiner and E.J. Begg, J. Chromatogr. B 807 (2004) 217.
[51] B. Hamilton, M. Hurbungs, J.-P. Vernoux, A. Jones and R.J. Lewis, Toxicon 40 (2002) 685.
[52] M.A. Poli, S.M. Musser, R.W. Dickey, P.P. Eilers and S. Hall, Toxicon 38 (2000) 981.
[53] H.C. Andersson, J. Hajslova, V. Schulzova, Z. Panovska, L. Hajokova and J. Gry, Food Add. Contam. 16(10) (1999) 439.
[54] F. Musshoff, B. Madea and J. Beike, Forensic Sci. Int. 113 (2000) 389.
[55] K. Tsujikawa, T. Kanamori, Y. Iwata, Y. Ohmae, R. Sugita, H. Inoue and T. Kishi, Forensic Sci. Int. 138 (2003) 85.
[56] T.C. Goosen, D. Cillié, F.G. Bailey, C. Yu, K. He,. P.F. Hollenberg, P.M. Woster, L. Cohen, J.A. Williams, M. Rheeders and H.P. Dijkstra, Clin. Pharm.Therap. 76(6) (2004) 607.
[57] E. Järvenpää, M. Jestoi and R. Huopalahti, Phytochem. Anal. 8 (1997) 250.
[58] M.Waksmundzka-Hajnos, A. Petruczynik, A. Dragan, D. Wianowska, A.L. Dawidowicz and I. Sowa, J. Chromatogr. B 800 (2004) 181.
[59] F. Papathanasiou, S.H. Mitchell and B.M.R. Harvey, J. Sci. Food Agric. 79 (1999) 32.
[60] B.J. Phillips, J.A. Hughes, J.C. Phillips, D.G. Walters, D. Anderson and C.S.M. Tahourdin, Food Chem. Toxicol., 34 (1996) 439.
[61] M. Sengül, F. Keles and M.S. Keles, Food Control 15 (2004) 281.
[62] K. Edgell, E.J. Erb, J.E. Longbottom and V. Lopez-Avila, J. AOAC Int. 75(5) (1992) 858.
[63] H. Miwa, J. Chromatogr. A 881 (2000) 365.
[64] L. Huber, Applications of diode-array detection in high performance liquid chromatography. Hewlett-Packard Co. Publication Number 12-5953-2330 (1989).
[65] M.G. Knize, R. Sinha, N. Rothman, E.D. Brown, C.P. Salmon, O.A. Levander, P.L. Cunningham and J.S. Felton, Food Chem. Toxicol. 33 (1995) 545.
[66] K.A. Lefebvre, S. Bargu, T. Kieckhefer and M.W. Silver, Toxicon 40 (2002) 971.
[67] A.M. Pupin and M.C.F. Toledo, Food Chem. 55(2) (1996) 185.

[68] L. Pensado, E. Blanco, M.C. Casais, M.C. Mejuto, E. Martinez, A.M. Carro and R. Cela, J. Chromatogr. A 1056 (2004) 121.
[69] M. Pascale, A. Visconti, G. Avantaggiato, M. Pronczuk and J. Chelkowski, J. Sci. Food Agric.79 (1999) 2094.
[70] V. Gökmen and J. Acar, J. Chromatogr. A 847 (1999) 69.
[71] S. Kermasha, M. Goetghebeur, J. Dumont and R. Couture, Food Res. Int. 28(3) (1995) 245.
[72] M.J. Nozal, J.L. Bernal, L. Toribio, J.J. Jiménez and M.T. Martin, J. Chromatogr. A 917 (2001) 95.
[73] M. Zappala, B. Fallico, E. Arena and A. Verzena, Food Control 16 (2005) 273.
[74] M. Kokkonen, M. Jestoi and A. Rizzo, International J. Food Microbiol. 99 (2005) 207.
[75] R. Krska, R. Schuhmacher, M. Grasserbauer and P.M. Scott, J. Chromatogr. A 746 (1996) 233.
[76] D.P. Overy, K.A. Seifert, M.E. Savard and J.C. Frisvad, Int. J. Food Microbiol. 88 (2003) 69.
[77] G.J. Soleas, J. Yan and D.M. Goldberg, J. Agric. Food Chem. 49 (2001) 2733.
[78] C.G. Zambonin, L. Monaci and A. Aresta, Food Chem. 78 (2002) 249.
[79] K. Koutsoumanis, K. Lampropoulou and G.-J.E. Nychas, J. Food Protec. 62(4) (1999) 398.
[80] A.R. Krizek, J.S. Smith and R.K. Phebus, J. Food Protec. 58(3) (1995) 284.
[81] J.S. Smith, P.B. Kenney, C.L. Kastner and M.M. Moore, J. Food Protec. 56(6) (1993) 497.
[82] L. Cárdenes, J.H. Ayala, A.M. Afonso and V. González, J. Chromatogr. A 1030 (2004) 87.
[83] B. Janoszka, U. Blaszczyk, L. Warzecha, M. Strózyk, A. Damasiewicz-Bodzek and D. Bodzek, (2001): Clean-up procedures for the analysis of heterocyclic aromatic amines (aminoazaarenes) from heat-treated meat samples. J. Chromatogr. A, 938:155-165.
[84] C.M. Lan and B.H.Chen, Food Chem. Toxicol. 40 (2002) 989.
[85] F. Toribio, L. Puignou and M.T. Galceran, J. Chromatogr. A 836 (1999) 223.
[86] L. Warzecha, B. Janoszka, U. Blaszczyk, M. Strózyk, D. Bodzek and C. Dobosz, J. Chromatogr. B 802 (2004) 95.
[87] R.K. Buick, C. Barry, I.M. Traynor, W.J. McCaughey and C.T. Elliott, J. Chromatogr. B 72 (1998) 71.
[88] A.L. Cinquina, P. Roberti, L. Giannetti, F. Longo, R. Draisci, A. Fagiolo and N.R. Brizioli, J. Chromatogr. A 987 (2003) 221.
[89] R. Draisci, L. Giannetti, L. Lucentini, L. Palleschi, G. Brambilla, L. Serpe and P. Gallo, J. Chromatogr. A 777 (1997) 201.
[90] M.E. Ferdin, R.G. Kvitek, C.K. Bretz, C.L. Powell, G.J. Doucette, K.A. Lefebvre, S. Coale and M.V. Silver, Toxicon 40 (2002) 1259.
[91] P.E. Núñez and A.C. Scoging, Int. J. Food Microbiol. 36 (1997) 39.
[92] P. Vale and M.A.M. de Sampayo, Toxicon 39 (2001b) 893.
[93] V. Schulzova, J. Hajslova, R. Peroutka, J. Gry and H.C. Andersson, Food Add. Contam. 19(9) (2002) 853.
[94] S.M. Kakar, M.F. Paine, P.W. Stewart and P.B. Watkins, Clin. Pharmacol. Therap.75(6) (2004) 569.
[95] P.C. Stevenson, M.S.J. Simmonds, M.A. Yule, N.C. Veitch, G.C. Kite, D. Irwin and M. Legg, Phytochem. 63 (2003) 41.
[96] T. Väänänen, P. Kuronen and E. Pehu, J. Chromatogr. A 869 (2000) 301.
[97] P.D. Brown, J.G. Tokuhisa, M. Reichelt and J. Gershenzon, Phytochem. 62 (2003) 471.
[98] M.C. López-Blanco, B. Cancho-Grande and J. Simal-Gándara, J. Chromatogr. A 963 (2002) 117.

[99] H. Hayashi, S. Hattori, K. Inoue, K. Sarsebaev, M. Ito and G. Honda, Biol. Pharm. Bull. 26(6) (2003a) 867.
[100] H. Hayashi, S. Hattori, K. Inoue, O. Khodzhimatov, O. Ashurmetov, M. Ito and G. Honda, Biol. Pharm. Bull. 51(11) (2003b) 1338.
[101] Y.Jiang, H.-T. Lu and F. Chen, J. Chromatogr. A 1033 (2004) 183.
[102] G. Lunn and L.C. Hellwing, Handbook of derivatization reactions for HPLC. A Wiley-Interscience Publication, New York, USA, 1998.
[103] M.C. Herrera, R.C. Prados-Rosales, J.L. Luque-García and M.D. Luque de Castro, Anal. Chim. Acta 463 (2002) 189.
[104] P. Dilkin, C.A. Mallmann, C.A.A. de Almeida and B. Correa, J. Chromatogr. A 925 (2001) 151.
[105] L. Cárdenes J.H. Ayala, V. González and A.M. Afonso, J. Chromatogr. A 946 (2002) 133.
[106] C. García, V. González, C. Cornejo, H. Palma-Fleming and N. Lagos, Toxicon 43 (2004) 121.
[107] L. Puech, S. Dragacci, E. Gleizes and J.-M. Fremy, Food Add. Contam. 16(6) (1999) 239.
[108] J.S. González, M.R.Vieytes, J.M. Vieites and L.M. Botana, J. Chromatogr. A 793 (1998) 63.
[109] J.S. González, F. Leira, M.R. Vieytes, J.M. Vieites, A.M. Botana and L.M. Botana, J. Chromatogr. A 876 (2000) 117.
[110] C. Sánchez-Brunete, B. Albero and J.L. Tadeo, J. Food Protec. 67(11) (2004) 2565.
[111] N. Kishikawa, M. Wada, N. Kuroda, S. Akiyama and K. Nakashima, J. Chromatogr. B 789 (2003) 257.
[112] H. Kaneda, M. Takashio, T. Osawa, S. Kawakishi, S. Koshino and T. Tamaki, J. Food Sci. 61(1) (1996) 105.
[113] A. Anastasio, R. Mercogliano, L. Vollano. T. Pepe and M.L. Cortesi, J. Agric. Food Chem. 52 (2004) 4452.
[114] A. Barranco, R.M. Alonso-Salces, I. Crespo, L.A. Berrueta, B Gallo, F. Vicente, M. Sarobe, J. Food Protec. 67(12) (2004b) 2786.
[115] O. Dafflon, L. Scheurer, H. Koch and J.O. Bosser, Mitteilungen aus Lebensmitteluntersuchung und Hygiene 91(2) (2000) 158.
[116] M.J. Fernandez, C. Garcia, R.J. Garcia-Villanova and J.A. Gomez, J. Agric. Food Chem. 44 (1996) 1785.
[117] M.S. García Falcón, S. González Amigo, M.A. Lage Yusty, M.J. López de Alda Villaizán and J. Simal Lozano, J. Chromatogr. A 753 (1996) 207.
[118] M.S. García-Falcón, S. González Amigo, M.A. Lage Yusty and J. Simal Lozano, Food Add. Contam. 16(1) (1999) 9.
[119] M.S. García-Falcon and J. Simal-Gándara, Food Add. Contam. 22(1) (2005) 1.
[120] B. Janoszka, L. Warzecha, U. Blaszczyk and D. Bodzek, Acta Chromatogr. 14 (2004a) 115.
[121] B. Janoszka, L. Warzecha, U. Blaszczyk and D. Bodzek, Acta Chromatogr. 14 (2004b) 129.
[122] M.A. Lage Yusty and J.L. Cortizo Daviña, Food Control 16 (2005) 59.
[123] J.F. Lawrence and D.F. Weber, J. Agric. Food Chem. 32 (1984a) 789.
[124] J.F. Lawrence and D.F. Weber, J. Agric. Food Chem. 52 (1984b) 794.
[125] C.J. Musial and J.F. Uthe, J. Assoc. Off. Anal. Chem.69(3) (1986) 462.
[126] B. Saint-Aubert, J.F. Cooper, C. Astre, J. Spiliotis and H. Joyeux, J. Food Comp. Anal. 5 (1992) 257.
[127] S. Schlemitz and W. Pfannhauser, Food Add. Contam. 13(8) (1996) 969.
[128] F. van Stijn, M.A.T. Kerkhoff and B.G.M. Vandeginste, J. Chromatogr. A 750 (1996) 263.
[129] K. Takatsuki, S. Suzuki, N. Sato and I. Ushizawa, J. Assoc. Off. Anal. Chem.68(5) (1985) 945.

[130] S. Vazquez Troche, M.S. García Falcón, S. González Amigo, M.A. Lage Yusty and J. Simal Lozano, Talanta 51 (2000) 1069.
[131] C. Viau, A. Diakité, A. Ruzgyte, B. Tuchweber, C. Blais, M. Bouchard and A. Vyskocil, J. Chromatogr. B 778 (2002) 165.
[132] H. Vuontisjärvi, M. Keinänen, P. Vuorinen and K. Peltonen, Polycyclic Aromatic Compounds 24 (2004) 333.
[133] H.Y. Yabiku, M.S. Martins and M.Y. Takahashi, Food Add. Contam. 10(4) (1993) 399.
[134] A. de las Heras, A. Schoch, M. Gibis and A. Fischer, Eur. Food Res. Technol. 217 (2003) 180.
[135] T. Sakai, A. Habiro and S. Kawahara, J. Chromatogr. B 726 (1999) 313
[136] E. Bisson, L. Byass, A. Garner and R.C. Garner, Food Agric. Immunol. 6 (1994) 331.
[137] C. Dall'Asta, G. Galaverna, A. Biancardi, M. Gasparini, S. Sforza, A. Dossena and R. Marchelli, J. Chromatogr. A 1047 (2004) 241.
[138] S. Dragacci, F. Grosso and J. Gilbert, J. AOAC Int. 84(2) (2001) 437.
[139] G. Filek and W. Lindner, J. Chromatogr. A 732 (1996) 291.
[140] L.A.B. Leoni, L.M. Valente Soares and P.L.C. Oliveira, Food Add. Contam. 17(10) (2000) 867.
[141] K.A. Scudamore and S.J. MacDonald, Food Add. Contam. 15(4) (1998) 401.
[142] E.K.Tangni, S. Ponchaut, M. Maudoux, R. Rozenberg and Y. Larondelle, Food Add. Contam. 19(12) (2002) 1169.
[143] L.G.M.T. Tuinstra, P.G.M. Kienhuis, W.A. Traag, M.M.L. Aerts and W.M.J. Beek, J. High Resolut. Chromatogr. 12 (1989) 709.
[144] A. Visconti, M. Pascale and G. Centonze, J. AOAC Int. 84(6) (2001) 1818.
[145] C. Ruiz-Capillas and A. Moral, J. Food Sci. 66(7) (2001) 1030.
[146] S.R. Vale and B.A. Glória, J. AOAC Int. 80(5) (1997) 1006.
[147] Z. Öner, O. Sagdic and B. Simsek, Eur. Food Res. Technol. 219 (2004) 455.
[148] M.T. Galceran, P. Pais and L. Puignou, J. Chromatogr. A 719 (1996) 203.
[149] A. Ristic, M. Cichna and G. Sontag, J. Chromatogr. B 802 (2004) 87.
[150] V. Cerkvenik, D.Z. Doganoc, V. Skubic, W.M.J. Beek and H.J. Keukens, Eur. Food Res. Technol. 213 (2001) 72.
[151] F. Imperiale, J. Sallovitz, A. Lifschitz and C. Lanusse, Food Add. Contam. 19(9) (2002) 810.
[152] L. Knold, M. Reitov, A.B. Mortensen and J. Hansen-Møller, J. AOAC Int. 85(2) (2002) 365.
[153] T. Nagata, F. Miyamoto, Y. Hasegawa and E. Ashizawa, J. AOAC Int. 86 (2003) 490.
[154] H. Pouliquen and M.L.Morvan, Food Add. Contam. 19(3) (2002) 223.
[155] J.M. van de Riet, N.N. Brothers, J.N. Pearce and B.G. Burns, J. AOAC Int. 84(5) (2001) 1358.
[156] B.Aase and A. Rogstad, J. Chromatogr. A 764 (1997) 223.
[157] T.N. Asp, S. Larsen and T. Aune, Toxicon 43 (2004) 319.
[158] P. Ciminiello, E. Fattorusso, M. Forino, S. Magno, R. Poletti, M. Satake, R. Viviani and T. Yasumoto, Toxicon 35(2) (1997) 177.
[159] A. Gago-Martínez, S.A. Moscoso, J.M. Leão Martins, J.-A. Rodriguez Vázquez, B. Niedzwiadek and J.F. Lawrence, J. Chromatogr. A 905 (2001) 351.
[160] C. Hummert, J.L. Shen and B. Luckas, (1996): Automatic high-performance liquid chromatographic method for the determination of diarrhetic shellfish poison. J. Chromatogr. A 729 (1996) 387.
[161] K.J. James, M. Gillman, M. Lehane and A. Gago-Martinez, J. Chromatogr. A, 871 (2000) 1.
[162] S.S. Kelly, A.G. Bishop, E.P. Carmody and K.J. James, J. Chromatogr. A 749 (1996) 33.
[163] A. Kungsuwan, O. Arakawa, M. Promdet and Y. Onoue, Toxicon 35(8) (1997) 1341.
[164] J.F. Lawrence, C. Menard and C. Clerox, J. AOAC Int. 78(2) (1995) 514.

[165] J.F. Lawrence, S. Roussel and C. Ménard, J. Chromatogr. A 721 (1996) 359.
[166] J.F. Lawrence and B. Wong, J. Chromatogr. A 755 (1996) 227.
[167] J. M. Leão, A. Gago, J.A. Rodríguez-Vázquez, E.C. Aguete, M.M. Omil and M. Comesaña, J. Chromatogr. A 798 (1998) 131.
[168] Y. Oshima, J. AOAC Int. 78(2) (1995) 528.
[169] P. Vale and M.A.M. de M. Sampayo, Toxicon, 39 (2001a) 561.
[170] I.C. Yen, L.R. de Astudillo, J.F. Soler and A. La Barbera-Sanchez, Toxicon 44 (2004) 743.
[171] M.A. Bacigalupo, R. Longhi and G. Meroni, J. Food Comp. Anal. 17 (2004) 665.
[172] A. Abad, M.J. Moreno, R. Pelgri, M.I. Martínez, A. Sáez, M. Gamón and A. Montoya, J. Chromatogr. A 833 (1999) 3.
[173] A. Caballo-López and M.D. Luque de Castro, J. Chromatogr. A 998 (2003) 51.
[174] D. Chaput, J. Assoc. Off. Anal. Chem.71(3) (1988) 542.
[175] C.P. Sanz, A.E. Fernández, Z.S. Ferrera and J.J.S. Rodríguez, J. AOAC Int. 85(1) (2002) 44.
[176] P.J. Marriott, P.D. Carpender, P.H. Brady, M.J. McMormick, A.J. Griffiths, T.S.G. Hatvani and S.G. Rasdell, J. Liq. Chromatogr. 16(15) (1993) 3229.
[177] P.G. Sim, R.K. Boyd, R.M. Gershey, R. Guevremont and W.D. Jamieson, Biomed. Environ. Mass Spectrom. 14 (1987) 375.
[178] P. Simko, J. Chromatogr. B 770 (2002) 3.
[179] H.P. van Egmond, K.M. Jonker, M. Poelman, P. Scherpenisse, A.G. Stern, A.G., P. Wezenbeek, A.A. Bergwerff and H.J. Van den Top, Food Add. Contam. 21(4) (2004) 331.
[180] R. Pocklington, J.E. Milley, S.S. Bates, C.J. Bird, A.S.W. de Freitas and M.A. Quilliam, Int. J. Environ. Anal. Chem. 38 (1990) 351.
[181] J.S. Lee, T. Yanagi, R. Kenma and T. Yasumoto, Agric. Biol. Chem. 51(3) (1987) 877.
[182] M.A. Quilliam, J. AOAC Int. 78(2) (1995) 555.
[183] ISO 14501:1998: Milk and milk powder – Determination of aflatoxin M_1 content – Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography.
[184] ISO 15141-1:1998: Determination of ochratoxin A in cereals and cereal products – Part 1: High performance liquid chromatographic method with silica gel clean up.
[185] ISO 15141-2:1998: Determination of ochratoxin A in cereals and cereal products – Part 2: High performance liquid chromatographic method with bicarbonate clean up.
[186] ISO 16050:2003: Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products – High-performance liquid chromatographic method.
[187] M. Jiménez, J.J. Mateo and R. Mateo, J. Chromatogr. A 890 (2000) 473.
[188] H. Petterson, SMT4-CT-96-2047 (2001).
[189] J. Schultheiss, D. Jensen and R. Galensa, J. Chromatogr. A 880 (2000) 233.
[190] U. Gerbl, M. Cichna, M. Zsivkovits, S. Knasmüller and G. Sontag, J. Chromatogr. B 802 (2004) 107.
[191] L.A. Lin, J. Chromatogr. 632 (1993) 69.
[192] D. Briones-Reyes, L. Gómez-Martinez and R. Cueva-Rolón, Food Chem. 100 (2006) 693.
[193] S. De Saeger, L. Sibanda and C. Van Peteghem, Anal. Chim. Acta 487(2) (2003) 137.
[194] Commission decision 2002/657/EC of 12th August 2002. Official Journal of the European Communities L221 (2002) 8.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 14

Liquid chromatography-mass spectrometry

Cristina Blasco, Yolanda Picó

Laboratori de Bromatologia i Toxicologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

1. Introduction

Liquid chromatography (LC) is very effective in separating non-volatile and thermally labile compounds. Unfortunately, the lack of sensitive and selective LC detectors had, just until recently, prevented the widespread of this technique in food toxicant analysis. This drawback has been solved by combining LC with mass spectrometry (MS) [1, 2].

Organic contaminants comprise tens of thousands of molecules, many of which are still being identified and recognized using state-of-the-art mass analyzers [3-5]. LC-MS can solve one of the goals of analytical chemistry, as is to display and analyze most, or almost all, the toxicants that can be present in food [2, 3]. The increasing application of LC-MS in this field has been neck and neck with the developments and evolution of mass spectrometry.

Two decades ago, the appropriate interface of the LC to MS was a challenging task because this arrangement was considered as an 'unnatural marriage'. LC-MS combines an instrument that operates in the condensed phase with other that operates under vacuum. At the present, this problem has been solved with the atmospheric pressure ionization (API) sources, which have turned LC/MS into an ideal and widely used method for the analysis of food contaminants and residues owing to its superior specificity, sensitivity and efficiency. API techniques are compatible with reversed-phase eluent systems, taking into account the use of volatile solvents and additives in chromatographic separation, and preserving all the advantages of LC [6-8].

All these developments have been carried out using single quadrupole as the most common analyzer. However, recently, analytical chemists have began to realize that the different LC-MS interfaces were designed to provide a soft ionization process that leads to a mass spectrum with only a few ions that most of the times are not sufficient to recognize and quantify all the analytes of interest in the sample. Increasingly, therefore, other mass analyzers with different features began to make headway to help to solve the current analytical problems. These recently-introduced mass analyzers have been divided into two different groups: the first group comprises high resolution techniques, such as time-of-flight mass spectrometry (TOF-MS), and the second group includes techniques capable of carrying out tandem mass spectrometry, such as triple quadrupole (QqQ), ion trap (QIT) or the hybrid quadrupole time-of-flight (QqTOF) [9-12].

The power of these new technologies is that they provide structural information for identifying non-target contaminants (unknown compounds), increase the specificity of target-analytes identification and achieve high sensitive trace-level determination [4, 13]. At the same time, these instruments offer the highest degree of certainty in analyte identification, and provide unambiguous data that are in accordance with recent European Guidelines and Criteria to identify contaminants in different matrices [14-16]. Additionally, greater sample throughput can be achieved because extensive sample clean-up strategies can be considerably reduced by using selective liquid chromatography-mass spectrometric (LC-MS) detection [6, 17].

Anyhow, the increasing importance of LC-MS in the field of contaminants analysis in food is emphasized by the appearance of many review articles dealing with both, new perspectives of LC-MS [9, 18, 19] and the different aspects of its applications [2, 11, 16, 17, 20-26]. LC-MS are found in a very wide range of analytical applications within food toxicant analysis, for example, veterinary drugs residues, myco and phycotoxins, pesticide residue monitoring, and throughout the food production and food processing industry. Table 1 presents an overview of these applications to provide a good idea to the reader on the broad usefulness of the technique for determining food toxicants.

This chapter dealt with the use of LC coupled to MS to determine food toxicants. A brief introduction to the fundamental theory for the different mass analyzers illustrates possibilities and limitations of this technique. The analytical chain of LC/MS can be divided into three stages: separation, interface, and mass analysis; this chapter follows this division. References that are good overviews of each LC/MS technology, related applications, fundamentals, and instrumentation availability are included in the appropriate sections. The instrumental setup and the experimental settings, essentials for the successful coupling of LC and MS in the analysis of organic contaminants, are also outlined. The capacity to provide useful data for quantifying organic contaminants, and the possibility of obtaining structural information for identifying target and non-target compounds, are discussed. Contemporary applications of this technique in the last five years to study food toxicants are presented.

2. Liquid chromatographic separation

The major chromatographic mode to separate organic contaminants by LC-MS is reversed-phase (RPLC), using or not an ion-pair. Because of the robustness and ease of the method development, they cover more than 95 % of LC applications.

In RPLC, medium polarity, polar, and ionic organic contaminants are separated according to the differences in hydrophobicity by partitioning between an apolar stationary phase and a polar mobile phase. Most stationary phases are based on silica that has been chemically modified with octadecyl (C_{18} or ODS) or octyl (C_8 or OS) [27-30] chains.

Table 1

Summary of studies that have used LC-MS to determine food toxicants

Type of contaminant	Example of compound	Matrix	Applications	Ref.
Veterinary drugs				
Veterinary antibiotics	β-lactams, aminoglycosides, phenicols, fluoroquinolones, cephalosporins, macrocyclic lactones, nitrofuran metabolites, bacitracin, colistin	Milk, Kidney, Liver, Shellfish, Fish, Honey, Shrimp, Meat, Egg, Seafood	Identification and quantification for control purposes LC-QqQ-MS/MS LC-QIT-MS with quantification by MS and MS^2 Comparison with microbial assay Screening and confirmatory method	[28, 31-48]
Analgesic and anti-inflammatory drugs	Sulfonamide, benzodiazepines, malachite green	Egg, dietary supplements, finfish	LC-QqQ-MS/MS LC-QIT-MS/MS Fragmentation pathways Identification and quantification	[49-53]
β-agonist	Clenbuterol	Liver	Identification and quantification for control purposes	[54]
Hormone	Thyreostatic, anabolic	Muscle, crude meat, infant food	LC-MS LC-QqQ-MS/MS LC-TOF-MS	[55, 56]
Pesticides and TPs				
Ammonium quaternary	Chlormequat, mepiquat	Fruit, vegetables, juices, tomato, pear, wheat flour	LC-QqQ-MS/MS LC-QIT-MS with quantification by MS and MS^2 Normalized Methods	[57-60]
Neutral	Carbamates, phenylureas, benzoylureas, azoles, amides, triazoles, triazinones, benzimidazoles, morpholines, phenoxyalkanoids, dinitroanilides, daminozide, thiazoles, sulfonylurea	Fruit, vegetables, baby food	Searchable library of API product ion mass spectra Automated identification of isotopically labelled LC-MS LC-QIT-MS quantification by MS, MS^2, and MS^3 LC-QqQ-MS/MS LC-ToF-MS LC-QqToF-MS/MS	[61-84]
	Ethephon	Vegetables	LC-QqQ-MS/MS	[85]

Type of contaminant	Example of compound	Matrix	Applications	Ref.
Food packaging migrating products	Semicarbazide	Food, baby food	Identification LC-QqQ-MS/MS	[86-89]
	Perfluorochemicals			[90]
Monomers of plasticizer polymers	Bisphenol A diglycil ether, novolac glycidil eter,	Food simulants	Identification and structure elucidation	[91, 92]
	Epoxidized soybean oil (ESIBO)	Soybean oil	LC-QqQ-MS/MS	[93]
Mycotoxins	Aflatoxins, ochratoxin A, trichothecene, T-2 toxin, deoxynivalenol, sterigmatocystin, citrinine, ergot alkaloid	Wheat, feed, cereals, cereal based	LC-QqQ-MS/MS LC-QIT-MS with quantitative determination and structure elucidation (MS^2-MS^6) Comparison with a biological test	[94-96, 96-102]
Algae and fish toxins	Azaspirazind, nodularin, demethylnodularyn, domoic acid, microcystins, amnesic shellfish toxins, diarreic shellfish toxins,	Shellfish Fish	Unique ion combination to distinguish between isomers by MS^3 Comparison with triple quadrupole	[10, 103-109]
Heat induced decomposition products	Aminoimidazoazarenes, carbolines	Lyophilized meat extract, cooked meats, grill scrapings	Determination by MS and MS^2 Fragmentation patterns by MS^n Comparison with TQ	[110-112]
	Acrylamide	Foodstuffs, bakery products, french fries, chocolat, potato chips	Comparison of LC and GC LC-QIT-MS with quantitation by MS and MS^2	[113-115, 115-128]
	3-hydroxymethylfurfural	Baby food	LC-QqQ-MS/MS	[129]
	Aromatic amines	Aqueous food simulants	LC-QqQ-MS/MS	[130]
	Epoxidized soybean oil	Soybean oil	LC-QqQ-MS/MS	[93]
Food additives				

Type of contaminant	Example of compound	Matrix	Applications	Ref.
Dyes	Sudan I, Indigo carmine	Red vinegar, chili powder, chili containing food	LC-QqQ-MS/MS LC-MS	[131-134]
Flavour enhancers	Perchlorate, chlorate	Wheat, dietary supplements	LC-QqQ-MS/MS	[135]
Natural toxins	Quinolizidine alkaloids, phomopsins, milk allergens	Lupin seeds, lupin containing food, milk	LC-QqQ-MS/MS LC-QqTOF-MS/MS	[136, 137]

Aminopropyl, cyanopropyl, or phenyl silica can also be used for reversed-phase separations [138-140]. Other apolar stationary phases are based on synthetic polymers such as the styrene-divinylbenzene copolymer or carbon [141, 142]. The direct combination of LC with MS reduces the stress on chromatographic separation because of the mass selectivity and distinctive fragmentation patterns. However, chromatographic separation can be crucial in some cases, which in turn is directly related to the vast number (approximately 600) of different stationary phases available. This facilitates the solution of many separation problems simply by selecting appropriate stationary phases different selectivities to allow polar analytes separations or to improve peak shape of basic compounds, but on the other hand, identifying the most suitable column for a specific purpose can be far from straight forward.

New alternatives to conventional non-polar alkyl bonded phases have been introduced; the most popular are the embedded and endcapped phases. These are modification of the traditional C18 phase, with the addition of a functional non-polar or polar group, usually an amide or carbamic group, either within (embedded) or at the end (endcapped) of the alkyl chain, thus modifying the selectivity in comparison to conventional alkyl bonded phases.

An interesting example can be found in the case of two metabolites of the pesticide carbofuran, 3-ketocarbofuran and 3-keto-7-phenol carbofuran because in-source fragmentation of the former produces the precursor ion of the latter. Soler et al. [143] compared the performance of six different columns to choose the most appropriate. Poor peak shapes and deficient resolution were observed using most of the column checked. Complete peak overlap were obtained using columns designed for the analysis of polar compounds, whereas some separation of both compounds was obtained with columns prepared to reduce the interaction of the basic analytes. In the same way, a comparison of the performance of six narrow-bore reversed-phase liquid chromatography columns was carried out to propose a chromatographic column for the separation of 16 heterocyclic amines using LC–ESI-MS. The strong adsorption of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-9H-pyrido[2,3-b]indole (AαC) and 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC) on the Discovery column, giving very wide peaks that prevent their detection, forced us to reject it for further studies. Moreover, the Zorbax column was discarded because of the low values of peak symmetry and peak height, probably due to the absence of endcapped treatment in its stationary phase. Among the rest of columns, the TSK Gel® Semi-Micro ODS-80TS was selected because it provided the shortest equilibration time and permitted the highest injection volume and the lowest limits of detection [54].

As mobile phases, the most common solvents used in LC/API-MS are water, methanol, acetonitrile and mixtures of the above, with gradient elution. Buffers incorporated to the mobile phase chromatographic purposes should be volatile to avoid problems with the MS interface. The primary used ones are composed of acetic acid, formic acid, ammonium acetate, ammonium formate or ammonia [144-146]. Figure 1 displays the effect of the type

and concentration of buffer used for the separation and detection of sulfonamides with LC-MS. When the percentage of acetonitrile in the eluent was below 20 % (Figures 1A and C), the peaks of some sulfonamides were broad. This can be explained as intracolumn band broadening by the diffusion of sample band, because the standard mixture of five sulfonamides in 100 % acetonitrile was injected to the ODS column. This unwanted phenomenon could be resolved by using the same solvent as the mobile phase.

The ion pair reagents such as heptafluorobutiric acid, tributylamine or triethylamine are added to the mobile phase for improving chromatographic behavior and peak shape of the molecules ionized in solution [147, 148]. Table 2 summarizes the different mobile phases preferred for each type of contaminant, as well as the influencing factors and the recommendations to combine them with mass spectrometry.

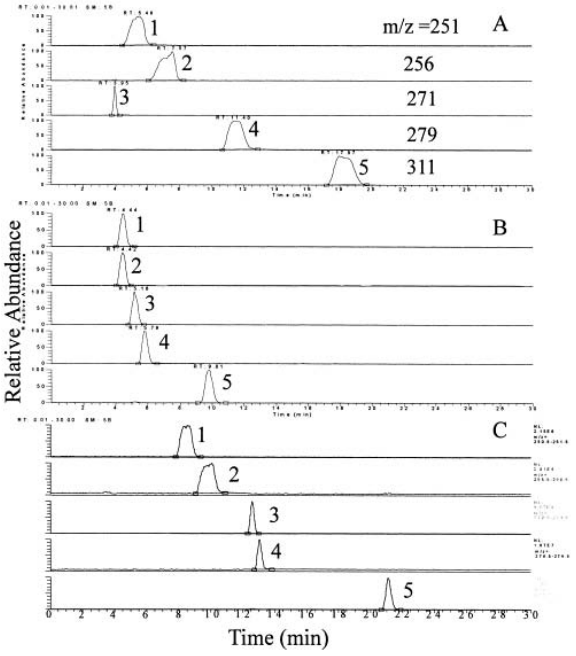


Figure 1. Extracted ion chromatograms of LC–MS for standard solution (2 μg/g) obtained with three different mobile phases. (A) CH_3CN–water–aqueous 0.1 *M* ammonium acetate (15:35:50, v/v), APCI. (B) CH_3CN–water–aqueous 0.7 *M* acetic acid (30:20:50, v/v), ESI. (C) Gradient from 10:90 to 25:75 (CH_3CN–aqueous 0.7 *M* acetic acid, v/v) for 3 min and isocratic with 25:75, ESI. (1) Sulfadiazine (SDZ), (2) sulfathiazole (STZ), (3) sulfamethizole (SMTZ), (4) sulfamethazine (SMZ), (5) sulfadimethoxine (SDM) (Reproduced from [149] with permission from Elsevier© 2003).

The mobile phase is important to obtain a good chromatographic separation, but it also affects the analyte ionization and the sensitivity of the mass spectrometer [70, 150]. The amount of organic modifiers can increase or decrease the ionization efficiency depending on the interface. The efficiency of the electrospray (ESI) process depends on the conductivity and surface tension of the liquid being nebulized. When the conductivity is too high (i.e-highly aqueous) it is difficult to produce a stable spray and to vaporize the droplets formed by the action of the high voltage and nebulizing gas. Because the surface tension of water is much higher than the surface tension of methanol or acetonitrile, the sensitivity is reduced when using more than 70-80 % of aqueous mobile phase. The aqueous-organic ratio is more significant when working at high flow rates since there is more solvent to be nebulized and vaporized. A very high organic content may also lower the sensitivity, especially if not additive is used, because the conductivity of pure organic solvent is too low. A small percentage of water in the mobile phase aids the droplet formation. Commonly, higher organic modifier percentages improve ionization in ESI and worsen it in atmospheric pressurec chemical ionization (APCI). Although solution chemistry is not as critical in APCI as in electrospray, the solvent properties still need to be considered for the best performance. Protic solvents, such as methanol, improve positive ionization. Addition of volatile buffers, such as ammonium formate-formic acid or acetic acid-ammonium acetate, provide ion enhancement in basic analytes. They are compatible at concentration of 10 to 20 mM. The pH of the mobile phase determines the ionization state of the analytes, when working with acids, bases or amphoteric species, and therefore affects the response in LC/API-MS. The mobile phase pH also determines chromatographic selectivity for ionizable compounds.

Ion-exchange liquid chromatography (IELC) has been used to separate ionic species such as ammonium quaternary herbicides, biogenic amines and acrylamide [30, 115, 151]. Most applied columns have a packing material that bears sulfonic acid (strong cation exchange or SCX) side groups. Acrylamide is mainly determined by using a column that combines ion exchange with size exclusion chromatography [151]. The need for strong salts, counter ions and buffers imposes additional complications. These complications are well illustrated in the typical chromatographic separation that is shown in Figure 2 for a standard mixture of seven amines by UV, suppressed conductivity and MS detection using selected ion monitoring (SIMs), using the gradient elution program described in the left side (concentrations relative to the LOQ of the suppressed conductivity detection) [152]. The standard mixture contains those polyamines and biogenic amines expected to be naturally found in meat products (polyamines) and also other amines that are related to microbial growth (putrescine, cadaverine, histamine and tyramine). In this case, all the considered amines were separated in 40 min (total run time). It is to be stressed that tyramine cannot be detected by MS if this device follows a suppressed conductivity detector because tyramine is removed by the suppressor. Tyramine could be detected by MS only when the separator column is directly connected to the mass detector.

Table 2

Liquid chromatographic separation

Mobile phase constituents	Influencing factors	Observations	Compounds	Ref.
Organic modifier Methanol Acetonitrile	• Surface tension and dielectric constant of the solvent • Gas phase basicity or acidity (proton affinity) of the solvent • Elutropic strength of the solvents (at lower elute compounds at higher-percentage of organic solvent)	•The use of methanol instead of acetonitrile is encouraged •Higher organic solvent percentages improve sensitivity using ESI	-Steroid hormones -Trichothecenes -Non ionic pesticides: carbamates, phenylureas, benzoylureas, azoles, amines, triazoles, morpholines, thiazoles -Veterinary drugs -Food packaging migrating materials	[69, 71, 72, 75, 83, 84, 99, 100, 153-157]
Salt addition Ammonium acetate Ammonium formate	• Volatile buffers • Salt concentrations, necessary to obtain acceptably sharp and well-retained peaks for the target compounds. • Salt composition	•Ammonium acetate and ammonium formate are applicable at concentrations lower than 10 mM for ESI and 100 mM for APCI • Presence of ammonium can cause signal suppression of acidic compounds	-Yessotoxins -Aflatoxins, citruline, ochratoxin A, fumonisins -Ammonium quaternary herbicides -Daminozide -Pesticides -Food packaging materials -Veterinary drugs	[31, 55, 57, 82, 86, 94-96, 115]
pH modification Acetic acid Formic acid Tripropylamine Ammonium hydroxide	• Acids or bases used as mobile phase have to be volatile • pH influences separation as well as the ionization of the analytes in the interface. Some times in contrary ways	•Post column addition of acid or basic solution to improve ionization • "wrong way-around results" (intense $[M+H]^+$ ions in spraying basic solutions and intense $[M-H]^-$ sprayed from acidic solutions)	-Algae and fish toxins -Macrolide antibiotics -Tetracyclines -Heterocyclic amines	[16, 21, 34, 50, 56, 106, 108, 158, 159]
Ion-pairing reagents Heptafluorobutiric acid Tributylamine Triethylamine	• ion pairing reagents have to be volatiles • Ion-par formation increases the hydrophobicity and the retention on the stationary phase	•Use of volatile ion pairing reagents	-Ammonium quaternary herbicides -Acrylamide	[30, 59, 160-162]

However, in this case you should find another eluent because the eluent contains methane sulfonic acid (MSA), which is not compatible with the mass spectrometric detector. A test with a MS compatible acid – such as formic acid – enabled tyramine detection; but this eluent did not allow a full separation of all the other amines. Furthermore the relative high concentration of formic acid (30 mM) – necessary for the tyramine elution – reduces in mass spectrometry the sensitivity and the ionization of the analyte (tyramine).

Chromatographic conditions for IC-MS determination

Column	**IonPac CG17 50 mm × 2 mm; IonPac CS17 250 mm × 2 mm**	
	Time	**MSA (mM)**
Eluent	−7	3
	0	3
	6	3
	26	30
	40	30
Temperature (°C)	40	
Flow rate (mL min^{-1})	0.38	
Injected volume (μL)	5	
Detection	Suppressed conductivity, CSRS Ultra-II, external water mode	
	UV 276 nm	
	MS ESI+: 3.0 kV, cone 50 V, probe temperature: 400 °C, dwell time: 0.2 s	
SIM	Analyte	*m*/z
	[Putrescine + H]$^+$	89
	[Cadaverine + H]$^+$	103
	[Histamine + H]$^+$	112
	[Phenethylamine + H]$^+$	122
	[Agmatine + H]$^+$	131
	[Spermidine + H]$^+$	146

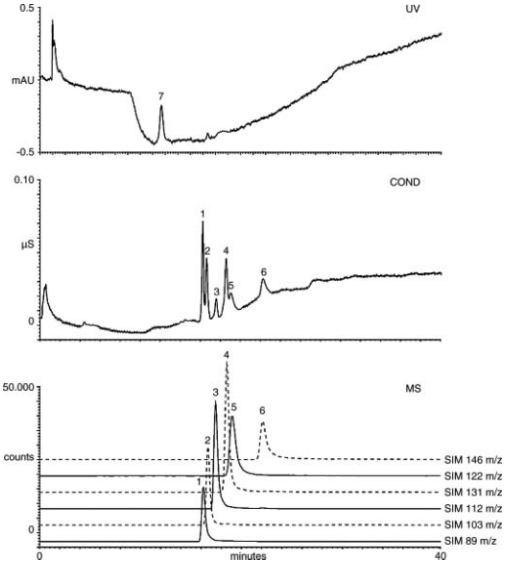


Figure 2. Chromatograms of a standard solution of biogenic amines (0.5 mg L^{-1} each). Chromatographic conditions are reported in the table of the left. Peaks: 1, putrescine; 2, cadaverine; 3, histamine; 4, agmatine; 5, phenetylamine; 6, spermidine; 7, tyramine (Reproduced from [152] with permission from Elsevier© 2005).

3. Interfacing systems

The ioization of the target compounds takes place in the interface. Together, ESI and APCI provide efficient ionization for very different types of molecules including polar, labile, and high molecular mass toxicants [163, 164]. Other LC-MS interfaces (for example, thermospray, particle-beam) have influence modern LC–MS, but have been moved out quickly from research laboratories. Also, a promising new ionization source, atmospheric-pressure photoionization (APPI), was introduced just a few years ago, but are not yet largely used. Evidently, acid compounds are ionized in NI instead basic ones, which are ionized in PI mode [11, 12].

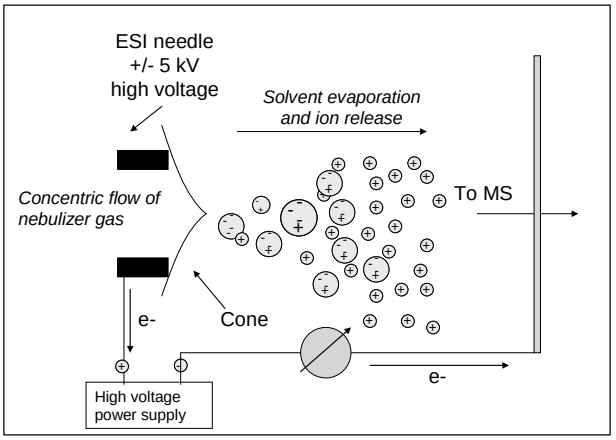


Figure 3. Scheme of the electrospray process.

Actually, the large majority of applications are done with electrospray and APCI ionisation. ESI is a very soft ionization technique, especially well suited for ionic compounds and compounds with a high molecular weight. The spray is generated by electrically charging the liquid to a very high voltage. The charged liquid in the nozzle becomes unstable as it is forced to hold more and more charge. Soon, the liquid reaches a critical point, at which it can hold no more electrical charge, and at the tip of the nozzle, the droplets explode by coulomb repulsion into yield highly charged molecules. The capillary is held at high voltage in atmospheric pressure to generate the spray. Sometimes the spraying is supported by a make up flow, which allows the use of higher flow rates. Liquid-phase chemistry plays a key role in the ion formation on ESI [1, 82, 138, 161, 165-167].

In APCI, a stable spray is generated by heating an aerosol from the liquid effluent of the LC with a sheath flow of gas at atmospheric pressure. Ions are generated by a corona discharged in the spray, forming chemical ionization plasma. APCI is better suited for non-ionic compounds of moderated molecular weight. Chemical ionization occurs in the vapor state to form the $[M+H]^+$ ion. The corona-discharge needle in the APCI source produces a stream of electrons, which ionizes the solvent of the mobile phase. According to the current theory on APCI ionization, in the PI mode the $CH_3OH_2^+$ and H_3O^+, present in the vapor state, transfer protons to the weakly basic pesticides in the vaporized state in line with their proton affinity, whereas in the NI mode the electronegative compounds attach an electron, and become negatively charged. APCI interface can produce thermal degradation of thermolabile compounds.

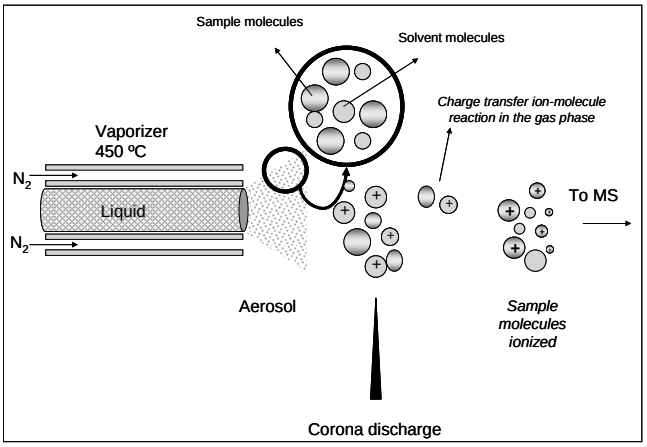


Figure 4. Scheme of the APCI process.

There are some basic rules to select the ionization mode and interface. However, the literature also shows that there is not a unique alternative because most organic contaminants are complex molecules that contain basic sites that can easily be protonated, and electronegative atoms that have tendency to attract electrons, which makes difficult to predict their course according to the theory of ionization. A diagram called the "ionization continuum flow" shows that proton affinity in the gas phase and polarity in solution (pKa) are useful for selecting APCI or ESI (Figure 4). [150]

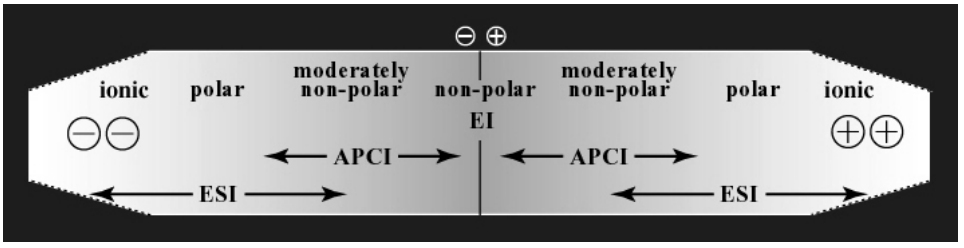


Figure 4. Continuous flow diagram (Reproduced from [150] with permission from American Chemical Society© 2000).

However, there are a great number of food toxicants that can be determined by both APCI and ESI with an adequate sensitivity [168-171]. A summary of the literature is shown in Table 3.

Table 3. Detection of various classes of organic contaminants using LC-QIT-MS by different ionization modes and interfaces

Compound	Characteristics	Ref
Veterinary drugs		
Aminoglucosides	Determinable with ESI in PI mode	[44, 172]
β-lactams	Most sensitive using ESI in PI but also determinable by ESI in NI, and APCI in PI with less sensitivity	[34, 173, 174]
Macrolide antibiotics	Very sensitive using ESI in PI mode and also determinable by APCI in NI mode	[175]
Fluoroquinolones	Determinable with ESI in PI mode	[28]
Sulfonamide	Determinable with ESI in PI mode	[50, 176, 177]
Tetracycline	Determinable by all APCI and ESI in PI and NI modes more sensitive by ESI in PI mode	[176, 178]
Macrocyclic lactones	Determinable by APCI in NI mode	[179]
Phenicols	Determinable by ESI in PI mode	[28, 38]
β-agonists	Determinable by ESI in PI mode	[27, 180]
Pesticides		
Anionic or acidic		
Phenoxyacid	Determinable in NI mode, more sensitive by ESI that by APCI	[165, 181]
Halogenated phenols	Determinable in NI mode, more sensitive by ESI that by APCI	[181]
Neutral		
Imidazoles, benzoimidazoles	More sensitive by PI mode than by NI mode, and more sensitive by APCI than by ESI	[61, 63, 83, 165, 182, 183]
Carbamates	Determinable by APCI in PI and NI modes and ESI in PI modes. Equal sensitive by APCI and ESI in PI modes. Less sensitive by APCI in NI mode	[61, 76, 83, 168, 181, 183]
Organophosphates	Determinable by ESI and APCI in PI mode and by APCI in NI mode	[1, 63]
Anilide herbicides	Determinable in PI mode. More sensitive ESI than APCI	[83, 165]
Benzyolureas and phenylurea, sulfonylurea	Most sensitive by APCI in PI mode. Equal sensitive in ESI by PI and NI. Little sensitive by APCI in NI mode	[140, 165, 170, 171, 181, 183, 184]
Triazines	Determinable in PI mode. More sensitive by APCI than by ESI	[165, 181, 184]
Nicotinoids		
Acaricide		
Pyrethryn	Determinable by ESI in PI mode	[83]
Macrocyclic lactones	Determinable by ESI in PI mode	[185]
Cationic (ammonium quaternary herbicides)	Determinable by ESI in PI mode	[30, 138, 161, 162, 166, 167, 171, 186]
Mycotoxins		

Compound	Characteristics	Ref
Aflatoxins, citrinine, ochratoxin A, fumosins, trichothecenes	Determinable by ESI in PI mode	{186,404, 187, 189
Heat induced decomposition products		
Heterocyclic amines	Determinable in PI mode. More sensitive by APCI than by ESI	[110, 111, 187, 188]
Acrylamide	Determinable in PI mode. More sensitive by ESI than by APCI	[113, 189-191]
Algae and fish toxins		
Microcystins*, nodularins, domoic acid, yessotoxins, azapiracids	Determinable by ESI in PI mode. * Also determinable by ESI in NI mode	[105, 107-109, 192, 193]

Atmospheric pressure photoionization (APPI) is an interesting addition to API techniques for increasing the range of LC-MS applications to less polar compounds. APPI operates as APCI, but it uses photons in the vacuum UV region instead of a discharge of electrons. The APPI ionizes a molecule (M) by photoabsorption and electron ejection to form the molecule radical $[M]^+$. In the presence of water vapor or protonic solvents, $[M]^+$ can extract an H^- atom to form a protonated molecule $[M + H]^+$. The addition of a dopant, such an acetone or toluene, can increase the ionization yield of the target compounds. There are two different geometries of the source: the in-line geometry and the orthogonal [143]: (i) the in-line geometry source, which always requires a dopant to increase efficiency of ion formation, has the discharge lamp placed straight on the stainless steel heated nebulizer probe and some simple modifications to position an electrical connector for the high-voltage supply of the discharge lamp and (ii) the orthogonal geometry source, in which the heated nebulizer and the photo-ionization lamp are, respectively, perpendicular and in-line with respect to the mass spectrometer's ionic path. Such a source can be indistinctly be used with or without a dopant. Figure 5 shows a schematic diagram of the orthogonal source.

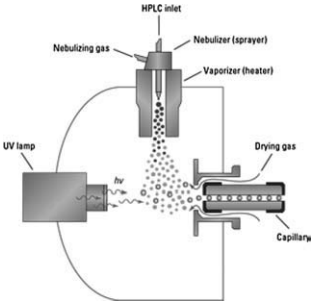


Figure 5. Scheme of the APPI interface (courtesy of Agilent)

Compounds, which show lower first ionization potential than the energy of the photons, are ionized. APPI-MS is an important complimentary technique to APCI for low and non-polar analytes. Sensitivity is similar by both interfaces. However, an advantage of using APPI for carbamate determination in fruits and vegetables is the low matrix effect observes that makes unnecessary the use of matrix-matched standards [194, 195]. Takino et al. [196] also demonstrated that the APPI technique is an ideal ionization technique for determining patulin in apple juice because of its sensitivity and high selectivity, and remark, again, the low matrix effect observed. As shows Figure 6, the apple juice matrix led to alterations in the chromatograms, including some additional peaks and rise in the base line. However, the intensity of these additional peaks and the overall baseline with APPI were lower than those with APCI, and caused no interference. Furthermore, the changes in retention time and peak intensity with APPI and APCI were 0.6, 4.7 % and 0,7, 52,4 %, respectively. These results pointed out that APPI could offer higher selectivity and lower matrix effect than APCI.

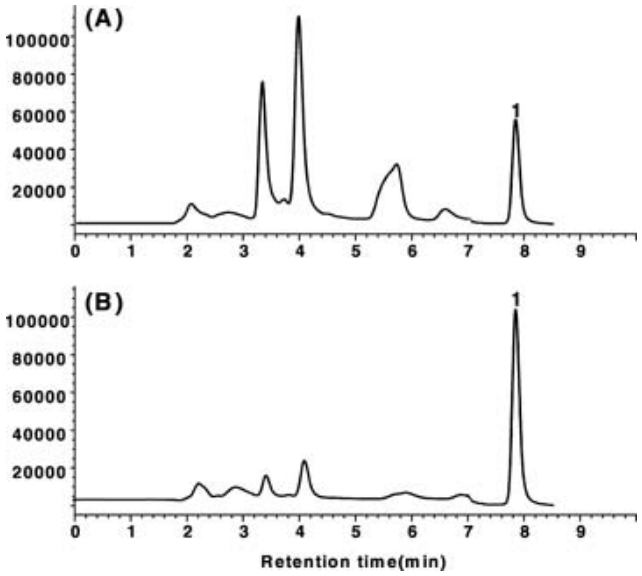


Figure 6. SIM chromatograms for patulin (peaks marked "1") in apple juice at 20 ng mL^{-1} with (A) APCI and (B) APPI (Reproduced from [196] with permission from Wiley© 2003).

3.1. Matrix effects

A feature of the LC-MS specificity is its ability to simplify or, even to eliminate, sample preparation and chromatographic separation attaining a large sample throughput. In spite of

this, the sample complexity and the number of compounds that co-eluted with the analytes generate a very important problem in LC-MS –the matrix interferences. These interferences are caused by the coextractants present in the injected sample, which may cause enhancement or suppression of the analyte signal [18].

The matrix effect is related with the ionization source and is attributed to the influence of matrix components in the affectivity of the ionization processes in the API interfaces — causing a positive or negative effect in the amount of ions formed from the target analytes. ESI interface is more susceptible than APCI to matrix signal-suppression effects, which has been widely discussed. However, the information about the effects of this type of interferences on APCI interface is barer.

The exact mechanism of matrix interference phenomena is not well-known given rise to different theories on the origin of matrix effect. Most instituted one states that the organic compounds present in the sample in concentrations greater than 105 M may compete with the analyte for an access to the droplet surface for gas phase ion emission, and that the gas-phase basicity or acidity of co-eluting matrix components may be higher than that of the analyte of interest, and thus proton transfer probably occurs in the ionization process, decreasing the ion intensity of the analyte. Another hypothesis refers to the radius of droplets from which gas-phase ions are emitted. If samples contain non-volatile matrix components, droplets are prevented from reaching their critical radius and surface field, hence the ionization efficiency decreases and the ion signal for an analyte is reduced. Matrix components may also influence the effectivity of the ion formation in the ionization process by altering the surface tension of the electrospray droplets and by building adduct ions or ion pairs with the analytes. The basic or acidic character of the matrix components could promote the formation of the protonated or deprotonated molecular ions of the analytes during the ionization process, causing enhancement of the signal. There is still a lot to be done to understand the mechanisms and to properly predict matrix effects.

These effects originates that the response of an analyte in pure solvent standard varies significantly from that in matrix samples. They can be easily detected by comparing the response obtained from a standard solution and that from a spiked pre-treated sample (post-extraction spike). An example of matrix suppression is illustrated for the pesticide triflumisol in different matrices (Figure 7). Triflumisol peaks acquired by LC-ESI-MS in selected reaction monitoring (SRM) mode in solvent or post-extraction spiked extracts of eggplant, lettuce, and pepper are compared. Co-eluting matrix components in the pepper extracts almost completely suppress the analyte response, while for eggplant and lettuce extracts less, but still significant, suppression is observed [197].

Practically, matrix effects created a problem that has to be solved when accurate results are required, as is that these effects must be reduced or compensated by any of the approaches summarized in Table 5. There are two general approaches to deal with matrix effects in quantitative analysis. First, one may eliminate the sample constituents responsible for the

matrix effects. This would imply improvement of the sample pre-treatment and/or the chromatographic separation (efficiency and/or resolution). Although this would in principle be the best approach, it may be difficult to achieve, especially in multiresidue analysis, where a variety of interferences may be involved. Alternatively, one may reduce or eliminate the influence that matrix effects have on the accuracy and/or precision of the method. The latter may be achieved by one or a number of the following measures: change to another ionization method, change the mobile-phase composition, and/or use adequate standardization. In many cases, both approaches must be combined to achieve adequate quantitative results. However, the easiest way to eliminate the matrix suppression/enhancement phenomena, without any further laborious sample clean-up and/or complicated chromatographic separation, is to apply an appropriate calibration technique that compensates for the matrix effects. This is the most widelaccepted and used approach in the literature [198].

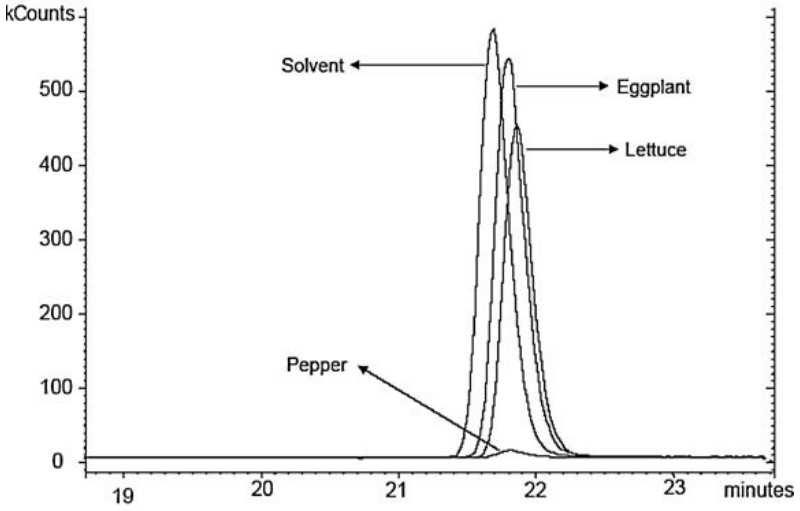


Figure 7. Matrix suppression in the LC-ESI-MS analysis of triflumisol in different matrices. The compound was isolated by liquid extraction with ethyl acetate, evaporation to dryness, and reconstitution in methanol (Reprinted from [197] with permission from Elsevier© 2004).

Table 4

Procedures for compensating matrix effects

Procedure	Merits	Drawback	Applications	Ref
REDUCTION OF THE MATRIX CONSTITUENTS				
More selective extraction or more extensive clean-up	Effective to eliminate the matrix effects	Laborious and time consuming Possible loss of the analytes during several consecutive clean-up steps	Seventeen Polar Pesticides in fruits Carbamates, benzimidazoles, azoles, benzoylurea in apple sample Chlormequat in fruits Carbofuran in potato β-lactam antibiotics in milk Clenbuterol in bovine liver Fusarium mycotoxins in maize	[183] [171] [30] [168] [174] [180] [80]
To inject less amount of sample by diluting the extract or by disminishing the injected volume	Effective to eliminate the matrix effect Simplicity	Increase the LOD	Not reported recently to determine toxic compounds in food	--
CORRECT EFFECTS ON ACCURATE AND PRECISION				
Improvement of the LC-MS method				
Efficiency by LC-LC	Increase peak capacity	Increase the analysis time Complexity of the system	Not reported recently to determine toxic compounds in food	--
Modification of mobile phase composition	Easy to apply	Response of the standard can be significantly suppressed by the buffer.	Pesticides Several contaminants	[82, 199] [70, 200]
Change the ionization mode				
Appropriate calibration				
External matrix-matched standards	Economical Easy to apply	No general regression curve for different food and environmental matrices Extremely time-consuming	Chlormequat in pears N-methylcarbamates in baby food Seventeen Polar Pesticides in apples and apricots	[167] [76] [183] [61]

Procedure	Merits	Drawback	Applications	Ref
			Carbendazim, hexythiazox, imidacloprid, methiocarb and thiabendazole in oranges	[171]
			Carbamates, benzimidazoles, azoles, Benzoylurea in apple sample	[139]
			Daminozide in apples	[34]
			β-lactam antibiotics in kidney	[172]
			Gentamicin and neomycin in milk	[201]
			Cephapirin (CEPH) in milk	[176]
Internal standards				
Isotopically labeled internal standard	Retention time identical or very close to the retention time of the analyte	Rather expensive (especially in multicomponent analysis).	Chlormequat in pears, grapes, purées and juices (d4-chlormequat)	[30]
		Isotopically labeled standards not available	Clenbuterol-d6 in liver samples	[27]
			Acrylamide	[189]
			Heterocyclic amines in food	[187]
Eco-peak technique	Possibility of monitoring the signal reduction during the analysis and to compensate it by relating sample peak area to this internal standard.	Low peak capacity of LC as compared to GC to double the number of peaks in the chromatogram	Carbamates, benzimidazoles, azoles, benzoylurea in apple sample	[171]
Post-column addition	Internal standard (e.g. structure analogue of analyzed compound (s)	Additional pump is necessary for routine batch analysis	Not reported recently to determine toxic compounds in food	

4. Mass analyzers

4.1. Single quadrupole

The quadrupole has been the most common analyzer in laboratories until recently. It is not only the standard analyzer for GC–MS (which will not be discussed here) but also is part of various designs of LC–MS instruments. Quadrupoles are not used only as mass analyzers; they also are implemented frequently as ion transfer optics and collision cells. The quadrupole consists of four parallel metal rods. Each opposing rod pair is connected together electrically and a radio frequency (RF) voltage is applied between one pair of rods, and the other. A direct current voltage is then superimposed on the RF voltage. Figure 8 shows a scheme of the single quadrupole. Ions travel through the quadrupole in between the rods. Only ions of a certain *m*/z will reach the detector for a given ratio of voltages, other ions have unstable trajectories and will collide with the rods. This allows selection of a particular ion, or scanning by varying the voltages.

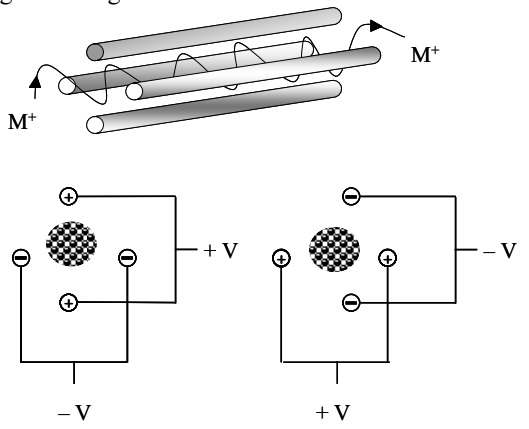


Figure 8. Scheme of a single quadrupole

In full-scan experiment, the DC and RF components are ramped at a constant ratio, and ions entering from the ion source are enabled to pass through the rod assembly successively. For a specific combination of DC and RF, only a very small *m/z* range can pass through, the size of which determines the resolution of the instrument. Quadrupoles usually are operated at unit resolution throughout the mass range (often up to *m/z* 4000), allowing the separation of *m/z* 150 and 151 or *m/z* 1500 and 1501, for example. The sequential detection in the full-scan mode of any quadrupole analyzer results in a low duty cycle, typically of the order of 0.1%, depending upon the monitored *m/z* range. This is translated in low sensitivity that makes this mode inappropriate to determine organic contaminants because they are commonly at trace levels in the sample.

The duty cycle and the resulting sensitivity is much higher in the SIM mode of the quadrupole (close to 100%). In this case, the DC and RF potentials are held constant, so only a specific *m/z* ratio can pass through. One also can jump between different voltages, to allow more than one *m/z* ratio to be detected sequentially, with a corresponding reduction in duty cycle. However, one drawback of this mode is that only a limited number of ions can be monitored, for each compound because ESI and APCI were designed to provide a soft-ionization process that leads to a mass spectrum with only a few ions. The poor fragmentation of molecules is translated in deficient specificity because isobaric interferences (compounds with the same m/z relation) or multiple-component spectra are frequently observed in extracts of complex matrices such as food.

Single quadrupole instruments usually are limited to measuring intact species generated by the ionization source, resulting in less selectivity. In any case, this mass analyzer has been the most applied in mass analysis for many decades because it is relatively inexpensive, rugged are particularly sensitive in SIM mode, and ideal for trace target applications. However, either, the lack of fragment ions or that those fragment ions could come from several compounds which coeluted in the same peak in LC–MS, making structure assignments difficult (similarly, quantitative analysis in the SIM mode would be difficult when isobaric interferences contribute to the chromatographic peaks). Figure 9 shows a typical example of isobaric interferences. Carbaryl and thiabendazole are two pesticides that have a very different structure but the same molecular weight. These pesticides generally coeluted in isocratic LC conditions.

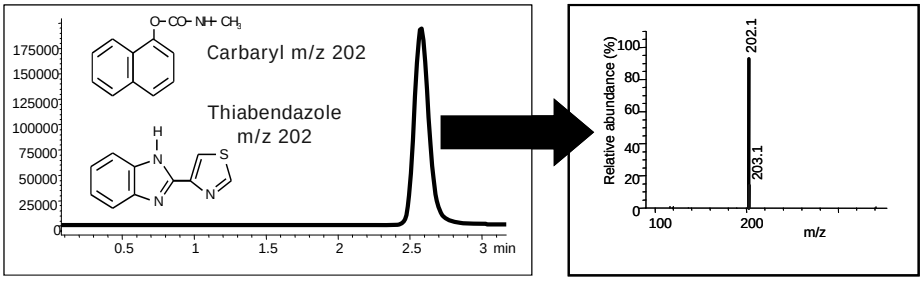


Figure 9. LC-MS chromatogram of a mixture of carbaryl and thiabendazole using LC-MS and appliying a cone voltage (known also as fragmentor voltage) of 20 V.

In a single-quadrupole instrument, operating with an ESI or APCI source, selectivity can be increased when fragmentation is induced in the ion source region by increasing the voltage. This process is known as collision induced dissociation (CID). This procedure refers to the activation of ions in the region between the ion source and the analyzer, in which fragmentation can be initiated by collisions with residual gas molecules at intermediate pressures ("in-source CID"). The generated fragment ions can be used for limited structure

elucidation or confirmation purposes. Figure 10 located the fragmentor voltage within the ionization source, and illustrates how increasing these fragmentor or cone voltage thiabendazol and carbaryl can be distinguised by the obtention of a characteristic fragment ion of each molecule, *m*/*z* 175 for thiabendazole and *m*/*z* 145 for carbaryl.

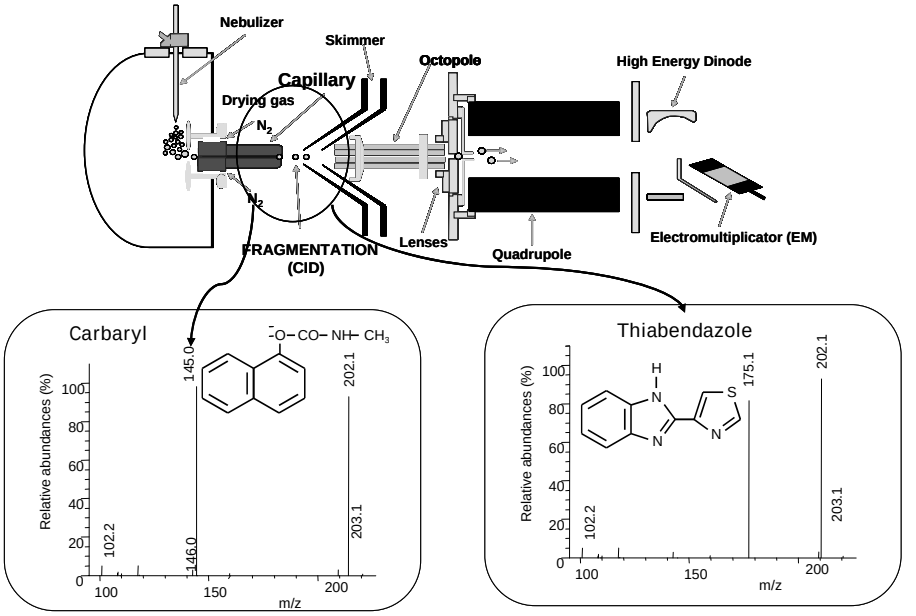


Figure 10. LC-MS chromatogram of a mixture of carbaryl and thiabendazole using LC-MS and appliying a cone voltage (known also as fragmentor voltage) of 100 V.

Single quadrupole instruments usually are limited to measuring intact species generated by the ionization source, resulting in incomplete selectivity in comparison to many of the advanced instruments described in the following sections. Typical applications of single quadrupoles, when the EU criteria (2002/657/EC) or related ones have to be meet, include screening and quantification. However, a triple quadrupole or an ion-trap should be used anyway for confirmation purposes. LC-single quadrupole based methods are advantageous because samples can be analyzed after a simple cleanup. However, these methods do not perform well in difficult matrices such as coffee. As good example of the lack of selectivity of the single quadrupole to determine acrylamide in coffee is shown in Figure 11. The presence of interfering ions in the aqueous extract, even after SPE cleanup, prevents an accurate and reliable quantification of acrylamide [202].

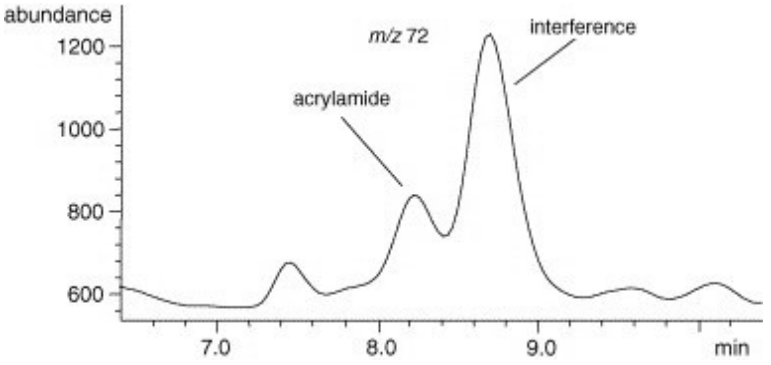


Figure 11. LC–MS chromatogram of acrylamide in coffee. Chromatographic conditions: column, Inertsil ODS-3; mobile phase, 0.01 mM acetic acid in 0.2% aqueous solution of formic acid and 0.2% acetic acid in acetonitrile (98:2, v/v) at 0.6 ml/min (Reproduced from [202] with permission from Elsevier© 2006).

4.2. Time-of-flight

Linear TOF mass spectrometer is very simple. It consists only of an ion-accelerating region, a flight tube, and a detector. In theory, all ions experience the same potential difference during acceleration and, consequently, have the same kinetic energy at the start of the flight tube, and thus different velocities depending upon their mass. Therefore, their arrival time at the detector is proportional to their mass and they reach the detector in order of increasing mass. The major deficiency of a simple linear TOF instrument is its insufficient mass resolution, resulting from flight time variations of ions of the same *m/z*. As a result, modern TOF instruments commonly employ reflectons to enhance resolution [203, 204].

A reflectron TOF (RETOF) is used to focus ions of the same *m/z* but of different kinetic energy. There are different designs of reflectrons but often they consist of a series of rings or grids. It is located after the drift tube, creating a retarding field that the ions penetrate. Depending upon their kinetic energy, they enter this field at different depths and then are reflected back into the flight tube, where they drift to the detector, which is placed close to the ion source. Consider the case of ions of the same *m/z* but significant energy spread, which would be detected as broad peaks in the linear TOF, the ion with a higher kinetic energy penetrates deeper and spends more time in the reflectron. The slow ion, on the other hand, returns to the flight tube faster, but with the same lower kinetic energy. The faster ion will catch up with the slow one at the detector, thereby improving mass resolution greatly [7, 205-210].

The pulsed nature of TOF analysis makes the coupling with electrospray -a continuous source of ions- difficult. The necessary focusing of the ions coming from the ESI source is usally carried out by an orthogonal acceleration (oa) TOF type. The ions are focussed into the ortogonal accelerator as a narrow ion beam and a slice of it is pushed down into the flight tube [206].

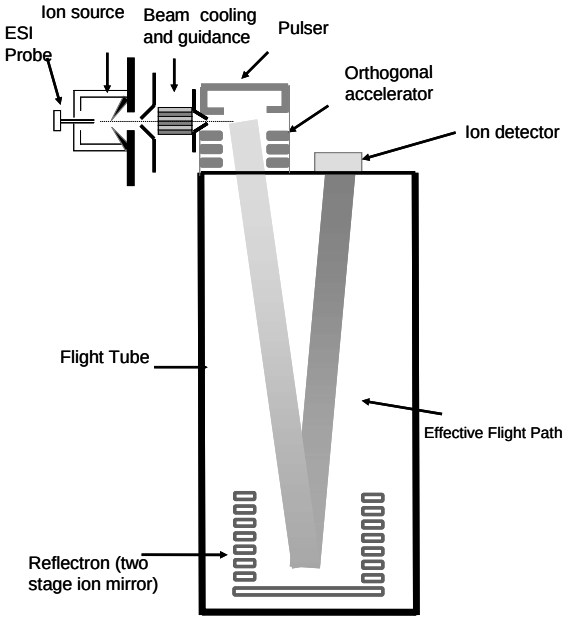


Figure 12. Scheme of TOF.

LC-TOF-MS instruments present several advantages. The high mass range that can be analyzed (with a linear TOF instrument) and the high ion transmission attained the quasisimultaneous detection of all ions, resulting in high sensitivity "full spectrum" analyses (TOF-MS is not a scanning instrument). This characteristics make this instrument just right in qualitative applications such as the identification of non-tatgeted and/or unknown compounds, in which the acquisition of an entire mass spectrum is required. In addition, LC-TOF-MS offer improved selectivity due to the high-resolution power linked to the capability to provide exact mass chromatograms over nominal mass chromatograms (1 Da mass range), which attains identificatication of mass interferences with analytes having the same nominal mass and chromatographic retention time [206, 210].

A nice example of identification of non-targeted pesticides and their metabolites was reported by Thurman et al. [68] who use this technique to identify (withouth the initial use of

standards, different post-harvest fungicides and their metabolites. In summary, the method involves accurate mass identification of the molecule and its isotopic clusters, database searches, and MSn pathway elucidation, followed by standard identification when possible. Figure 13 (insert) displays the total ion chromatogram of a lemon extract. A chlorine-containing suspected specie was found in this TIC at the retention time of 22.7 min. As can be seen in Figure 13 A in the accurate mass spectrum, there are two main peaks: m/z 376 and 308. The isotopic pattern and the difference between both signals evidence the presence of three chlorine atoms in the studied specie. Using a large accuracy error threshold and including a minimun and a maximum number of chlorine atoms of three in the elemental composition calculator tool, only two elemental composition matched with the m/z input: $C_{15}H_{17}N_3O_2Cl_3$ and $C_8H_{17}N_9SCl_3$. Using "The Merck Index" data base, a unique match with the second formula, corresponding to prochloraz, was found. In the same lemon extract, an ion with the same isotopic patterm (three chlorine atoms) at the retention time of 16.9 min (Figure 13 B) appears.

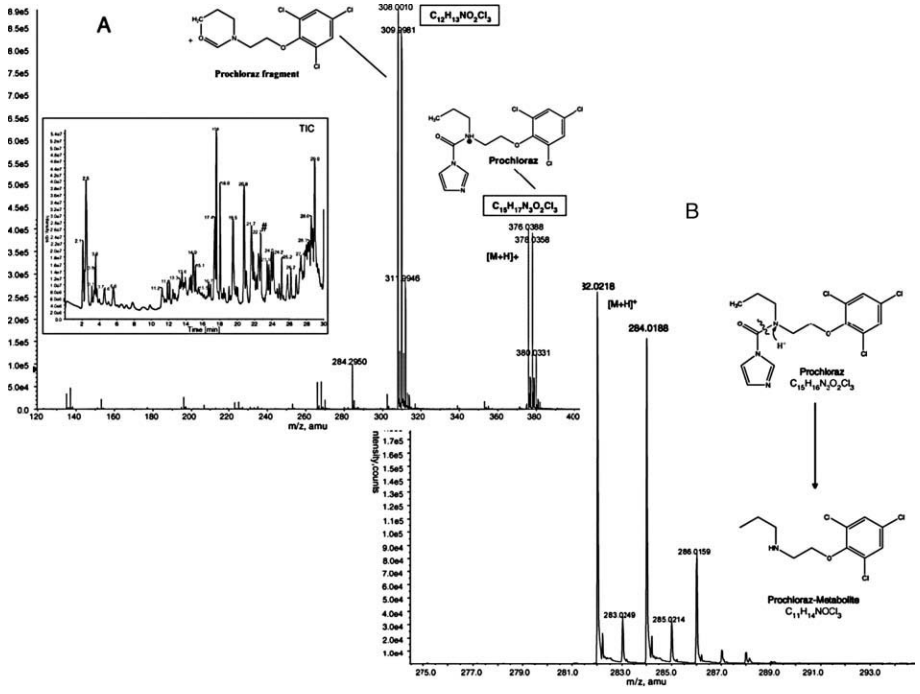


Figure 13. (A) Accurate mass spectrum of prochloraz, identified in the studied lemon extract at tR 22.7 min; (inset) TIC of the lemon extract and (B) Accurate mass spectrum of the proposed prochloraz degradation product identified in the studied lemon extract at Tr 16.9 min. (Reproduced (modified) from [68] with permission from Elsevier© 2005)

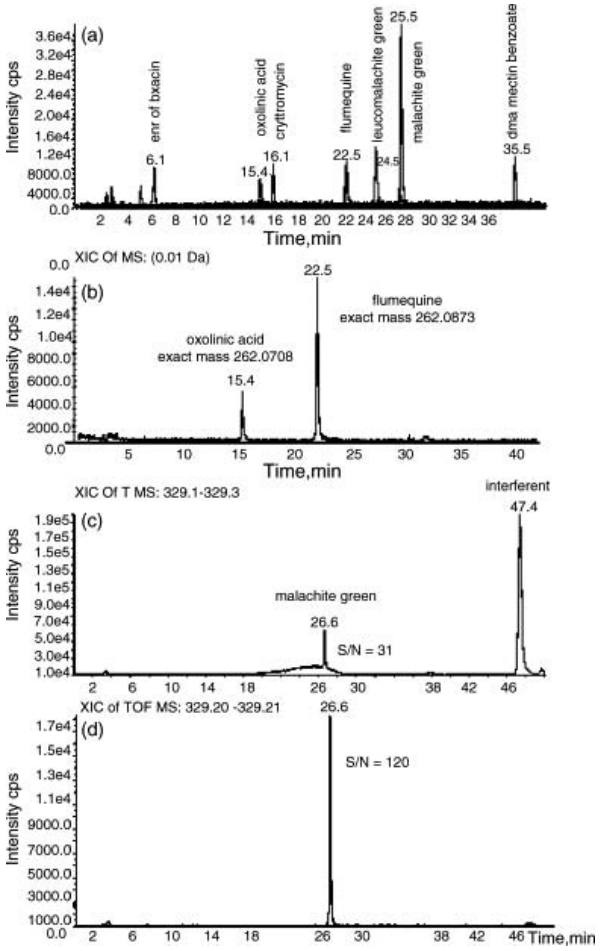


Figure 14 (a) Extracted ion chromatogram (EIC) using a mass window of ±0.01 Da for target analytes in extract of salmon spiked at 15 μg/l; (b) detection of analytes with the same nominal mass (oxolinic acid, *m*/*z* 262.0709 and flumequine, *m*/*z* 262.0873) EIC using a mass window of ±0.01 Da; (c) detection of MG and matrix interference in EIC (±0.2 Da); (d) EIC of MG (±0.01 Da) (Reproduced from [211] with permission from Elsevier© 2006.

Another interesting application of LC-TOF-MS was the simultaneous confirmation by accurate mass measurements and quantitative determination of antibiotic residues in food. Figure 14 (a) shows overlapped extracted ion chromatograms (EIC) using a mass window of 0.01 Da around the masses of interest, which correspond to an extract of salmon spiked at 15 μg/l. With this improved selectivity, compounds that are resolved at different retention time, but have the same nominal mass, such as oxolinic acid and flumequine (*m*/*z* 262) are discriminated as it is shown in Figure 14 (b) by exact mass 262.0709 and 262.0873, respectively. Figures 14 (c) and (d) show an additional example corresponding to malachite green (MG) and matrix interference, where enhanced selectivity is demonstrated by the EIC reconstructed using a mass window of ±0.01 Da (Figure 14 (d)) over the EIC with ±0.2 Da (Figure 14 (c)) for *m*/*z* 329. Thus, MG and matrix interference, which are resolved with different retention times, are discriminated by exact mass measurement (Figure 14 (d)) using a mass window of 0.01 Da. On the other hand, the selectivity is related with an enhanced S/N ratio, which is observed when narrow mass windows from ±0.2 to ±0.01 Da are applied and is showed in Fig. 2(d) for MG. In order to compute errors, an average mass spectrum was taken across the largest peak in the EIC, and masses for each analyte were compared against the theoretical mass.

The disadvantage of TOF is its limited dynamic range; that is, the ratio of the maximum to the minimum observable ion intensities or concentrations over which a linear response is obtained from the detector. This is the direct result of the types of detectors that are employed for handling the large number of high-resolution spectra, making the application of TOF instruments to quantitative analyses less attractive, but not impossible as demonstrates the number of applications of LC-TOF-MS to quantitive determination of food toxicants [7, 206, 207]

Finally, it should be remarked, as it is exemplified in some of the spectra reported, CID fragments obtained in TOF-MS analysis can provide useful structural information for confirmation of residues. Since MS spectra are recorded at high resolution, the use of TOF for confirmatory methods could fulfil the EU legislation criteria, based on the use of IPs [208, 209].

4.3. Tandem mass analyzers

4.3.1. Triple quadrupole

The most common mass analyzer for quantitative analyses is the *triple quadrupole* (QqQ). This MS–MS instrument consists of three consecutive (Q_1 q_2 Q_3 ; Q refers to a mass-resolving quadrupole, q to an rf-only quadrupole). This configuration achieves additional ion activation in q_2 , after the target ion has been selected in Q_1 . The second quadrupole q_2 is operated in the rf-only mode, in which only an rf voltage is applied to the quadrupole rods (no dc component), thus effectively becoming a wide-band pass for the ions. It can be filled

with a neutral gas such as N_2 or Ar, acting as a collision gas. The ions leaving Q_1 are accelerated into q_2 with offset voltages between 0 and 100 V. The results of the *collision-induced dissociation* (CID) can be analyzed with the mass analyzer Q_3 . This is the most common form of an MS–MS experiment on a triple-quadrupole mass spectrometer, and it is called a *product ion scan*. In addition, several other selective MS–MS modes are available, depending on what mode Q_1 and Q_3 are used. The high versatility allows to employ various selective screening strategies (i.e. neutral-loss, precursor-ion, and product ion scan modes) [204, 212, 213].

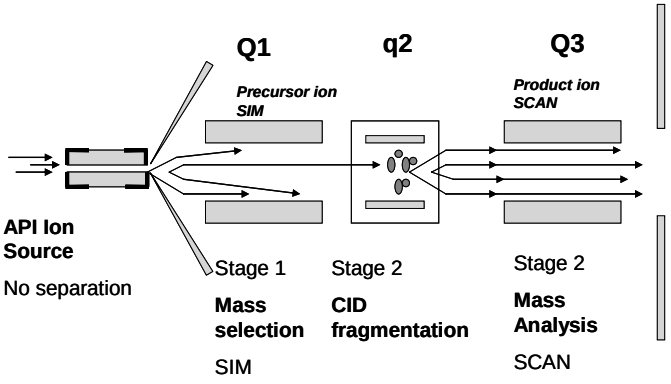


Figure 15. Scheme of a QqQ

The precursor ion scan is the other way around the product ion scan. The third quadrupole is set to select a specific product ion formed in q2 , and the first quadrupole is scanned for all precursor ions forming the chosen fragment. A neutral loss scan experiment means that Q1 and Q3 are scanned over the desired mass range. Both quadrupoles are synchronized to maintain a constant mass difference between the selected masses for all scan [213].

Selected reaction-monitoring mode (SRM) - single or multiple (MRM) - enhance the detection limit in analytical procedures. Q1 and Q3 quadrupoles are both fixed at a single mass. Because the lack of sensitivity of the other modes, only multiple reaction monitoring (MRM) have a general use. MRM filter the precursor and the product ion. The advantage is that can perform this operation for multiple precursor and products ion obtained high sensitivity and selectivity. The disadvantage is that the spectrum can not be obtained [58, 204, 212, 213].

Figure 16 shows one of the most common applications conducted with triple-quadrupole instruments, the quantitative analysis of pesticides and their metabolites in fruits and vegetables [211]. This example highlights the large number of analytes that can be

simultaneously determined (more than 73 in this case). In these analyses, the *MRM mode* is almost always applied, allowing enhanced sensitivity and selectivity by circumventing isobaric interferences from food. LC-MS/MS with QqQ in SRM has become so far, the most widely used technique for the quantitation of toxic compounds in food because, under these conditions, a high sensitivity is achieved. Nowadays, a QqQ mass spectrometer is capable to detect approximately 100 analytes simultaneously with sufficient sensitivity for determination at μg/kg level. The use of time windows programme (periods) is not necessary unless the number of analytes to be analyzed within one run is significantly increased, or the compound pesents a very low response. Because of the high sensitivity achieved by MS/MS, gradient elution on a small reversed-phase analytical column is usually sufficient for identification. However, for confirmation purposes, at least two transitions must be recorded, and then an increase in the LODs occurs because the second transition is less abundant. Usually, transitions from the most abundant precursor to the most abundant product ions are selected. Small fragments with *m/z* ratios of <80 were generally omitted if alternative product ions are available. Figure 17 illustrates other application to determine veterinary drug residues, which remarks the low quantificatication limits achievable. In this case, besides to be highly specific, the ESI-MS/MS detector provides sensitivity for analyzing aminoglycoside antibiotics in milk at levels well below tolerance levels [32].

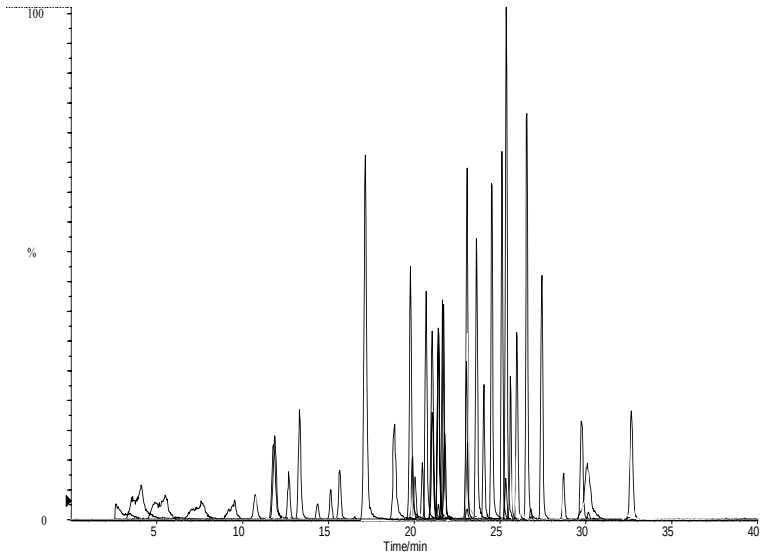


Figure 16. Combined MRM chromatogram of a matrix-matched standard at 0.05 mg/kg prepared in orange (Reproduced from [211] with permission from Wiley© 2005)

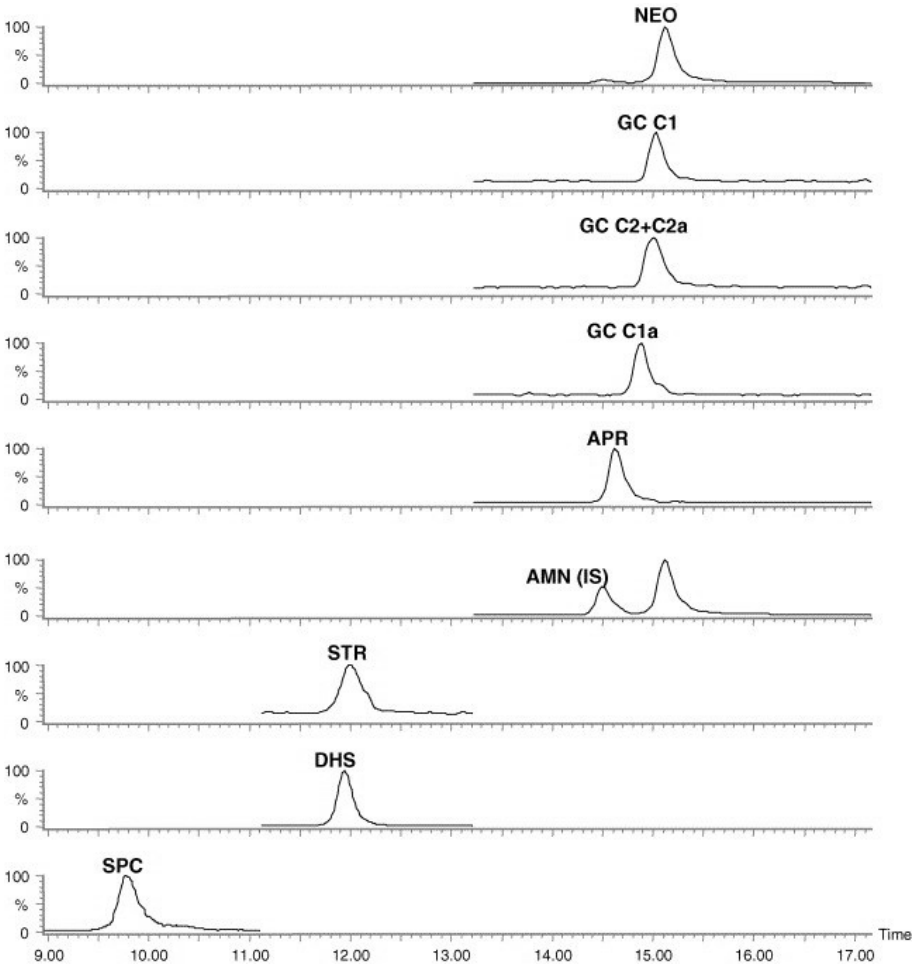


Figure 17. MRM LC-ESI/MS/MS from analysis of a bovine milk sample spiked with aminoglycoside antibiotics at one half of the tolerance level set by the European Union. Spectinomycin (100 ng/ml) (SPC), 100 ng/ml dihydrostreptomycin (DHS), 100 ng/ml streptomycin (STR), aminosidine (AMN) is the surrogate internal standard added to milk at 100 ng/ml level, 50 ng/ml of each gentamicin (GC), 750 ng/ml neomycin B (NEO). Apramycin (APR) is not permitted for veterinary use in European Union and it was added arbitrarily at the 50 ng/ml level (Reproduced from [32] with permission of Elsevier© 2005).

4.3.2. Quadrupole ion-trap

Nowadays, there are two versions of the quadruple ion trap. The traditional one, which is the three dimensional version of the linear quadrupole mass filter, and the most recently developed quadrupole linear ion trap. The former is the most applied in the field of food toxicant analysis because it was already marketted years ago and is a relatively affordable instrument. The latter is a new developed device with very promising features that has not been applied to food toxicant analysis yet [214].

In a QIT, ions are subjected to forces applied by an rf field but the forces occurs in the three dimensions instead of just two [215, 216]. This mass analyzer consists of three electrodes: one ring electrode between two hyperbolic endcap electrodes, which form a three-dimensional trap. The oscillating potential difference established between the ring and endcap electrodes forms a substantial quadrupolar field (up to 6 kV of rf voltage can be applied to produce the trapping and the scanning). The ions pass in and out of the traps by holes in the endcapds [216, 217]. A scheme of the QIT is shown in Figure 18.

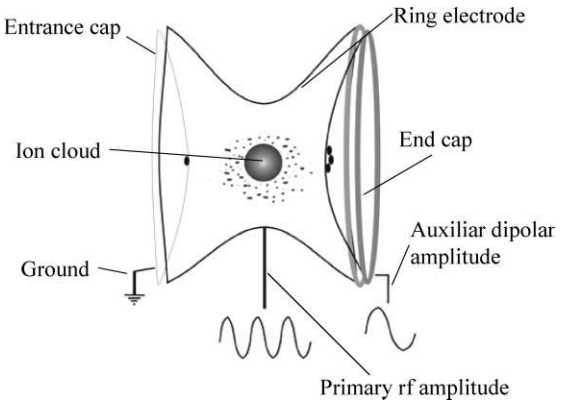


Figure 18. Scheme of a Quadrupole ion trap of classical setup with a particle of positive charge, surrounded by a cloud of similarly charged particles. The electric field is generated by a quadrupole of endcaps and a ring electrode.

QIT instrument has the advantage, over other tandem mass analyzers, that multiplies the stages of mass analysis umpteenth times by preselecting an ion, and analyzing the induced fragments, which helps to elucidate fragmentation pathways [7]. This is designated as multi-stage mass spectrometry, or MS^n, analysis, where n-1 indicates the number of fragmentation steps used [13, 204, 215, 216, 218].

Using mass-selective ejection, a QIT takes a sample, ionizes it, and then traps ions over a large mass range of interest, simultaneously. The ions in a QIT have a kinetic energy of ca. 1 eV, and can reside in the trap from milliseconds to hours. An increment in the dc potential will isolate ions of a particular m/z by ejecting all other ions from the trap. A subsequent

increase of the rf potential will eject the stored ions from the trap and send them to the detector, where the mass spectrum will be recorded [30, 61, 219].

Helium, at a pressure of approximately 10-3 torr, is used as a collision gas in the QIT. Helium gas has the effect of collisionally damping the ion motion causing their migration into a tighter ion cloud to the center of the ion trap. The closer the ions are to the center of the trap, the more uniform is the ejection process, because of the quadrupolar field that is more homogeneous and leads to improve the detection [13, 204, 216].

The principal advantages of the QIT in chemical analysis are:

- High sensitivity of full-scan in MS and MS/MS modes. QIT achieves full scan mass spectrum of a wide range of *m/z* enough sensitive to detect organic contaminants at trace levels, which facilitates identification of unknown compounds [61, 219].
- Tandem mass spectrometry experiments are available by performing sequential mass analysis measurements. Precursor ion isolation, fragmentation and product ion analysis all take place in the trapping volume by separating the events in time, rather than in space. Supplementary frequencies applied to the end cap electrodes are used both to eject unwanted ions during the precursor ion isolation and to excite precursor ions to carry out CID [61].

These features make a QIT an attractive option to detect organic contaminants in food. For the determination of contaminants that are completely unknown, data dependent full-scan MS and MS^n are proposed by some authors [68, 69].

QIT has also demonstrated to be useful for differenciating isomers of a compound, as illustrated by Lehane et al. [108] who proposed an LC-MS^3 method for determining azaspiracids in mussels. The azaspiracids, AZA4 and AZA5 are positional isomers of a cyclic heptapeptide that differ only in the position of an hydroxyl group in the molecule. The MS^3 spectrum provides a characteristic fragmentation pattern for each compound, resulting from cleavage of the A-ring at C9-C10. This framgentations attains the distinction between isomeric hydroxilated azaspiracids, AZA4 and AZA5, as can be observed in Figure 20, which shows the LC-MS^3 chromatogram with the separation of both isomers and the corresponding mass spectra.

However, the QIT also suffers from some drawbacks:

- Low resolution, and mass shift. Owing to the low mass resolution of the ion trap, different assignments could be proposed for some fragments, especially when nitrogen was involved.
- Limited dynamic range (i.e., it can not handle samples in which the ion abundances vary greatly and the range of ion traps is limited to ~106, when there are too many ions in the trap, space charge effect led to diminish performance).
- Inability to trap product ions below m/z 50 and the existence of an upper limit on the ratio between the precursor mass and the lowest trapped fragment ion mass, which is approximately 0.3 depending on the qz value. The fragment ions with masses in the lower

third of the mass range will not be detected. Actually, these drawbacks are relevant for the determination of small organic molecules as a majority of food or environmental contaminants, such as acrylamide. However, Figure 21 demonstrates that using improved clean-up procedures and carefully optimizing the LC-MS conditions, it is possible to determine acrylamide in foods using LC-QIT-MS.

- Limited number of product ions that can simultaneously be isolated and fragmentated in the trap, which, consequently limits the number of compounds determinable.

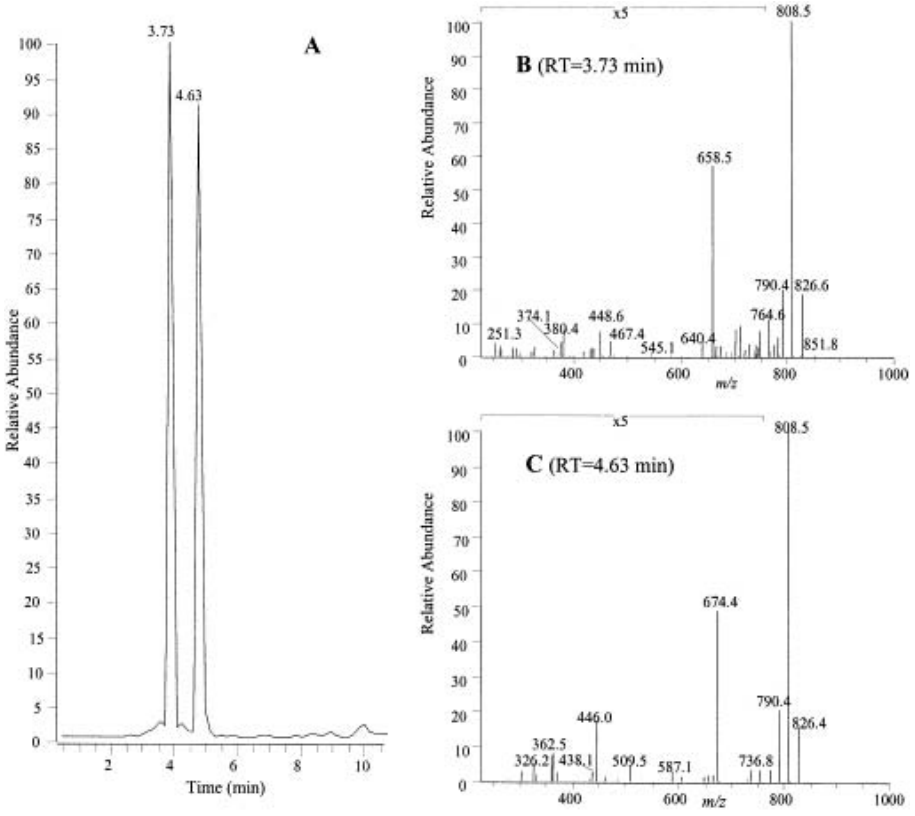


Figure 20. (A) Chromatogram showing the separation of the isomers, AZA4 and AZA5, which was obtained using LC–MS3 (method 1); AZA4 (3.73 min) and AZA5 (4.63 min). (B) and (C) are the mass spectra corresponding to AZA4 and AZA5, respectively (Reproduced from [108] with permission of Elsevier© 2002).

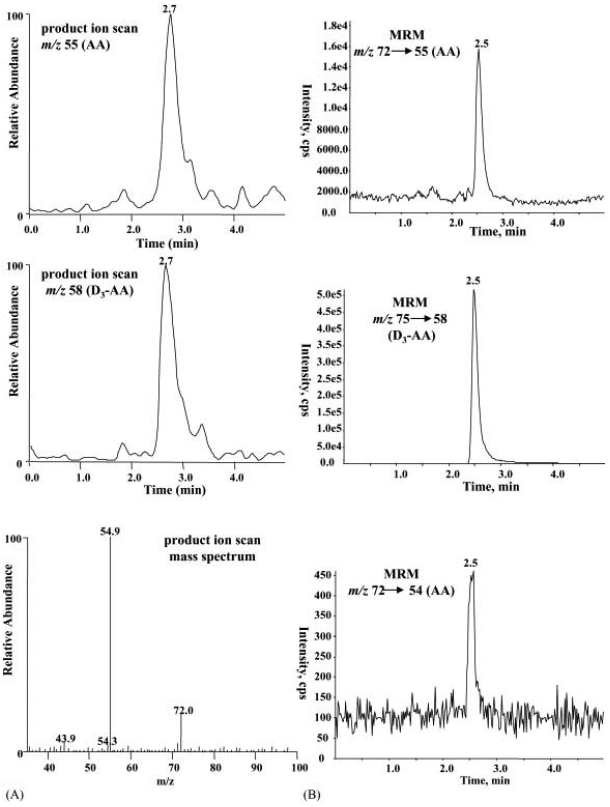


Figure 21. Chromatograms of acrylamide in two food samples: (A) crisp bread sticks, ion trap (product ion scan); (B) pastry product (*sobaos Martínez*), triple quadrupole (MRM) (Reproduced from [114] with permission from Elsevier© 2006).

Linear ion traps (LITs) have become very popular recently, either as a stand-alone MS or as front-end stages prior to FT-ICR, or as Q_3 in a triple-quadrupole. Rf-only quadrupoles are widely used as collision cells and ion transfer optics. In the latter application, ions passing through the quadrupole are confined in the center of the quadrupole axis at low gas pressures, thus increasing transmission efficiency. Often, other multipoles such as hexapoles or octopoles are used for the same purpose. This effect is called *collisional cooling* or *collisional focusing*. Furthermore, by adding trapping electrodes at the beginning and the end of such a quadrupole, one can store ions in the device. The hybrid quadrupole-linear ion trap (QqLIT) design allows an additional stage of MS-MS after the regular triple-quadrupole CID.

However, these designs have been introduced in the market a short time ago at very high prices and there is no application to food analysis [214].

4.3.3. Hybrid quadrupole time-of-flight.

The implementation of a quadrupole mass filter prior to the TOF tube is another tandem mass spectrometer that is becoming used in the field of food toxicant analysis. In fact, this can be taken one step further, by adding a second, radio frequency (rf)-only quadrupole as a collision cell (Q_1 q_2 TOF). The TOF analyzer then is used to provide accurate mass data for fragment ions formed in q_2 .

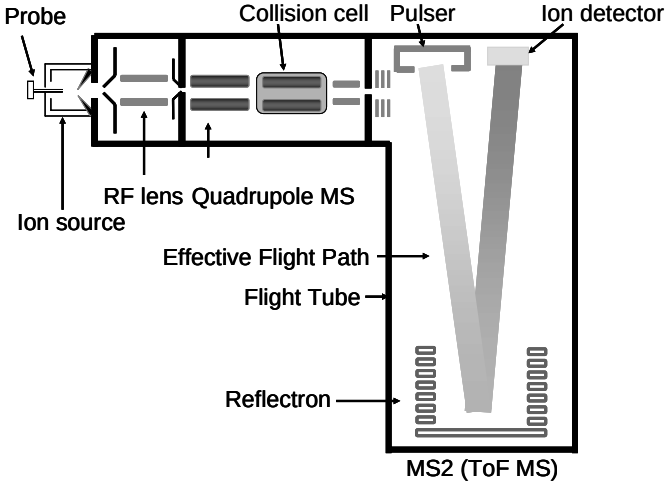


Figure 22. Scheme of a QqTOF

QToF can work in single MS as well as MS/MS operation modes. In the former, the first quadrupole is operated in band pass mode and the analysis is performed on the "high end" ToF analyzer. For MS/MS, a precursor ion is selected in the first quadrupole, the second produces collision-induced dissociation and the mass analysis of the fragment ions is performed in the ToF analyzer. By virtue of its tandem MS capabilities, product ion full spectra are obtained and any ion can be selected to reconstruct an ion chromatogram. Contrarily to QqQ instruments, the high resolution analysis allows the construction of accurate, sub-unit mass interval ion chromatogram, which results in a better signal to noise ratio.

QToF-MS is a very attractive tool due to the combination of high sensitivity, high resolution, and high mass accuracy for both precursor and product ions, which is performed

when the instruments are used in information-dependent acquisition experiments. In addition, data acquisition is so fast that multiple precursors from co-eluting peaks can be monitored simultaneously. Figure 22 illustrates the potential of capillary liquid chromatography (micro lC)-quadrupole/time-of-flight mass spectrometry (Q-TOF MS) for the confirmation of Sudan I, II, III and IV azo-dyes as contaminants in hot-chilli food products.

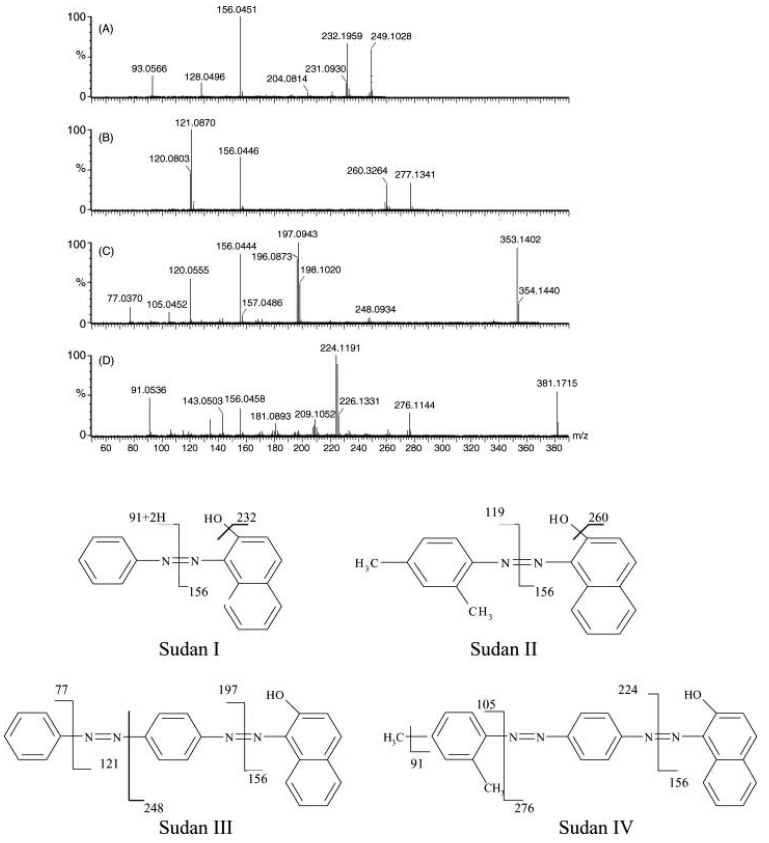


Figure 23. Q-TOF MS–MS product–ion spectra of standard solution (0.1 μg/ml) mixture of (A) Sudan I, (B) Sudan II, (C) Sudan III and (D) Sudan IV and fragmentation pathways of the four azo-dyes (Reproduced from [220] with permission of Elsevier© 2004).

5. Applications

5.1. Pesticide residues

Pesticide residue determination by LC-MS is one of the most developed fields of analysis. Almost of types of ionization sources and mass analyzers have been checked and applied to this field [2, 6, 11].

Several methods have been developed to analyze a unique pesticide or very few structurally related compounds. These methods are adapted to compounds that have special characteristics, such as being ionic, preventing their incorporation into a traditional multi-residue procedures. The most typical example is represented by the ammonium quaternary herbicides growth regulators [57, 166, 167, 186, 221, 222].

LC/MS methods are commonly developed to carry out both simultaneously, multi-class and multiresidue analysis [75, 83, 84, 131, 223, 224]. Several methods have been reported for a number fungicides, carbamates, dithiocarbamates, triazines, macrocyclic lactones, neonicotinoids, organophosphorus, phenylureas, and benzoylureas [61-63, 82, 197].

The majority of the methods have been developed for their application to fruits and vegetables. Applications of LC-ESI-MS/MS for analysis of pesticides in infant foods are scarce. In a study of Wang et al. [225], 13 pesticides were determined in apple based infant food. Only one paper reports a simple and rapid assay for analyzing residues of carbamate insecticides in bovine milk [226].

LC-QqQ-MS/MS has facilitated the enlargement of the number of pesticides that can simultaneously be determined to more than 50. Klein and Alder [227] proposed a simple method based on methanol extraction and SPE. The method known as QuEChERS utilizes acetonitrile for extraction, anhydrous $MgSO_4$:NaCl to induce partitioning of acetonitrile extract from the water of the sample, and dispersive SPE to cleanup. It has been validated for more than 229 pesticides [223, 224, 228] and has been used with fat-containing foods, such as milk, eggs, avocado, and animal tissues. Another method based on ethyl acetate extractions only requires a solvent exchange step prior to LC-MS/MS analysis [73]. Hetherton et al. [211] developed a method that also used acetonitrile extraction, which achieves satisfactory results even without any clean up.

The use of QIT to analyze multiple pesticide residues is limited to several multi-class pesticides in fruits [61-63, 83, 84, 229], and infant formulae [76] because its limitation in the number of product ions that can be isolated at the same time. QIT requires the use of of several time windows. Although according to the manufacturer instruction around 30 ions can be simultaneously fragmented, the reality is that to obtain sufficient data points per chromatographic peak, no more than three compounds in a time widow can be acquired since the QIT requires longer cycle-time than QqQ [183].

Another important issue on pesticide control to ensure food safety, which is still a challenge for the analyst, is the identification of unknown-target pesticides and metabolites. The high resolving power of mass spectrometric techniques, such as TOF-MS, has been applied successfully to identify and quantify target and not target pesticides as well as unknown metabolites[3, 66, 68, 69, 83, 84, 229]. There are two reports that describe an interesting identification scheme, using a combination of LC-MS/TOF and LC-MS QIT with searching of empirical formulas generated through accurate mass and a ChemIndex or Merck Index databases [68, 69]

5.2. Veterinary drugs

The analysis of veterinary drugs, which includes antihelmintics, antibiotics (aminoglycosides, β-lactam antibiotics, macrolides, peptides, suphonamides and trimetroprin, tetracyclines, quinolones, chloramphenicol, malachite green), coccidiostats (nitroimidazoles, nitrofurans), hormones (anabolic steroids, corticosteroids, tyreostats), β-agonists and tanquilizers, is monopolized by QqQ in MRM because the new criteria established by the European Union for performing confirmatory analysis of veterinary drugs in animal food-products [21, 27, 32-34, 36, 38, 39, 41, 43, 44, 56, 172-174, 177, 179, 180, 201, 230]. Quality criteria for the identification of organic residues and contaminants in animal and animal products are based on the use of identification points (IPs). The system of IPs balances the identification power of the different mass analyzers. The minimum number of IPs for forbidden compounds is set to four, for compounds with MRLs a minimum of three IPs are required for confirmation. According to the report, any MS technique or combination of techniques may be employed to attain the number of IPs needed for the identification of a compound. The number of IPs achieved by MS detection depends on the technique used. Tables 5 and 6 show the relationship between mass spectrometry technique and IPs earned, as well as examples of the number of IPs.

The combination of LC-QqQ allows unequivocal identification of traces of antibiotics and antibacterial agents in complex biological matrices, such as honey, eggs, milk and meat. The sensitivity is QqQ in MRM is particular useful for confirming the presence of banned substances that require limits of detection as low as possible [23, 50, 149].

QIT instruments are also used in some instances [54]. For example, van Hoof et al. analyzed eight quinolones in bovine muscle, milk and aqua culture products using ESI in PI mode with this mass analyzer. Detection was performed using three segments, in which only 2-4 precursor ions were acquired obtaining LOQs between 36 and 110 μg/kg using product-ion scan. For accurate confirmation of two quinolones, oxonilic acid and flumequine, which exhibit only the product ion *m*/*z* 244 in the MS/MS spectrum. MS^3 spectra were obtained in an additional run.

Table 5. MS techniques and IPs obtained per ion.

MS technique	IPs obtained per ion
Low resolution mass spectrometry (LR)	1
LR-MS^n precursor ion	1
LR-MS^n transition products	1.5
HRMS	2
HR-MS^n precursor ion	2
HR-MS^n transition products	2.5

Footnotes:

(1) Each ion may only be counted once

(2) Different analytes can be used to increase the number of IPs only if the derivatives employ different reaction chemistries.

(3) Transition products include both product and second product ions.

Table 6. Examples of the number of IPs earned for a range of techniques and combinations thereof (n= an integer)

Technique(s)	Number of ions	IPs
LC-MS	N	n
LC-MS-MS	1 precursor and 2 product ions	4
LC-MS-MS	2 precursor ions, each with 1 product ion	5
LC-MS-MS-MS	1 precursor, 1 product ion and 2 second product ions	5.5 2 n
HRMS	N	

QIT has also been used for screening and confirmation of drug residues in different foods. The presence of antibiotics in eggs and milk has been identified using product-ion scan MS/MS with limits of confirmation at low ppb levels.

Another interesting mass analyzer that has already been applied in this field is TOF-MS. Hernando et al. [211] proposed a method for simultaneous confirmation by accurate mass measurement and quantitative determination of antibiotic, fungicide and parasiticide residues in edible portions of salmon. Confirmation of chemotherapeutants has been based on the systems of IPs. A validation study is presented evaluating the accuracy in terms of precision (λ_{ppm} 0.83-1.15) and trueness (0.22-0.70 Da). Limits of detection and limits of quantification

were in the ranges of 1-3 and 3-9 μg/kg, respectively, which are well below the MRLs established in current EU legislation (100-200 μg/kg).

5.3. Food packaging migrating products

The application of LC–MS in the study of food-contact materials has been focused on two aspects (i) the monitoring of known compounds and (ii) the comprehensive analysis of unknown migrant.

The determination of semicarbazide [88, 89], hydroxymethyl furfural [129], bisphenol A, diglycil ether [92] and epoxidized soybean oil (ESIBO) [93] has been widely described, mainly in infant food and also in another types of food and food simultants. This analysis is relatively simple. Protocols and reuslts are quite well-established.

The search for unknowns poses difficulties such as product concentrations are low; large amounts of low-MW material (monomers/oligomers) can obscure large parts of a chromatogram; polar analytes may give poor peak shapes or no peaks at all (because of column activity); products are non-volatile (cannot be analyzed by GC) or too volatile (cannot be analyzed by LC–MS). These challenges must be met by a combination of strategies to separate the analytes from the polymer matrix (e.g., by headspace-GC), to concentrate solvent extracts, and to optimize separations (gradient profiles in LC, different columns in GC). Preferably, both GC and LC should be used to cover the entire range of potential migrants [90, 91]. The screening of unknown has been reported for paper and paper board packaging as well as for plastic.

5.4. Mycotoxins

The most widely spread classes of mycotoxins: patulin (produced from *Penicillium*, *Aspergillus*, and *Byssochlamys* molds), aflatoxins (produced from several species of *Aspergillus* molds), ochratoxin A (produced from *Penicillium* and *Aspergillus* molds), zearelenona, trichothecenes, and fumonisins (all produced from *Fusarium* molds) [22].

The use of LC-MS to determine mycotoxins is not as widespread as to determine other organic contaminants because most of the mycotoxins are not too sensitive molecules by LC-MS and tend to form sodium adducts that are difficult to fragment being difficult to meet the confirmation criteria. However, the selectivity of MS is unchallenged if compared to common LC detection methods and accuracy and precision are generally high. The sensitivity issue is the real problem to determine mycotoxins by LC-MS. It is also important to bear in mid that LC-fluorescence attain sensitivities hardly reached only by QqQ detectors [96-98].

In contrast, when toxins are not volatile and/or do not bear suitable chromophores or fluorophores, LC-MS is the unique method to perform specific analysis without requiring any

derivatization procedure. For trichothecenes and fumonisins, LC-MS methods are unrivalled for the quantitative determination [80, 94, 95, 101].

Many examples of mycotoxin determination if food matrices have been reported which made use of LC-MS. These methods are outstanding confirmatory methods also when other detection methods are available. This is clear in the case of patulin, aflatoxins, ochratoxin A and zerealenone, which are usually extracted, analyzed and quantified with other detectors than mass spectrometers. For these classes of toxins, MS detection is not strictly necessary, with the exception of complex matrices containing close interferences, unless it is mandatory to confirm the exact identity of the analyte [25, 29, 99, 100, 153, 231].

5.5. Algae and fish toxins

Phycotoxins or algal toxins are produced by marine dinoflagellates. For some of them, a restrictive MRL value (0.1 μg/kg) have been established. These toxins include a large number of substance, which are still under study. Both ESI and APCI ionization sources have been used for coupling to MS. ESI is recommended for analysis of azaspiracids, compounds easily ionized at low pH in positive method as well as for microcystins, compounds of high molecular weight [105, 158].

Single quadrupole has attained the simultaneous determination of eleven toxins, including anatoxin, saxitoxin, domoic acid, nodularin, microcystins, okadaic acid and dinophysistoxins. The absolute limit of detection reported for anatoxin was 0.5 ng [104].

However, recent misidentification of phenylalanine for anatoxin has highlighted the inadequacies of single stage MS. The use of tandem mass spectrometry MS/MS using QqQ, QIT and QqTOF has been widely reported to identify these compounds [103-106, 108, 158, 192, 193]. The determination of this toxins have involved the more sophisticated instruments because there are no available standards for many of these compounds. The lack of standards makes identification and confirmation more difficult than for other compounds.

5.6. Heat induced decomposition products

Some toxic compounds such as acrylamide and heterocyclic amines can originate during thermal treatment of cooking of some food products.

Acrylamide has been determined using either GC-MS or LC-MS with no obvious method-dependent differences in results obtained between the two approaches. However, the main advantage on LC-MS based methods is that acrylamide can be analyzed without prior derivatization, which considerable simplifies and expedite the analysis. Many laboratories has developed its “own” LC-MS or LC-MS/MS technique but one of the limitations of these techniques is the difficulties of applying an universal clean-up approach valid for many different food matrices that avoid interferences from co-extractives [113, 189, 190].

An ESI source in positive mode in combination with QqQ analyzers , is the instrument most frequently utilized to obtain LODs ranged from 5 to 60 μg/kg, depending on the food

sample. To achieve lower LODS (0.6 μg/kg), Belkasi et al. [190] used extensive clean-up procedures, wherein anion-exchange, cation-exchange and carbon based cartridges where connected in tandem.

Acrylamide protonated ion is obtained in the API source, and fragmentation of this precursor ion in a collision cell reveals a prominent product ion at m/z 55 due to the loss of NH_3, while other transitions exhibit very low intensities. In complex food samples, such as coffee or cocoa, high background level of interfering co-extractives undergoing the same transition as acrylamide (m/z 72 → 55) are observed. In these settings, the transition m/z 72 → 54 proved to be an alternative due to its higher specificity. However, it should be noted that higher injection volumes or analyte enrichment are necessary to achieve adequate sensitivity [60, 114].

Another heat induced decomposition products are heterocyclic amines (HAs), the major mutagens found in cooked foods. Has are formed during high-temperature cooking (125-250 ºC) of protein rich foods, such as meat and fish [130, 152].

Both ESI and APCI ionization techniques have been used for analysing Has in food, with ESI generally attained better sensitivity. Barceló-Barrachina et al. [110] reported LODs of 0.1-3.6 μg/kg for the analysis of Has in lyophilized meat extract using ESI and of 0.8-10 μg/kg using APCI. In both cases, a QIT in product ion scan was used to conduct the analysis. QqQ instruments are preferred for analysis of Has, since low LODs are obtained in MRM mode. A comparison of QqQ and QIT in the analysis of HAs in meat showed that LODs in QqQ (0.0.2-0.1 μg/kg) were 19-360 times lower than those obtained with the QIT.

5.7. Additives

LC-MS has provided the ultimate method for the structural elucidation or confirmation of the identity of dyes. The sulfonic group of the dyes is strongly acidic and, therefore, completely dissociated in aqueous solution, so that the formation of $R\text{-}(SO_3)_n]^{n-}$ ions is very easy. Suitable liquid chromatography/mass spectrometry (LC/MS) conditions were examined for Amaranth, Red 2G (R2G), Azo Rubine (Azo), Fast Red E (FRE) and Brilliant Blue FCF, which were detected in Akasu, a red vinegar made in Hong Kong from sake lees, on both thin layer chromatography (TLC) and photodiode array high-performance liquid chromatography (PDA-HPLC). Molecular-related ions for each dye were detected with excellent sensitivity by LC/MS using electro-spray ionization with negative ion mode, capillary voltage 3.00 kV, cone voltage 50 V and desolvation temperature 400 degrees C. LC/MS analysis of refined Akasu under these conditions enabled us to obtain clear mass spectra of R2G, Azo and FRE, which were present at trace levels in the Akasu. The results were consistent with those from TLC and PDA-HPLC [133].

These experiments suggested that LC/MS analysis is applicable for confirmation of dyes in food. Raw materials, intermediates and subsidiary colours in Food Yellow No. 5 (Sunset Yellow FCF) were determined using liquid chromatography/mass spectrometry (LC/MS)

with electrospray ionization. A gradient consisting of acetonitrile and 0.04% aqueous ammonium carbonate solution was used for the HPLC mobile phase. Quasi-molecular ions of impurities were used as monitor ions. It was necessary to use fragment ions of the sodium salts of 6-hydroxy-5-phenylazo-2-naphthalenesulphonic acid (SS-AN) and 4-(2-hydroxy-1-naphthylazo) benzenesulphonic acid (2N-SA) as monitor ions because the compounds are not resolved by chromatography and have the same molecular weight. An accurate method based on the use of reversed-phase (RP) liquid chromatography-tandem mass spectrometry interfaced with electrospray (LC-ESI-MS/MS) was devised for the determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli food samples. Also a micro LC-ESI QqTOF MS method was developed as a powerful confirmation alternative. Complementary information to that obtained by LC-ESI-QqQ-MS was obtained. The high resolution of the A-TOF-MS mass analyzer allowed to obtain unambiguous confirmation of Sudan I in real samples by means of the accurate mass by MS and MS/MS experiments [132, 133, 220, 232].

5.8. Toxic food constituents

Food constitutes the most complex part of our chemical environment. Naturally occurring substances in food, such as goitrogens and estrogens, are toxicants. However, there is a difference of degree between toxicity and hazard, and the hazard to man in normal good health from naturally occurring toxicants in food is usually slight, because of (a) the small concentrations and (b) antagonistic interactions between toxicants which provide "safety in numbers." Hazard from foods may indeed exist under some circumstances, such as the presence of abnormal contaminants, ingestion of abnormal amounts, or abnormal susceptibility existing in some disease states.

The determination of toxic food constituents is the lesser developed field of analysis within food toxicant analysis, restricted to some toxic alcaloids [136] and some allergens [137].

6. Conclusions and future trends

The relatively high number of publications on the analysis of toxic substances in food samples by LC coupled to MS/MS shows that this technique has become a powerful tool in the quality control of food products and the safeguarding of human health. However, analysis of these compounds is not a simple task due to the complexity of the matrices and the low concentrations of the analytes present in the samples.

ESI is the most frequently utilized ionization source because of its higher sensitivity for toxic compounds because most of them are readily ionizable in liquid phase. However, for some compounds, better results are obtained using APCI suggesting that both API sources should be considered.

Matrix interferences are unresolved question yet. The tendency is to perform an appropriate quantification by isotopical dilution when a small number of compounds are determined or using matrix-matched standard when a large number of target analytes are simultaneously analyzed.

QqQ is the instruments most commonly used in this field because the lower LODs achievable permit compliance with the strict MRLs established by govering authorities. Although both, QqQ and QIT are frequently used in this field. Aspects regarding confirmation of food toxicants must also be mentioned. QIT product-ion scan can confirm analytes while in triple quadrupole, at least two transition precursor-product ions have to be monitored.

Routine determination of food toxicants in food-products is always carried out by well established techniques such as QqQ and QIT. More recent approaches such as TOF and linear traps as well as their combinations with quadrupoles to attain tandem mass spectrometry QqTOF and QLIT are gaining acceptance but their use is not generalized yet.

References

[1] L.Goodwin, J.R.Startin, D.M.Goodall, B.J.Keely, Rapid Commun. Mass Spectrom. 17 (2003) 963.
[2] M.Careri, F.Bianchi, C.Corradini, J. Chromatogr. A 970 (2002) 3.
[3] I.Ferrer, E.M.Thurman, Trends Anal. Chem. 22 (2003) 750.
[4] W.M.A.Niessen, Analusis 28 (2000) 885.
[5] W.G.Fong, H.A.Moye, J.N.Seiber, J.P.Toth, Pesticide residues in foods. Methods, techniques and regulations. John Wiley & Sons, New York, 1999.
[6] Y.Picó, G.Font, J.C.Moltó, J.Mañes, J. Chromatogr. A 882 (2000) 153.
[7] E.T.Furlong, I.Ferrer, P.M.Gates, J.D.Cahill, E.M.Thurman, in I.Ferrer, E.M.Thurman (Editors), Liquid chromatography/mass spectrometry, MS/MS and time of flight MS: Analysis of emerging contaminants. American Chemical Society, Washington DC, 2003, p. 175.
[8] A.C.Hogenboom, W.M.A.Niessen, U.A.Th.Brinkman, J Sep Sci 24 (2001) 331.
[9] V.Andreu, Y.Pico, Current Anal. Chem. 1 (2005) 241.
[10] Liquid chromatography/mass spectrometry, MS/MS and time-of-flight MS. Global view publishing, Pittsburgh, PA, 2004.
[11] Y.Picó, C.Blasco, G.Font, Mass Spectrom. Rev. 23 (2004) 45.
[12] S.D.Richarson, Anal. Chem. 72 (2004) 3337.
[13] B.R.Larsen, Analusis 28 (2000) 941.
[14] Directorate General Health and Consumer Protection, European Commission, Brussels, 2004, p. 1.
[15] Directorate General Health and Consumer Protection, European Commision, Brussels, 2003, p. 1.
[16] L.Debrauwer, S.Chevolleau, D.Zalko, A.Paris, J.Tulliez, Sciences des Aliments 25 (2005) 273.
[17] L.Mondello, G.Dugo, P.Dugo, LC-GC Eur Recent applications in LC-MS (2002) 12.
[18] J.Hajslova, J.Zrostlikova, J. Chromatogr. A 1000 (2003) 181.

[19] J.Henion, E.Brewer, G.S.Rule, Anal. Chem. (1998) 640A.
[20] W.L.Budde, Mass Spectrom. Rev. 23 (2004) 1.
[21] A.Gentili, D.Perret, S.Marchese, Trends Anal. Chem. 24 (2005) 704.
[22] J.Gilbert, E.A.Vargas, J. Toxicol. Tox. Rev. 22 (2004) 381.
[23] S.I.Kotretsou, Crit. Rev. Food Sci. Nut. 44 (2004) 173.
[24] O.Nuñez, E.Moyano, M.T.Galceran, Trends Anal. Chem. 24 (2005) 683.
[25] S.Sforza, C.Dall'Asta, R.Marchelli, Mass Spectrom. Rev. 25 (2006) 54.
[26] A.A.M.Stolker, U.A.T.Brinkman, J. Chromatogr. A 1067 (2005) 15.
[27] K.De Wasch, H.De Brabander, D.Courtheyn, Analyst 123 (1998) 2701.
[28] S.B.Turnipseed, J.E.Roybal, A.P.Pfenning, P.J.Kijak, Anal. Chim. Acta 483 (2003) 373.
[29] A.Landreau, Y.F.Pouchus, C.Sallenave-Namont, J.F.Biard, M.C.Boumard, T.R.du Pont, F.Mondeguer, C.Goulard, J.F.Verbist, J. Microbiol. Met. 48 (2002) 194.
[30] R.Castro, E.Moyano, M.T.Galceran, J. AOAC Int. 84 (2001) 1903.
[31] H.M.Ashwin, S.L.Stead, J.C.Taylor, J.R.Startin, S.F.Richmond, V.Homer, T.Bigwood, M.Sharman, Anal. Chim. Acta 529 (2005) 103.
[32] S.Bogialli, R.Curini, A.Di Corcia, A.Lagana, M.Mele, M.Nazzari, J. Chromatogr. A 1067 (2005) 93.
[33] C.K.Fagerquist, A.R.Lightfield, S.J.Lehotay, Anal. Chem. 77 (2005) 1473.
[34] C.K.Farguerquist, A.R.Lightfiel, Rapid Commun. Mass Spectrom. 17 (2003) 660.
[35] J.K.Finzi, J.L.Donato, M.Sucupira, G.De Nucci, J. Chromatogr. B 824 (2005) 30.
[36] A.F.Forti, G.Campana, A.Simonella, M.Multari, G.Scortichini, Anal. Chim. Acta 529 (2005) 257.
[37] T.Goto, Y.Ito, S.Yamada, H.Matsumoto, H.Oka, J. Chromatogr. A 1100 (2005) 193.
[38] W.Hammack, M.C.Carson, B.K.Neuhaus, J.A.Hurlbut, C.Nochetto, J.S.Stuart, A.Brown, D.Kilpatrick, K.Youngs, K.Ferbos, D.N.Heller, J. AOAC Int. 86 (2004) 1135.
[39] J.A.Hernández-Arteseros, J.Barbosa, R.Compano, M.D.Prat, J. Chromatogr. A 945 (2002) 1.
[40] Codex Alimenarius Commission, 2003.
[41] D.Hurtaud-Pessel, E.Verdon, J.Blot, P.Sanders, Food Add. Contam. 23 (2006) 569.
[42] R.Ishii, M.Horie, M.Murayama, T.Maitani, J. Food Hyg. Soc. Japan 47 (2006) 58.
[43] A.Kaufmann, P.Butcher, Rapid Commun. Mass Spectrom. 19 (2005) 3694.
[44] A.Kaufmann, P.Butcher, P.Kölbener, Rapid Commun. Mass Spectrom. 17 (2004) 2575.
[45] H.T.Ronning, K.Einarsen, T.N.Asp, J. Chromatogr. A 1118 (2006) 226.
[46] D.W.M.Sin, C.Ho, Y.C.Wong, S.K.Ho, A.C.B.Ip, Anal. Chim. Acta 535 (2005) 23.
[47] B.Toussaint, M.Chedin, G.Bordin, A.R.Rodriguez, J. Chromatogr. A 1088 (2005) 32.
[48] S.B.Turnipseed, W.C.Andersen, C.M.Karbiwnyk, J.E.Roybal, K.E.Miller, Rapid Commun. Mass Spectrom. 20 (2006) 1231.
[49] A.Capiello, G.Famiglini, P.Palma, F.Mangani, Anal. Chem. 74 (2002) 3547.
[50] K.Klagkou, F.Pullen, M.Harrison, A.Organ, A.Firth, G.J.Langley, Rapid Commun. Mass Spectrom. 17 (2004) 2373.
[51] E.Mikami, T.Goto, O.A.Tsutomu, H.Oka, H.Kanamori, J. Health Sci. 51 (2005) 278.
[52] P.Scherpenisse, A.A.Bergwerff, Anal. Chim. Acta 529 (2005) 173.
[53] O.Van den hauwe, F.Dumoulin, C.Elliott, C.Van Peteghem, J. Chromatogr. B 817 (2005) 215.
[54] P.R.Kootstra, C.J.P.F.Kuijpers, K.L.Wubs, D.van Doorn, S.S.Sterk, L.A.van Ginkel, R.W.Stephany, Anal. Chim. Acta 529 (2005) 75.
[55] P.E.Asea, J.D.MacNeil, J.O.Boison, J. AOAC Int. 89 (2006) 567.

[56] A.Gentili, M.Sergi, D.Perret, S.Marchese, R.Curini, S.Lisandrin, Rapid Commun. Mass Spectrom. 20 (2006) 1845.
[57] L.Alder, J.R.Startin, J. AOAC Int. 88 (2005) 1762.
[58] M.Careri, L.Elviria, A.Mangia, I.Zagnoni, Rapid Commun. Mass Spectrom. 16 (2002) 1821.
[59] CEN, Brussels, 2002, p. 1.
[60] S.Riedeker, H.Obrist, N.Varga, R.H.Stadler, J. Chromatogr. A 966 (2002) 15.
[61] C.Blasco, G.Font, Y.Picó, J. Chromatogr. A 1043 (2004) 231.
[62] C.Blasco, M.Fenández, Y.Picó, G.Font, J.Mañes, Anal. Chim. Acta 461 (2002) 109.
[63] C.Blasco, G.Font, J.Mañes, Y.Picó, Anal. Chem. 75 (2003) 3606.
[64] J.M.Bonmatin, I.Moineau, R.Charvet, C.Fetcher, M.E.Colin, E.R.Bengsch, Anal. Chem. 75 (2003) 2027.
[65] P.Degelmann, S.Egger, H.Jurling, J.Muller, R.Niessner, D.Knopp, J. Agric. Food. Chem. 54 (2006) 2003.
[66] C.Ferrer, M.J.Gomez, J.F.Garcia-Reyes, I.Ferrer, E.M.Thurman, A.R.Fernandez-Alba, J. Chromatogr. A 1069 (2005) 183.
[67] M.J.Taylor, K.Hunter, K.B.Hunter, D.Lindsay, S.Le Bouhellec, J. Chromatogr. A 982 (2002) 225.
[68] E.M.Thurman, I.Ferrer, J.A.Zweigenbaum, J.F.Garcia-Reyes, M.Woodman, A.R.Fernandez-Alba, J. Chromatogr. A 1082 (2005) 71.
[69] E.M.Thurman, I.Ferrer, A.R.Fernandez-Alba, J. Chromatogr. A 1067 (2005) 127.
[70] T.L.Constantopoulos, G.S.Jackson, C.G.Enke, J. Am. Soc. Mass Spectrom. 10 (1999) 625.
[71] T.Goto, Y.Ito, S.Yamada, H.Matsumoto, H.Oka, H.Nagase, Anal. Chim. Acta 555 (2006) 225.
[72] F.Hernandez, O.J.Pozo, J.V.Sancho, L.Bijlsma, A.Barreda, E.Pitarch, J. Chromatogr. A 1109 (2006) 242.
[73] C.Jansson, T.Pihlström, B.G.Österdahl, K.E.Markides, J. Chromatogr. A 1023 (2004) 93.
[74] J.L.Josephs, M.Sanders, Rapid Commun. Mass Spectrom. 18 (2004) 743.
[75] M.Liu, Y.Hashi, Y.Y.Song, J.M.Lin, J. Chromatogr. A 1097 (2005) 183.
[76] B.Mickova, J.Zrostlikova, J.Hajslova, P.Rauch, M.J.Moreno, A.Abad, A.Montoya, Anal. Chim. Acta 495 (2003) 123.
[77] H.G.J.Mol, R.C.J.van Dam, O.M.Steijer, J. Chromatogr. A 1015 (2003) 119.
[78] D.Perret, A.Gentili, S.Marchese, M.Sergi, G.D'Ascenzo, J. AOAC Int. 85 (2002) 724.
[79] O.Pozo, J.M.Marin, J.V.Sancho, F.Hernández, J. Chromatogr. A 992 (2003) 133.
[80] D.Royer, H.U.Humpf, P.A.Guy, Food Add. Contam. 21 (2004) 678.
[81] P.G.Schermerhorn, P.E.Golden, A.J.Krynitsky, W.M.Leimkuehler, J. AOAC Int. 88 (2005) 1491.
[82] A.Asperger, J.Efer, T.Koal, W.Engelwald, J. Chromatogr. A 937 (2001) 65.
[83] C.Soler, J.Mañes, Y.Picó, J. Chromatogr. A 1048 (2004) 41.
[84] C.Soler, J.Manes, Y.Pico, J. Chromatogr. A 1088 (2005) 224.
[85] J.M.Marin, O.J.Pozo, J.Beltran, F.Hernandez, Rapid Commun. Mass Spectrom. 20 (2006) 419.
[86] S.Barnabas, S.Miklos, Magyar Allatorvosok Lapja 127 (2005) 178.
[87] S.V.C.De Souza, R.G.Junqueira, R.Ginn, J. Chromatogr. A 1077 (2005) 151.
[88] R.Ginn, L.Wilson, S.V.C.De Souza, M.B.De la Calle, J.Barbosa, B.Berendsen, I.Bockborn, M.Brandtner, P.Delahaut, T.Doering, P.Fuerst, C.Griffin, T.Gude, A.Janosi, A.Jaus, G.Kennedy, M.Mandix, E.M.Hilla, S.Plonevez, A.Posyniak, L.Saari, M.van Bruijnsvoort, E.Verdon, R.Wohlfarth, J. AOAC Int. 89 (2006) 728.

[89] G.O.Noonan, C.R.Warner, W.Hsu, T.H.Begley, G.A.Perfetti, G.W.Diachenko, J. Agric. Food. Chem. 53 (2005) 4680.
[90] T.H.Begley, K.White, P.Honigfort, M.L.Twaroski, R.Neches, R.A.Walker, Food Add. Contam. 22 (2005) 1023.
[91] R.S.Garcia, A.S.Silva, I.Cooper, R.Franz, P.P.Losada, Trends Food Sci. Tech. 17 (2006) 354.
[92] U.Berger, M.Oehme, J. AOAC Int. 83 (2000) 1367.
[93] M.Suman, S.La Tegola, D.Catellani, U.Bersellini, J. Agric. Food. Chem. 53 (2005) 9879.
[94] S.Biselli, L.Hartig, H.Wegner, C.Hummert, Lc Gc Europe 17 (2004) 25.
[95] S.Biselli, C.Hummert, Food Add. Contam. 22 (2005) 752.
[96] I.K.Cigic, M.Strlic, A.Schreiber, M.Kocjancic, B.Pihlar, Analytical Letters 39 (2006) 1475.
[97] E.M.Han, H.R.Park, S.J.Hu, K.S.Kwon, H.Lee, M.S.Ha, K.M.Kim, E.J.Ko, S.D.Ha, H.S.Chun, D.H.Chung, D.H.Bae, J. Microbiol. Biotechnol. 16 (2006) 643.
[98] G.Haubl, F.Berthiller, R.Krska, R.Schuhmacher, Anal. Bioanal. Chem. 384 (2006) 692.
[99] M.Klotzel, U.Lauber, H.U.Humpf, Mol. Nut. Food Res. 50 (2006) 261.
[100] M.Klotzel, B.Gutsche, U.Lauber, H.U.Humpf, J. Agric. Food. Chem. 53 (2005) 8904.
[101] C.Paepens, S.De Saeger, L.Sibanda, I.Barna-Vetro, M.Anselme, Y.Larondelle, C.Van Peteghem, J. Agric. Food. Chem. 53 (2005) 7337.
[102] U.Berger, M.Oehme, F.Kuhn, J. Agric. Food Chem. 47 (1999) 4240.
[103] A.Braña-Magdalena, M.Lehane, C.Moroney, A.Furey, K.J.James, Food Add. Contam. 20 (2003) 154.
[104] M.Bruno, M.Fiori, D.Mattei, S.Melchiorre, V.Messineo, F.Volpi, S.Bogialli, M.Nazzari, Nat. Pro. Res. 20 (2006) 827.
[105] M.Fernández-Amandi, A.Furey, M.Lehane, H.Ramstad, K.J.James, J. Chromatogr. A 976 (2002) 329.
[106] K.Jorgensen, S.Scanlon, L.B.Jensen, Food Add. Contam. 22 (2005) 743.
[107] K.Karlsson, V.Sipiä, H.Kankaanpää, J.Meriluoto, J. Chromatogr. 784 (2003) 243.
[108] M.Lehane, A.Braña-Magdalena, C.Moroney, A.Furey, K.J.James, J. Chromatogr. A 950 (2002) 139.
[109] P.Muñiz Ortea, O.Allis, B.M.Healy, M.Lehane, A.N.Shuilleabhain, A.Furey, K.J.James, Chemosphere 55 (2004) 1395.
[110] E.Barceló-Barrachina, E.Moyano, L.Puignou, M.T.Galceran, J. Chromatogr. A 1023 (2004) 67.
[111] F.Toribio, E.Moyano, L.Puignou, M.T.Galceran, J. Mass Spectrom. 37 (2002) 812.
[112] R.J.Turesky, J.Taylor, L.Schnackenberg, J.P.Freeman, R.D.Holland, J. Agric. Food. Chem. 53 (2005) 3248.
[113] A.Becalski, B.P.Y.Lau, D.Lewis, S.W.Seaman, W.F.Sun, Adv. Exper. Med. Biol. 561 (2005) 271.
[114] E.Bermudo, E.Moyano, L.Puignou, M.T.Galceran, Anal. Chim. Acta 559 (2006) 207.
[115] A.Claus, G.M.Weisz, D.R.Kammerer, R.Carle, A.Schieber, Mol. Nut. Food Res. 49 (2005) 918.
[116] E.Dybing, P.B.Farmer, M.Andersen, T.R.Fennell, S.P.D.Lalljle, D.J.G.Muller, S.Olin, B.J.Peterson, J.Schlatter, G.Scholz, J.A.Scimeca, N.Slimani, M.Tornqvist, S.Tuijtelaars, P.Verger, Food Chem. Toxicol. 43 (2005) 365.
[117] B.L.Eberhart, D.K.Ewald, R.A.Sanders, D.H.Tallmadge, D.V.Zyzak, M.A.Strothers, J. AOAC Int. 88 (2005) 1205.
[118] A.Cappiello, G.Famiglini, Anal. Chem. 67 (1995) 412.
[119] R.Matissek, M.Raters, Adv. Exper. Med. Biol. 561 (2005) 293.

[120] F.J.Mestdagh, B.De Meulenaer, C.Van Poucke, C.Detavernier, C.Cromphout, C.Van Peteghem, J. Agric. Food. Chem. 53 (2005) 6170.
[121] E.V.Petersson, J.Rosen, C.Turner, R.Danielsson, K.E.Hellenas, Anal. Chim. Acta 557 (2006) 287.
[122] Y.P.Ren, Y.Zhang, J.J.Jiao, Z.X.Cai, Y.Zhang, Food Add. Contam. 23 (2006) 228.
[123] J.A.Rufian-Henares, F.J.Morales, Food Chemistry 97 (2006) 555.
[124] H.Z.Senyuva, V.Gokmen, Food Add. Contam. 22 (2005) 204.
[125] R.H.Stadler, Adv. Exper. Med. Biol. 561 (2005) 157.
[126] H.W.Vesper, H.Licea-Perez, T.Meyers, M.Ospina, G.L.Myers, Adv. Exper. Med. Biol. 561 (2005) 89.
[127] R.Weisshaar, Eur. J. Lip. Sci. Technol. 106 (2004) 786.
[128] V.Yusa, G.Quintas, O.Pardo, P.Marti, A.Pastor, Food Add. Contam. 23 (2006) 237.
[129] V.Gokmen, H.Z.Senyuva, J. Agric. Food. Chem. 54 (2006) 2845.
[130] S.K.Mortensen, X.T.Trier, A.Foverskov, J.H.S.Petersen, J. Chromatogr. A 1091 (2005) 40.
[131] F.Ishikawa, M.Oishi, T.Shindo, M.Horie, A.Yasui, S.Ogino, K.Ito, J. Food Hyg. Soc. Japan 46 (2005) 228.
[132] M.Mazzetti, R.Fascioli, I.Mazzoncini, G.Spinelli, I.Morelli, A.Bertoli, Food Add. Contam. 21 (2004) 935.
[133] F.Puoci, C.Garreffa, F.Iemma, R.Muzzalupo, U.G.Spizzirri, N.Picci, Food Chemistry 93 (2005) 349.
[134] S.Tsuji, M.Nakano, M.Furukawa, K.Yoshii, Y.Tonogai, J. Food Hyg. Soc. Japan 46 (2005) 116.
[135] S.A.Snyder, R.C.Pleus, B.J.Vanderford, J.C.Holady, Anal. Chim. Acta 567 (2006) 26.
[136] H.Reinhard, H.Rupp, F.Sager, M.Streule, O.Zoller, J. Chromatogr. A 1112 (2006) 353.
[137] D.Weber, P.Raymond, S.Ben-Rejeb, B.Lau, J. Agric. Food. Chem. 54 (2006) 1604.
[138] H.G.J.Mol, R.C.J.van Dam, R.J.Vreeken, O.M.Steijer, J. AOAC Int. 83 (2000) 742.
[139] H.G.J.Mol, R.C.J.van Dam, R.J.Vreeken, O.M.Steijer, J. Chromatogr. A 833 (1999) 53.
[140] D.M.Drexler, P.R.Tiller, S.M.Wilbert, F.Q.Bramble, J.C.Schwartz, Rapid Commun. Mass Spectrom. 12 (1998) 1501.
[141] A.C.Hogenboom, P.Speksnijder, R.J.Vreeken, W.M.A.Niessen, U.A.Th.Brinkman, J. Chromatogr. A 777 (1997) 81.
[142] W.A.Minnaard, A.C.Hogenboom, U.K.Malmquist, P.Manini, W.M.A.Niessen, U.A.Th.Brinkman, Rapid Commun. Mass Spectrom. 10 (1996) 1569.
[143] A.Raffaelli, A.Saba, Mass Spectrom. Rev. 22 (2003) 318.
[144] J.E.Loyo-Rosales, I.Schmitz-Afonso, C.P.Rice, A.Torrents, Anal. Chem. 75 (2003) 4811.
[145] M.Cantero, S.Rubio, D.Perez-Bendito, J. Chromatogr. A 1046 (2004) 147.
[146] L.H.Levine, J.L.Garland, J.V.Johnson, Anal. Chem. 74 (2002) 2064.
[147] L.Lunar, S.Rubio, D.Perez-Bendito, J. Chromatogr. A 1031 (2004) 17.
[148] V.Andreu, Y.Pico, Anal. Chem. 76 (2004) 2878.
[149] D.H.Kim, D.W.Lee, J. Chromatogr. A 984 (2003) 153.
[150] E.M.Thurman, I.Ferrer, D.Barceló, Anal. Chem. 73 (2001) 5441.
[151] L.Castle, S.Eriksson, J. AOAC Int. 88 (2005) 274.
[152] G.Saccani, E.Tanzi, P.Pastore, S.Cavalli, A.Rey, J. Chromatogr. A 1082 (2005) 45.
[153] H.M.Muller, J.Reimann, U.Schumacher, K.Schwadorf, Mycopathologia 137 (1997) 185.
[154] V.Andreu, Y.Pico, Trends Anal. Chem. 23 (2004) 772.
[155] A.G.Frenich, I.M.Salvador, J.Martinez Vidal, T.Lopez-Lopez, Anal. Bioanal. Chem. 383 (2005) 1106.
[156] A.Sanusi, M.Millet, H.Wortham, P.Mirabel, Analusis 25 (1997) 302.

[157] L.Sun, H.K.Lee, J. Chromatogr. A 1014 (2003) 153.
[158] J.Chen, P.Xie, Environ. Toxicol. 20 (2005) 572.
[159] K.J.James, M.Gillman, M.F.Amandi, A.Lopez-Rivera, P.F.Puente, M.Lehane, S.Mitrovic, A.Furey, Toxicon 46 (2005) 852.
[160] J.C.Marr, J.B.King, Rapid Commun. Mass Spectrom. 11 (1997) 479.
[161] R.Castro, E.Moyano, M.T.Galceran, J. Chromatogr. A 914 (2001) 111.
[162] R.Castro, E.Moyano, M.T.Galceran, Chromatographia 53 (2001) 273.
[163] P.G.M.Kienhuis, R.B.Geerdink, Trends Anal. Chem. 19 (2000) 249.
[164] A.Lagana, A.Bacaloni, I.De Leva, A.Faberi, G.Fago, A.Marino, Anal. Chim. Acta 462 (2002) 187.
[165] R.Jeannot, H.Sabik, E.Sauvard, E.Genin, J. Chromatogr. A 879 (2000) 51.
[166] C.S.Evans, J.R.Startin, D.M.Goodall, B.J.Keely, Rapid Commun. Mass Spectrom. 15 (2001) 699.
[167] C.S.Evans, J.R.Startin, D.M.Goodall, B.J.Keely, J. Chromatogr. A 897 (2000) 399.
[168] G.S.Rule, A.V.Mordehai, J.Henion, Anal. Chem. 66 (1994) 230.
[169] A.J.Bell, D.Despeyroux, J.Murrell, P.Wats, Int J Mass Spectrom Ion Proc 165 (1997) 533.
[170] W.M.Draper, J. Agric. Food Chem. 49 (2003) 2746.
[171] J.Zrotlikova, J.Hajslova, J.Poutska, P.Begany, J. Chromatogr. A 973 (2002) 13.
[172] D.N.Heller, S.B.Clark, H.F.Righter, J. Mass Spectrom. 35 (2000) 39.
[173] D.N.Heller, M.A.Ngoh, Rapid Commun. Mass Spectrom. 12 (1998) 2031.
[174] D.M.Holstege, B.Puschner, G.Whitehead, F.D.Galey, J. Agric. Food Chem. 50 (2002) 406.
[175] S.Yang, K.H.Carlson, J. Chromatogr. A 1038 (2004) 141.
[176] S.Yang, J.Chan, K.H.Carlson, Rapid Commun. Mass Spectrom. 18 (2004) 2131.
[177] D.N.Heller, M.A.Ngoh, D.Donoghue, L.Podhorniak, H.Righter, M.H.Thomas, J. Chromatogr. B 774 (2002) 39.
[178] J.Zhu, D.D.Snow, D.A.Cassada, S.J.Monson, R.F.Spalding, J. Chromatogr. A 928 (2001) 177.
[179] L.Howells, M.J.Sauer, Analyst 126 (2001) 155.
[180] C.Crescenzi, S.Bayoudh, P.A.G.Cormack, T.Klein, K.Ensing, Anal. Chem. 73 (2001) 2171.
[181] D.Baglio, D.Kotzias, B.R.Larsen, J. Chromatogr. A 854 (1999) 207.
[182] P.Calza, S.Baudino, R.Aigotti, C.Baiocchi, P.Branca, E.Palizzetti, J Mass Spectrom 37 (2002) 566.
[183] J.Zrotlikova, J.Hajslova, R.Kovalczuk, R.Stepan, J.Poutska, J. AOAC Int. 86 (2003) 612.
[184] A.C.Hogenboom, W.M.A.Niessen, U.A.Th.Brinkman, J. Chromatogr. A 794 (1998) 201.
[185] M.L.Reyzer, J.S.Brodbelt, Anal. Chim. Acta 436 (2001) 11.
[186] C.S.Evans, J.R.Startin, D.M.Goodall, B.J.Keely, Rapid Commun. Mass Spectrom. 14 (2000) 112.
[187] F.Toribio, E.Moyano, L.Puignou, M.T.Galceran, J. Chromatogr. A 869 (2000) 307.
[188] S.Prabhu, M.J.Lee, W.-Y.Hu, B.Winnik, I.Yang, B.Bckeley, J.H.Hong, Anal. Biochem. 298 (2001) 306.
[189] J.S.Ahn, L.Castle, D.B.Clarke, A.S.Lloyd, M.R.Philo, D.R.Speck, Food Add. Contam. 19 (2002) 1116.
[190] A.Becalski, B.P.Y.Lau, D.Lewis, S.W.Seaman, J. Agric. Food. Chem. 51 (2003) 802.
[191] D.V.Zyzak, R.A.Sanders, M.Stojanovic, D.H.Tallmadge, B.L.Eberhart, D.K.Ewald, D.C.Gruber, T.R.Morsch, M.A.Strothers, G.P.Rizzi, M.D.Villagran, J. Agric. Food. Chem. 51 (2003) 4782.
[192] J.A.Zweigenbaum, J.D.Henion, K.A.Bettie, G.A.Codd, G.K.Poon, J. Pharm. Biomed. Anal. 23 (2000) 724.

[193] A.Furey, M.Lehane, P.Gillman, P.Fernández-Puente, K.J.James, J. Chromatogr. A 938 (2001) 167.
[194] N.Yoshioka, Y.Akiyama, K.Teranishi, J. Chromatogr. A 1022 (2004) 145.
[195] M.Takino, K.Yamaguchi, T.Nakahara, J. Agric. Food. Chem. 52 (2004) 727.
[196] M.Takino, S.Daishima, T.Nakahar, Rapid Commun. Mass Spectrom. 17 (2003) 1965.
[197] A.Agüera, S.López, A.R.Fernández-Alba, M.Contreras, J.Crespo, L.Piedra, J. Chromatogr. A 1045 (2004) 125.
[198] K.Bester, G.Bordin, A.Rodríguez, H.Schimmel, J.Pauwels, G.VanVyncht, Fresenius J. Anal. Chem. 371 (2001) 550.
[199] B.K.Choi, D.M.Hercules, A.I.Gusev, Fresenius J. Anal. Chem. 369 (2001) 370.
[200] B.K.Choi, D.M.Hercules, A.I.Gusev, J. Chromatogr. A 907 (2001) 337.
[201] D.N.Heller, D.A.Kaplan, N.G.Rummel, J.von Bredow, J. Agric. Food Chem. 48 (2000) 6035.
[202] V.Gokmen, H.Z.Senyuva, J. Chromatogr. A 1120 (2006) 194.
[203] J.Abian, G.Duran, D.Barceló, J. Agric. Food Chem. 41 (1993) 1264.
[204] F.Lemière, LC-GC Eur Guide to LC-MS (2001) 22.
[205] M.Guilhaus, D.Selby, V.Mlynski, Mass Spectrom. Rev. 19 (2000) 65.
[206] I.Bobeldijk, J.P.C.Vissers, G.Kearney, H.Major, J.A.Leerdam, J. Chromatogr. A 929 (2001) 63.
[207] R.B.Geerdink, W.M.A.Niessen, U.A.Th.Brinkman, J. Chromatogr. A 970 (2002) 65.
[208] A.C.Hogenboom, W.M.A.Niessen, D.Little, U.A.Th.Brinkman, Rapid Commun. Mass Spectrom. 13 (1999) 125.
[209] Maizels M., Budde W.L., Anal. Chem. 73 (2001) 5436.
[210] J.Vesella, F.Acobas, D.Benanou, J.L.Guinamant, Spectra Anal (2001) 32.
[211] C.L.Hetherton, M.D.Sykes, R.J.Fussell, D.M.Goodall, Rapid Commun. Mass Spectrom. 18 (2004) 2443.
[212] K.S.Chiu, A.Van Langehove, C.Tanaka, Biomed. Environ. Mass Spectrom. 18 (1989) 200.
[213] R.B.Geerdink, W.M.A.Niessen, U.A.Th.Brinkman, J. Chromatogr. A 910 (2001) 291.
[214] M.Petrovic, D.Barcelo, Anal. Bioanal. Chem. 385 (2006) 422.
[215] C.S.Creaser, J.W.Stygall, Trends Anal. Chem. 17 (1998) 583.
[216] G.Stafford, J. Am. Soc. Mass Spectrom. 13 (2002) 589.
[217] S.A.MacLuckey, G.J.Van Berkel, D.E.Goeringer, G.L.Glish, Anal. Chem. 66 (1994) 737 A-743 A.
[218] P.G.M.Kienhuis, R.B.Geerdink, J. Chromatogr. A 974 (2002) 161.
[219] C.Baumann, M.A.Cintora, M.Eichler, E.Linfante, M.Cooke, A.Przyborowska, J.M.Halket, Rapid Commun. Mass Spectrom. 14 (2000) 349.
[220] F.Calbiani, M.Careri, L.Elviri, A.Mangia, I.Zagnoni, J. Chromatogr. A 1058 (2004) 127.
[221] J.Hau, S.Riedeker, N.Varga, R.H.Stadler, J. Chromatogr. A 878 (2000) 77.
[222] R.K.Juhler, M.Vahl, J. AOAC Int. 82 (1999) 331.
[223] S.J.Lehotay, K.Mastovska, A.R.Lightfield, J. AOAC Int. 88 (2005) 615.
[224] S.J.Lehotay, A.De Kok, M.Hiemstra, P.van Bodegraven, J. AOAC Int. 88 (2005) 595.
[225] J.Wang, W.Cheung, D.Grant, J. Agric. Food. Chem. 53 (2005) 528.
[226] S.Bogialli, R.Curini, A.Di Corcia, A.Lagana, M.Nazzari, M.Tonci, J. Chromatogr. A 1054 (2004) 351.
[227] J.Klein, L.Alder, J. AOAC Int. 86 (2003) 1015.
[228] S.J.Lehotay, K.Mastovska, S.J.Yun, J. AOAC Int. 88 (2005) 630.
[229] C.Soler, J.Manes, Y.Pico, J. Chromatogr. A 1067 (2005) 115.

[230] M.D.Hernando, M.Mezcua, J.M.Suarez-Barcena, A.Fernandez-Alba, Anal. Chim. Acta 562 (2006) 176.
[231] J.L.Josephs, Rapid Commun. Mass Spectrom. 10 (1996) 1333.
[232] F.Calbiani, M.Careri, L.Elviri, A.Mangia, L.Pistara, I.Zagnoni, J. Chromatogr. A 1042 (2004) 123.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 15

Capillary electrophoresis

Agnes Fekete, Philippe Schmitt-Kopplin

GSF - National Research Center for Environment and Health, Institute for Ecological Chemistry, Molecular BioGeoanalysis, Ingolstädter Landstraße 1, D-85764 Neuherberg,Germany

1. Introduction

Recently, more than 500 methods have been developed for the determination of components of foods applying capillary electrophoresis (CE) since it has advantages of high separation efficiency, short analysis time, low sample and solvent consumption, low cost of the running and lower effect of matrices comparing with the other separation techniques. In addition, CE can separate compounds that have been traditionally difficult to handle by chromatographic techniques for example highly polar and water soluble substances. However, there are still some barriers to overcome. The major drawbacks in CE are the lower sensitivity and lower repeatability of the identification parameter (migration time) in comparison to the chromatographic techniques. To increase the method selectivity off-line and on-line preconcentration strategies are generally included into the method for trace analysis. Understanding the reason of the lower reproducibility, the theory of the separation that differs as of the chromatographic techniques are described. Because the components are separated in liquid, more parameters can be changed for tuning the optimum selectivity of the target substances. The type of the separation electrolyte and the ingredients are the main factor influencing the separation; therefore the CE techniques are divided according to the content of the separation medium which is also described briefly. In the second part of the review, developed methods for the determination of food contaminants namely antibiotics, pesticides and toxins and food components determinable with CE are summarized.

2. Principles of capillary electrophoresis

Capillary electrophoresis (CE) consists of a high-voltage power supply, two buffer reservoirs, a fused silica capillary and a detector. The basic set-up is usually completed with enhanced features such as multiple injection devices, autosamplers, sample and capillary temperature controls, programmable power supplies, multiplex capillaries, multiple detectors fraction collection and computer interfacing.

CE separation are usually performed in flexible fused silica capillary tube (length: from 20 to 100 cm; i.d.: 25 to 100 µm) filled with an appropriate buffer solution of defined pH and ionic strength (aqueous / non aqueous). A small volume of sample is introduced hydrodynamically (or less often electrokinetically) into the capillary to which an electrical potential is applied (Figure1). The sample amount (nanolitre range) needs to be kept lower than 3-4% of the column volume (microlitre range) to keep the separation efficiency high. Fraction collection is possible but allows only limited amounts of sample. Charged species of the sample exhibit different effective electrophoretic mobilities (field strength reduced velocities) and are thereby separated. Detection includes UV-Vis or laser induced fluorescence (LIF), electrochemistry, conductivity, mass spectrometry (MS). Although MS and LIF are prefferred due to the higher selectivity and sensitivity, spectrophotometry is the most applied detection system. Different separation principles can be applied; allowing separation of charged, neutral, polar or hydrophobic analytes as a function of the type of capillary column and separation buffer. Due to this high flexibility, CE is a promising separation technique for the determination of food components and contaminants which have versatile solubility and protonation/deprotonation ability.

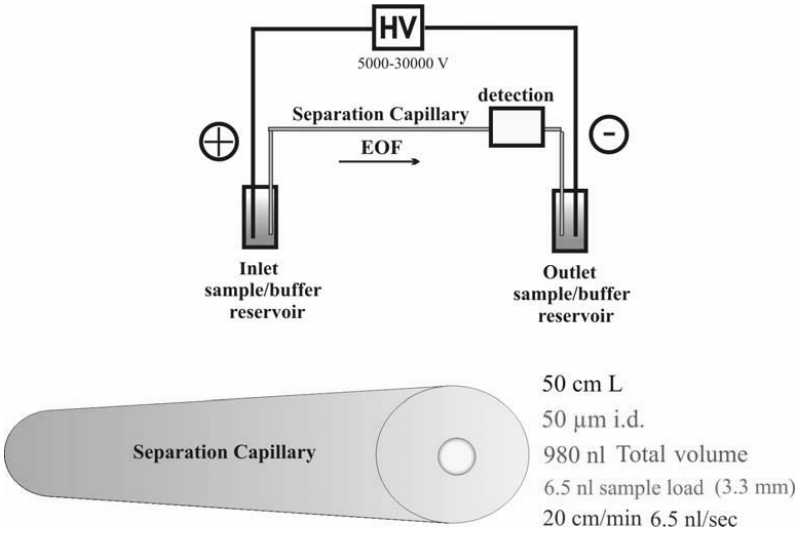


Figure 1. Simple setup of capillary electrophoresis.

2.1. The driving force in the capillary: the electroosmotic flow (EOF)

Origin of the electroosmotic flow. Electroosmosis is the fundamental process in CE that makes the method principally different to chromatography. It is a direct consequence of the surface charge on the walls of the uncoated fused silica capillary. This phenomenon of electrokinetics was described at first by Reuss nearly 200 years ago (1809). The capillary surface contains silanol groups (pK_a 3-5) that ionize as a function of the pH of the separation buffer. The dissociation to SiO^- produces a negatively charged wall on which an electrical double layer is established at the solid/liquid interface to preserve electroneutrality (Figure 2). The double layer was the basis of the theoretical modelling and developments in the 19th Century by v. Helmoltz, Gouy, Stern (to cite only them). Coating systems (such as polymers) can chemically alter the surface and also regulate the EOF if needed.

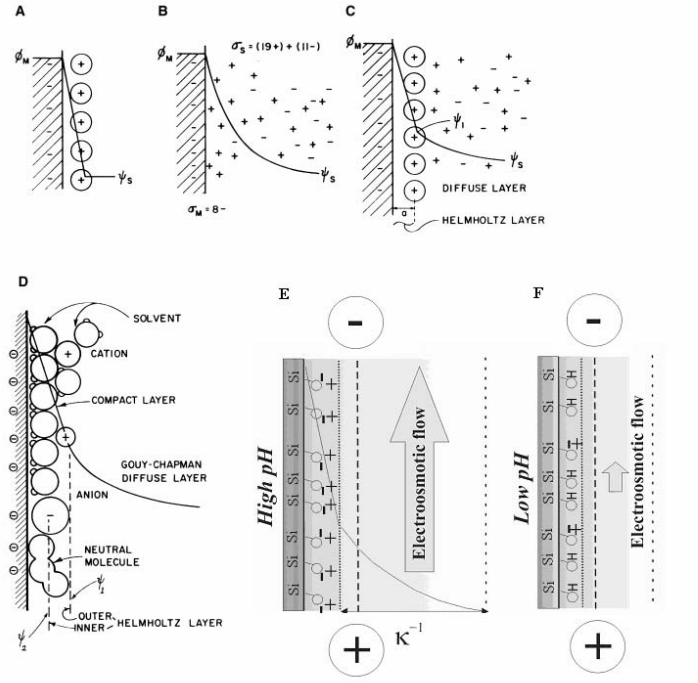


Figure 2. (A) Helmholtz and the capacitor model (B) Gouy and the diffuse layer model (C) Stern and the model as coupled diffuse layer charge distribution with corresponding drop of potential ψ (D) Overall model of the double-layer showing presence of solvent molecules (adapted from the *Encyclopedia of Surface and Colloid Science/Decker*), Schematic representation of the fused silica capillary surface at high (E) and low pH (F) values and consequences for the electroosmotic flow when a difference of potential is applied across the capillary.

The potential ψ drops to ψ_0/e at a distance of $x = \kappa^{-1}$, which is called the thickness of the diffuse double layer or also *Debye length;* κ^{-1} is dependent on the ionic strength I. An externally imposed tangential flow of the medium over the surface would lead to a distortion of the ions creating a "streaming potential". This process is reversible and when a potential is applied, the counter-ions and their associated solvating water molecules migrate towards the cathode resulting in a flow of solution. This flow called as *electroosmotic flow* is an electrically driven pump towards the detector.

The electroosmotic flow (μ_{eo}) is proportional to the zeta potential (ζ) and dependant on the chemistry, the viscosity (η) and the dielectric constant (ε) of the separation buffer. The zeta potential measured at the plane of shear close to the liquid-solid interface (slipping plane in Figure3) is related to the inverse of the charge per unit surface area, the number of valence electrons, and the square root of the concentration of the electrolyte, an increase in the concentration of the electrolyte decreases the EOF. Strongly adsorbed ions on the inner wall of the capillary or a decrease in pH (lowering the surface charge of the capillary) of the separation electrolyte have the same effect.

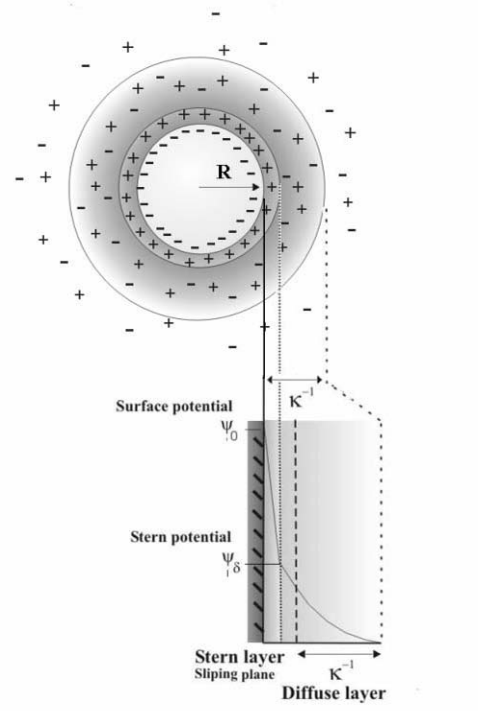


Figure 3. Schematic representation of a charged particle and its different charged layers; identical rules as in the previous Fig.2

2.2. Theory of zone electrophoresis for charged colloids and molecules

Basically the mobility of an analyte in free solution is defined as the ratio of its electric charge Z (Z = q.e, with e the charge if an electron and q the valance) to its electrophoretic friction coefficient f.

Charged colloidal particles in aqueous solutions will develop a solvation sphere, in which dipolar water molecules and oppositely charged ions accumulate near the charged surface of colloids, forming an electrical double layer. The potential across the surface of shear of the double layer is referred to as the zeta potential. The entire diameter of the colloid is composed of the diameter of the particle itself in addition of the thickness of the solvation sphere. Therefore, the thickness of the double layer can also contribute significantly to the electrophoretic mobility of a colloidal particle [1] (Figure3).

The electrostatic potential at the shear surface (ζ potential) controls to a large extent the mobility of the particle. An estimation of electrophoretic mobility is given by equation 1 [2] in the case of small potentials, assuming a rigid and non conducting spherical particle of Radius R, moving in a medium of viscosity η and permittivity ε, such as:

$$\mu = \frac{2 \cdot \varepsilon \cdot \zeta}{3 \cdot \eta} \cdot f(\kappa \cdot R) \qquad (1)$$

As already defined when describing the electroosmotic flow (EOF), κ is the reciprocal of the Debye length (electric double layer thickness), which is a function of the ionic strength of the solution. The Debye length (κ^{-1}) can be estimated with $\kappa^{-1} = 3.288.\sqrt{I}$ for a monovalent electrolyte at 25 °C (nm^{-1}; ionic strength *I* (mol/L)) i.e. can vary between 3.8 nm (for I = 6 mmol/L) and 17.6 nm (I = 0.3 mmol/L) [3]. It is obvious that the entire diameter of the particle is composed from the diameter of the rigid core and the contribution of the double layer. Therefore the thickness of the double layer will also determine the electrophoretic mobility of the particles [3].

The function f(κR), called *Henry function,* ranges from 1 at κR << 1 (*Hückel limit*) to 1.5 at κR >> 1 (*Smoluchowski limit*) as a function of the particle shape. As the particle migrates accompanied by a given amount of the surrounding liquid, the counter ions and the charged species it contains, may create *retardation* effects [4]. Furthermore the distortion of the diffuse layer in the applied field leads to a polarization so that the particle and the counter ions tend to be drawn back together. This is especially true for high ζ potentials and is known as a *relaxation* effect (4)]. The last effect is due to the distortion of the local electrical field by the particle. When the particle size is small in relation to the double layer (κR << 1), this effect is negligible (Hückel´s theory). When κR >> 1 this effect however needs to be taken into consideration (Smoluchowski´s theory). For example in the case of particles possessing very high ζ potentials (> 75 mV), the relaxation effect is no longer negligible and the dependence of the mobility on (κR) can be altered.

2.3. The most important techniques in brief

Many CE-techniques are routinely used in the analysis of biomolecules from small ions to macromolecules [5] and have been applied for the analysis of food quality and food toxicants. The presented CE methods are all based on similar principles of open tubular separations (excepting capillary electrochromatography) with eletrokinetically driven buffer flow. Some variations in the quality of the separation buffer are used to tune and induce interactions between the buffer components and the analytes improving the selectivity in the separation. The frequently used CE methods in food analysis are presented herein in brief.

2.3.1. Capillary zone electrophoresis (CZE)

By changing the quality of the separation buffer, optimized interactions with the sample and with some buffer constituents allow an increased selectivity in the separation of charged or neutral analytes. CZE allows the separation of anions and cations as a direct function of their effective charge to size ratio [6].

The addition of solvents to the running buffer up to 100% in nonaqueous buffers (non-aqueous CE - NACE), can additionally allow specific selectivity and increased solubility for some analytes and is mostly used for pharmaceuticals or plant secondary metabolites [7] with low water solubility. The selectivity is governed by their effective charge and thus by the separation buffer pH and indirectly the electroosmotic flow (EOF).

Here the definitions of apparent and effective electrophoretic mobility need to be developed. With the polarity setup of the anode being at the injection inlet and the cathode at the outlet, neutral sample will move towards the detector with the velocity of the EOF; cations will move to the cathode first with a higher apparent velocity (apparently faster) and anions will move against the EOF with a reduced apparent velocity (apparently slower) as shown in

Figure4. The peaks are detected with increasing times in the electropherogram. The electrophoretic mobility is defined as being the field strength reduced velocity of the ions in the capillary. With an applied electric field E across a capillary of total length L_t the field strength is E/L_t. After a time t following the injection, the analytes (anion, neutral, cation) will cross the detector situated at a length L_d from the inlet one after the other; the observed velocity (v) of the analyte is thus equal to L_d/t. The electrophoretic mobility calculated from the observed velocity is called apparent electrophoretic mobility (μ_{ap}). The effective electrophoretic mobility (μ_{ef}) takes account of the velocity of the buffer towards the detector (EOF, (μ_{eo})) and is thus the EOF-normalized electrophoretic mobility of the ions ($\mu_{ef} = \mu_{ap} - \mu_{eo}$); μ_{ef} is equal to zero for neutral analytes, negative for anions and positive for cations. The effective electrophoretic mobility is an absolute parameter independent of the applied field or the column length and only dependant of the charge and size of the analyte. A conversion of the electropherograms from the time-scale into the effective mobility scale (μ-scale) allows better reproducibility of separation patterns and of quantification parameters [8].

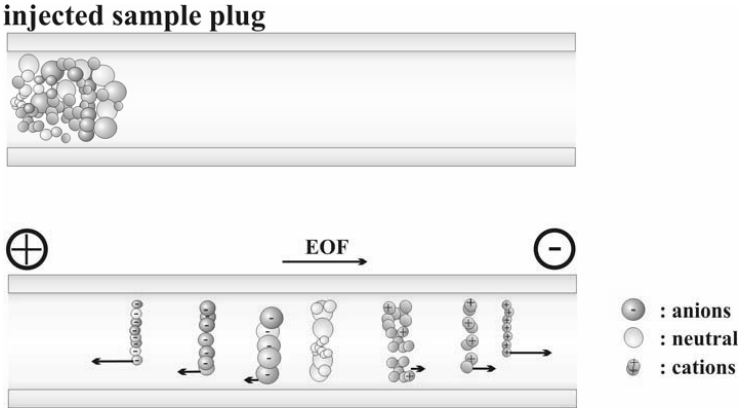


Figure 4. Illustration of separation by CZE

2.3.2. Micellar electrokinetic chromatography (MEKC)

Micellar electrokinetic chromatography (micellar capillary electrophoresis) is a technique introduced already 1984 by Terabe [9] – commercial CE systems were available only from 1988; the addition of charged surfactants (i.e. SDS or CTAB) to the separation buffer at concentration above their critical micellar concentration allow the separation of neutral or charged analytes (see Figure 5) as a function of their affinity to partition into micelles (hydrophobicity / K_{ow} dependant). A partial filling technique of MEKC needs to be used when coupled to mass spectrometry avoiding ion suppression due to the presence of surfactants in the electrospray ionization and a lower contamination of the mass spectrometer.

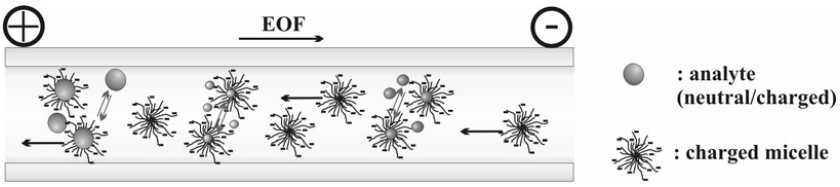


Figure 5. Separation theory based on MEKC

2.3.3. Chiral CZE and chiral MEKC

The addition of a chiral ligand (for example different substituted cyclodextrins (CD), antibiotics, sugar or peptide based ligands) to the CZE-separation buffer (Fig.6) allows the separation of ionic isomers (D/L, +/- enantiomers, stereomers). The separation of the isomers is based on different binding/partitioning constants between the chiral ligand and the D- or L-analyte. Non-racemic substances can also be separated based on their relative selective molecular binding occurring with the separation buffer constituents. The addition of a chiral ligand to the MEKC-buffer additionally permits the analysis of neutral isomers (enantiomers, stereoisomers). Similar results can be obtained when using charged cyclodextrines or other charged chiral ligands (i.e. chiral micelles).

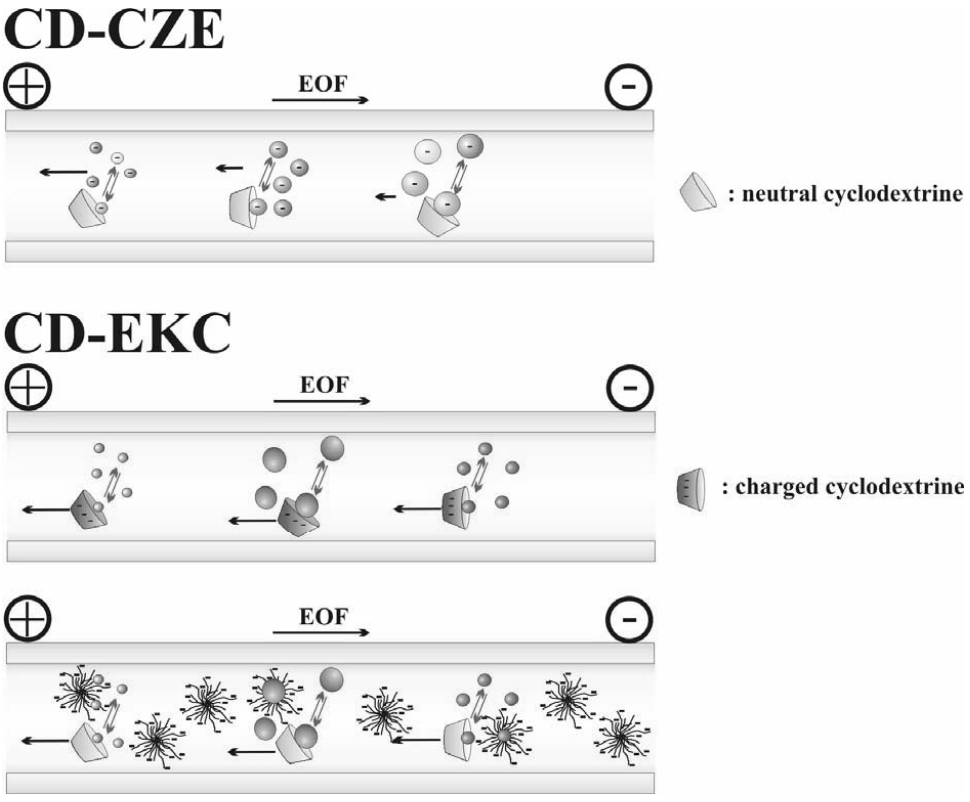


Figure 6. Illustration of chiral separation based on CZE and MEKC

2.3.4. Capillary gel electrophoresis (CGE)

Capillary gel electrophoresis allows the analysis of molecules based on their size. The separation is done in physical gels (diluted polymer solutions such as methylcellulose, polyethylene glycols) which molecular size and concentration determine the selectivity by sieving effects as shown in Figure7. This technique is the basis of the gene sequencing techniques used in modern molecular biology.

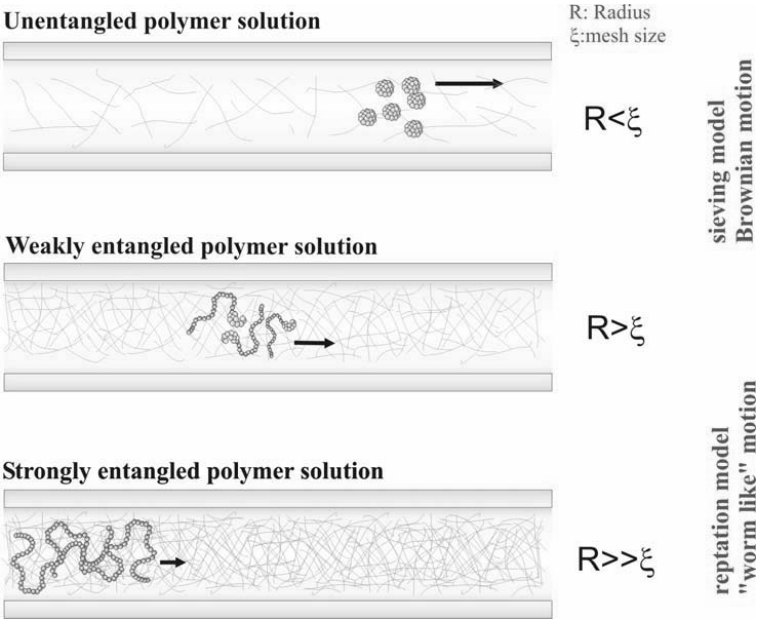


Figure 7. Separation theory based on CGE

2.3.5. Capillary electrochromatography (CEC)

Capillary electrochromatography is a relatively new coming capillary separation technique that uses the same fused silica capillaries as in CE except that they are filled such as in nanobore liquid chromatography (nanoLC) with silica based materials or with polymeric sorbents [10-12] as illustrated in Figure8. Due to the pH-dependant surface charge of the silica or the polymer, the EOF can acts as a pump such as in CZE, moving the analytes with the buffer towards the detector; the separation occurs by interactions of the analytes with the immobilized active surface (hydrophobic, hydrogen and/or ionic bonds.) combined with their own electrophoretic mobility: CEC can be considered as a hybrid of CZE and nanoLC. The separation of selected lignin/humic substance (HS) degradation compounds by capillary

electrochromatography with a methacrylate based monolithic column and a conventional column packed with 5µm octadecyl silica (ODS) particles was presented in [13] for the characterization of NOM. The effects of organic modifier concentration, pH of the mobile phase, ionic strength, applied voltage, and temperature on the separation of phenolic lignin monomers need to be investigated to determine the optimal separation conditions. The CEC of a soil fulvic acid with a monolithic column produced partly resolved broad bands; by means of NMR analysis a wide range of oxygen derived aromatic substitution patterns was found with prominent contributions from phenolic and carboxylic groups [13].

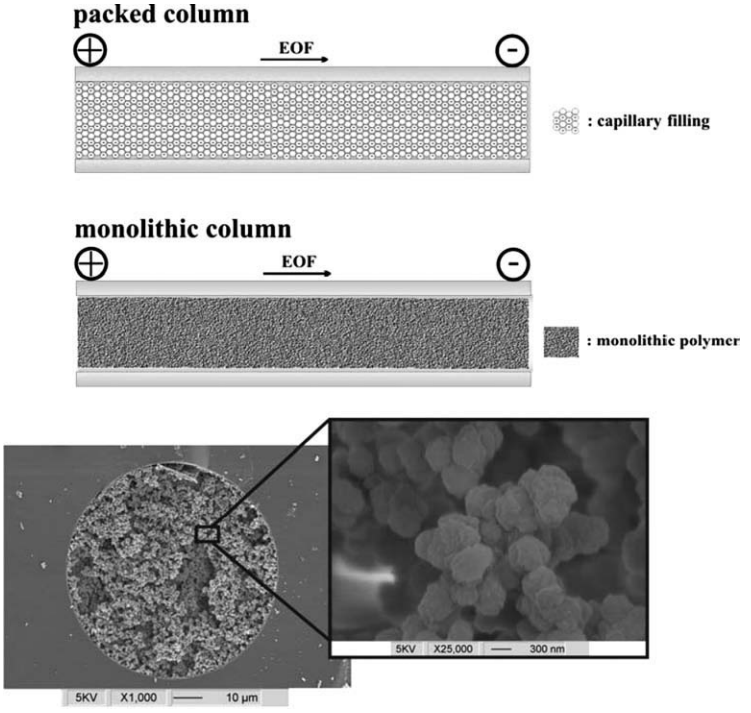


Figure 8. Illustration of capillaries used for CEC filled with silicagel and monolith.

3. Application of capillary electrophoresis in food analysis

The chemical and physical properties of the analytes, like dissociation coefficient (pK_a) and octanol/water partition coefficient (logP) determine mainly the type of the CE separation technique. Substances with protonisation/deprotonisation ability can be separated applying zone electrophoresis. CZE is the most used technique in food analysis since several

components are highly polar and contain amino, carboxy and/or hydroxyl groups. Apart from food contaminants including antibiotics used in veterinary practice, pesticides and toxins, other components present in food can be separated and detected by CE techniques. The concentration of the contaminants is usually very low and the presence of the matrices may have a big influence on separation (shift and co-migration). Selective sample preparation techniques are often needed due to the similar chemical-physical properties of analytes and matrices. The found applications for analysis of matrices can be classified as:

- inorganic ions and low molecular weight organic acids,
- biogenic amines used mostly for estimation of quality of sea products and wine,
- amino acids useful for studies of adulteration and food quality
- polyphenols and vitamins registered as the main components of functional food due to its antioxidant activity
- heterocyclic amines crucial for quality control due to their mutagenic effect,
- and proteins paying big role in adulteration control.

The differentiation between *food contaminants* and *food constituents* reflecting the food quality sometimes is difficult and only based on the concentration of the analytes in the different type of food. For example the type and amount of biogenic amines determines the quality of the wines; biogenic amines are also used for distinguish fresh and spoiled fish products. The possible CE-methods applied for in food analysis are summarized briefly herein. Reviews dealing with the analysis of different substance class in food are shown in Table 1.

3.1. CZE methods for food contaminants and components

Several capillary electrophoretic separations have been developed for the determination of antibiotics, herbicides and toxins, however, only a few articles deals with their determination from food as matrix [14].

3.1.1. Veterinary drugs

Most antibiotics including the group of (i) quinolones, (ii) tetracyclines, (iii) sulphonamides and (iv) miscellaneous antibactericides are amphoteric compounds allowing their selective separation in wide pH range since they are cations at acidic condition and anions at high pH.

Quinolones are a group of widely used synthetic antibacterial agents in human and veterinary medicine and they may be found in contaminated meat products. Their CE analysis has not been used for real samples in routine practice; the methods were always applied to spiked samples mostly from chicken muscle. Oxolinic acid, flumequine, norfloxacin, difloxacin, ciprofloxacin, marbofloxacin and danofloxacin have been separated with phosphate based separation electrolyte at pH 8-9 in presence of methanol used as organic modifier [15-17]. A typical electropherogram of 8 quinolones is shown in Figure 9 [18].

Table 1

General review on food analytic with use of CE

CE	Analyte	Goal, comment	Matrix	Ref
CE	DNA; Gliadines, glutenins and proteins; Lactoglobulines, caseine, CMP, furosine; Inorganic ions and organic acids; amino acids; phenols, flavonoids; vitamins	-review on analytic in food authenticity	Cereals, fruits, milk, meat, wine, honey	[101
CE	DNA; proteins and peptides, amino acids and amines; carbohydrates and gelling agents; inorganic and organic acids; phenolic compounds; vitamins; preservatives and colours; toxins and residues	-general review of published papers between 2002 and 2003	milk, cereal	[102]
CE	Proteins and amino acids; carbohydrates; vitamins; inorganic ions; additives; natural toxins; residues	-new detection systems -quality assessment;		[103]
CE	milk protein; amino acids; carbohydrates; inorganic ions and organic acids; phenolic compounds; vitamins; food additives; biogenic amines; toxins and residues	-general review -development and difficulties		[104]
CE	nucleic acid derivatives; carbohydrates; inorganic and organic anions; vitamins; phenolic acids; alditols and alcohols; amines; hop and beer bitter acids;	-analysis of beer components	Beer	[105]
CE	cereal proteins; amino acids; vitamins; carbohydrates; fatty acids; biogenic amines; veterinary products; fungicides	-adapting CE to routine application -possibilities of automatisation		[106]
MEKC	Enzymes, glucoamylases; amines	-evaluation of mutagenicity		[107]
CE	antibacterials, pesticides, toxins	-organic contamination in food		[14]
CE, HPLC	oxytetracycline, tetracycline, chlortetracycline, doxycycline	-analytic of tetracycline antibiotics -sample preparation methods		[21]
HPLC, CE	para-, diquat	-analytic of quaternary amine herbicides	potato and serum	[108]
GC, LC, CE	pesticides and metabolites	-sample preparation methods		[109]
GC, MEKC	polychlorinated biphenyls	-sample preparation methods		[110]
CE	inorganic anions and cations	-review on inorganic analysi	Beverage, food	[32]
CE, LC	heterocyclic amines	- HAs in cooked meat	meat, fish	[46]

CE	Analyte	Goal, comment	Matrix	Ref
CE, HPLC	catechins, caffeine, theanine, ascorbic acid; metal; ammonium, amines; organic acid	-review on analytic of tea	Tea	[84]
CE, HPLC	monosacharides, oligosaccharides ; alditols, alditol glycisides, polyols, amino sugars, deoxy sugars	-analytic of carbohydrates -sample preparation methods		[111]
CE	catechins, isoflavones, antocyanins, resveratrol, vitamins	-analytic of polyphenols in food	tea, soy, wine	[48]
CE HPLC	caffeine, theanine, catechin, epicatechin, epigallocatechin, (epi)gallocatechin gallate,	-review on analytic of tea catechins	Tea	[49]
CE, HPLC	cyanidin 3-glucoside, cyanidine 3-rutinoside, delphidin 3-glucoside,	-analytic of anthocyanins	currant, candy, juice, jelly	[51]
CE	cis and trans resveratrol	-separation of isomers	Wine	[50]
CE	Lac, cochineal, cardenia, monascus, elderberry pigments	-analysis of natural food pigments		[112]
CE	ascorbic acid, nicacin, thiamine	-water-soluble vitamin analytic	meat, milk	[113]
CE, GC, HPLC	sugars; carboxylic acids; amino acids	-separation techniques in food analytic	juices, citrus, apple, beer	[114]
CZE, MEKC	preservatives; antioxidants; sweeteners; colourings; nitrite, nitrate	-review on analytic of food additives until 2001	Beverage, vegetables	[52]
CE, CEC	Peptide; amino acids; organic acids; pesticides; sugar	-review on chiral separation	juices, wine, yogurt	[87]
CE-MS	peptides and proteins; amino acids; carbohydrates; polyphenols; toxins; pollutants	- food analytic applying CE-MS		[115]
CE	Proteins and peptides of animal origin	-analytic of on proteins	milk, egg, fish, meat	[116]
CE	Proteins		meat, milk cereal	[61]
CE	DNA	-combination with molecular methods	dairy products, meat	[95]

However, these substances are water soluble, NACE have been also developed [19] to increase the selectivity of the separation of seven quinolones from pig kidney extract. Ammonium acetate buffer solved in methanol and acetonitrile at ration of 1:1 was used as background electrolyte (BGE) in presence of hexadimethrine bromide acting as EOF reserve agent speeding up the analysis time.

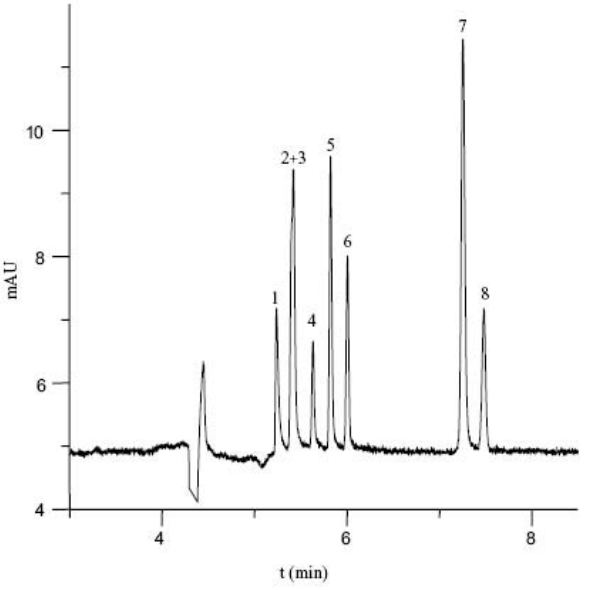


Figure 9. Electropherogram of a mixture of the quinolones. Electrophoretic conditions were 50mM phosphate buffer at pH 8.4, separation voltage at 20 kV and detection wavelength 260 nm. Migration order: (1) danofloxacin, (2 and 3) ciprofloxacin and sarafloxacin, (4) marbofloxacin, (5) enrofloxacin, (6) difloxacin, (7) oxolinic acid and (8) flumequine (Reproduced from [18] with permission from Elsevier© 2004)

Tetracycline antibiotics produced by *Streptomyces* are broad-spectrum antibiotics and they are administered to e.g. catfish to control bacterial infection. A CZE method using phosphate buffer at highly acidic pH (pH 2) has shown that oxytetracyclin is determinable from catfish and it is stable during the cooking process [20]. The method used UV-Vis detection at low wavelength, however, tetracyclines have native fluorescence due to the condensed ring that can be strongly intensified by application of alkaline earth ion in the separation electrolyte [21]. Good resolution of sulphonamides from pork extract have been achieved applying a phosphate buffer near to neutral pH showing the amphoteric character of this substance class [22]. Sensitive determination of seven sulphonamides has been achieved with combination of continuous flow system and CE-MS and the applicability was shown through spiked milk

samples [22; 23]. Adsorption of the cationic analytes onto the capillary wall was observed causing non-reproducible migration time, thus use of cationic coating was negligible. Capillary coating is a generally used method to govern the EOF when anions are in the interest of the determination or to prevent the adsorption of the sample components onto the inner surface of the fused-silica tube

3.1.2. Pesticides

Due to the diverse function of pesticide several methods applying CE have been determined; the most paper deals with the development and validation of these substances by CE. Pesticides like dithiocarbamates, acidic pesticides and some fungicides are determinable by CZE due to the ionisable group in the molecule, but the others like the triazines, urea derived, organic phosphorus substances and some multiple pesticides need to be separated with MEKC. Acidic pesticides including o-phenylphenol, ioxynil, haloxyfop, acifluoren und picloram were determined from orange juice as sample matrix applying CE-MS with off-line solid phase microextraction (SPME) used as selective sample preparation technique; the method describes the development and the performance characteristics [24]. CZE-MS have been used for the separation of pyrifenox, pirimicarb, cyprodinil and pyrimethanil function as multiple pesticides from orange juices with ammonium acetate based separation electrolyte at acidic pH [25]. Dithiocarbamates (ferbam, ziram, zineb, metham and maneb) have been selectively determined applying borate based BGE with pH of 9 from wheat extract, but no targets have been found from the examined samples [26].

3.1.3. Biological origin toxins

Toxins are third big substance class of food contaminants determinable by CE techniques. Naturally occurring toxins such as glycoalkaloids and microbial contaminants like food-born pathogens or toxin-producing microorganism have been determined by CE coupled to selective detection techniques like MS and fluorescence. Glycoalkaloids were determined from freeze dried potato extracts applying NACE coupled to MS and MS/MS [7; 27] and an electropherogram of standard solution of α-solanine, α-chaconine is shown in Figure10.

Domoic acid and paralytic shellfish poisoning (PSP) toxins were separated separately from contaminated mussel extracts by CE-UV [28]. Increasing the method sensitivity and selectivity, solid phase extraction (SPE) were included into the analysis process. Domoic acid contains amino and carboxylic acid groups thus it can be separated at wide pH range by CZE. Robust and fast separation was achieved by application of borate buffer in presence of methanol as organic modifier. PSP toxins were also separated with isotachophoretic (ITP) separation buffer containing morpholine at pH 5 [28].

Bacterial contaminations can be studied different ways applying CE. Lypopolysaccharides present in the wall of Gram-negative bacteria protonating at alkaline medium and have native fluorescence allowing their CE-LIF determination [29]. CE-LIF have been also used for the

determination of microorganisms from meat; however improvement of the method is required due to the board peaks of the targets and relatively long analysis time [30]. Improved separation was achieved for the determination of eight microorganism applying CZE electrolyte containing phosphate buffer in presence of calcium and myoinosytol hexakisphoshate that decrease the peak width due to the formation of protein-myoinosytol sandwich bonds [31]. Even though the method applicability was shown by spiked juice and corn flakes samples, no targets were found from the selected samples.

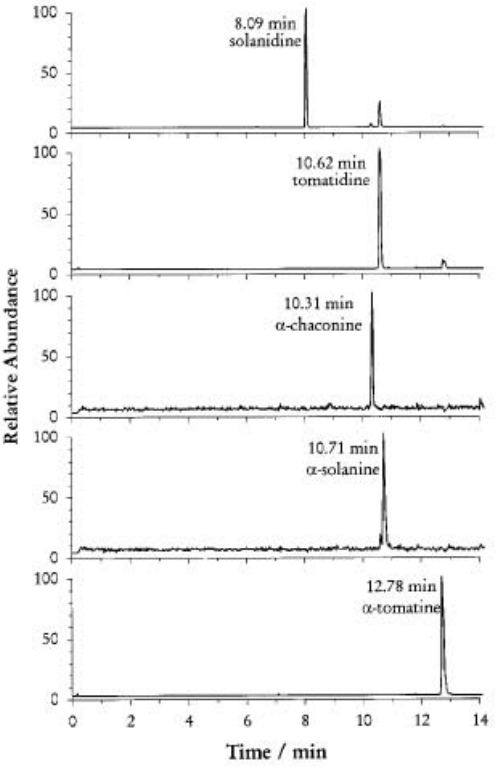


Figure 10: SIM traces by NACE-ESI-MS of a 1 mg/L standard mixture of glycoalkaloids and relative glycones. Electrophoretic conditions: buffer: ACN-MeOH (90:10 v/v) containing 50 mmol/L ammonium acetate and 1.2 mol/L acetic acid; uncoated fused-silica capillary, L_d = 80 cm, 50 µm ID; injection: pressure, 0.5 psi for 5 s; effective voltage: 25.5 kV; T = 20°C. ESI-needle voltage, V_{es} = +4.5 kV; spray current, sheath gas flow rate was set at (N_2) 0 AU; temperature of the aluminium capillary, T_{cap} = 180 °C and capillary voltage, 32 V; coaxial sheath liquid, methanol: water (1:1) with 1% of acetic acid at a flow rate of 2.5 µL/min. MS detection was performed in the SIM mode and the acquired masses were: 398, 416, 852, 868, 1034 for solanidine, tomatidine, α-chaconine, α-solanine, and α-tomatine, respectively (Reproduced from [7] with permission from Wiley© 2006).

Table 2

Selected CZE methods for food components

Detector	Analyte	Goals, comments	Matrix	Ref
ESI-MS	Procymidone, thiabendazole	-applying sample stacking and SPE	grape, tomato	[117]
ESI-MS/MS	dinoseb, pirimicarb, procymidone, pyriphenox, pyrimethanil, thiabenzadole	-application of SPE	Peaches, nectarines	[25]
MS, UV	pyrimethanil, pyriphenox, cyprodinil, cyromazine, pirimicarb	-application of SPME -use of chemometrics in optimization	orange juices, grape juice	[118]
DAD	Sulfamethazine, sufamerazine, sulfadiazine, sulfadimethoxine, sulfamonomethoxine,	-determination of sulfonamides used in veterinary practice; application of SPE	Meat	[22]
conductivity	Sulfite	-separation on PMMA chip	Wine	[119]
DAD	domoic acid as ASP toxin, GTX 1-5 as PSP toxin	-paralytic and amnesic shellfish toxin	contaminated mussel	[28]
DAD	*Versinia enterociliticia, Leuconostoc mesenteroides, Salmonella enteritidis, E. coli, Listeria monocitogenes*	-monitoring of bacterial contamination	juice, milk, corn flakes, baby food	[31]
UV	isocitric, citric, tartaric and malic acid	- study of adulteration	orange juice	[41]
UV	Nitrite, nitrate		meat, vegetables	[120]
Indirect UV	Oxalic-, malic-, aspartic-, glutamic-, quinic acid	-fingerprint at different tea infusions	tea infusion,	[40]
Indirect-UV	chloride, nitrate, sulfate, phosphate, tartarate, malate, succinate, citrate, acetate, lactate, ascorbate	-simultaneous determination of organic and inorganic acids	orange juice and wine,	[38]
DAD	Histamine	-study of the state of the deterioration	tuna fish	[121]
UV	histamine, tyramine	-comparison of CE and HPLC; use of SPE	fish, cheese, meat	[122]

Detector	Analyte	Goals, comments	Matrix	Ref
Indirect UV	methyl-, hist-, ethanol-, propyl-, isopropyl-, isoamyl-, tyr- and phenethylamine, purescine	-use of on-line SPE system	Wine	[123]
ESI-MS	20 amino acid	-analytic of free amino acid	infant food	[45]
UV	arginine, alanine, serine, aspargine, tryptophan, glutamic acid, phenylalanine, tyrosine, proline	-determination of free amino acids -fingerprints of different beers	orange juice, beer	[43]
UV	adenosine, guanosine, uridine	-fingerprints for quality control	*C. Mycelia*	[124]
ESI-MS	intact gellan gum	-analytic of polysacharides	fruit flavor drinks	[125]
Indirect UV	sucrose, sucralose, glucose, fructose	-applying chemometrics and SPE	juice, yoghurt	[60]
DAD	apigenin, baicalein, naringenin, luteolin, hesperetin, galangin, kaempferol, quercetin, myricetine	-use of SPE -comparison of the method	Wine	[126]
DAD	Caffeine,theo-, di- and enprophylline, theobromine	-analytic of alkylxanthines	chocolate	[127]
UV	Caffeine, adenine, theophylline, epigallocatechin-3- and epicatechin-3 gallate, epigallo- and epicatechin	-coupling of flow injection system (automation)	green tea	[53]
electrochem	*Trans*-resveratrol	-analytic of compounds with antioxidant activity	wine, chinese medicinal herb,	[128]
amperomet.	Chlorogenic-, genistic-, ferulic- and vanillic acid	-separation on microchip	red wine	[129]
ESI-MS	Cytochrome C, lysozyme from egg white, ribonuclease A	-applying physically adsorbed polymer coating	turkey and chicken egg white, beef	[130]
LIF	α-lactalbumin, β-lactalbumin, bovine serum albumin	-on-capillary derivatisation	Cheese from whey	[131]
UV	α-lactalbumin, β-lactalbumin	-adulteration of cows and goat milk products	cows and goat milk and cheese	[132]
UV	α-casein1, α-casein2, β-casein1, β-casein2	-casein concentration during the ripening time	Cheese (from ovine	[70]

Detector	Analyte	Goals, comments	Matrix	Ref
			milk)	
UV	para-κ-casein, β-lactoglobulin	-analysis of whey proteins and their degradation	milk, dairy products	[133]
UV	α-lactalbumins, β-lactoglobulins	-use of ITP based BGE	binary and ternary cheese	[69]
FL	DNA	-lab-on-microchip, including PCR -species identification	fish species	[134]

3.1.4. Inorganic ions and low molecular weight acids

Several minor components like halides, fat-soluble vitamins and metals are essential for human health but too high levels can lead to health diseases; in this case even endogenous compounds can be assigned to pollutants. Solutes like nitrate and nitrate may be present originally in the food extract, but can also originate from packing materials like paper as food contaminants. Thus the analysis methods applying CE for substances playing role in the food quality are summarized shortly (see Table 2).

One of the strong points of CE comparing with the other separation techniques is the simplicity and fastness of the determination of inorganic ions and low molecular weight acids and bases. Several methods have been developed for the determination of metals from different foods samples like milk, cola, vegetables and wheat flour [32]. Since they are cations in water, they can be separated by CZE applying buffer at acidic pH. However, their detection is more delicate since these molecules do not absorb light in the UV-Vis range therefore they can not be directly detected spectrophotometrically. Since spectrophotometric detection is commercially available in the CE instruments, methods using indirect-UV mode have been developed for the analysis of inorganic cations. It uses the addition of an absorbing co-ion into the separation electrolyte and negative peaks are detected at the migration zones of the charged and non-UV active solutes. Applying indirect-UV detection, the mobility of the UV-absorbing ion present in the separation electrolyte has to be similar as the targets in order to reduce fronting and tailing of the analyte peaks. The concentration of the UV-absorbing ion in the BGE influence the sensitivity of the method, however it may cause instability of the baseline at too high concentration. Indirect-UV detection imposes severe limitations on the choice of electrolytes, their concentrations and additives. Moreover, this technique is the least selective and therefore the most subject to interferences. For the determination of ammonia, alkali, alkali-earth and other metals like manganese, nickel, zinc, cupper and chrome from different food extracts imidazole or a commercially available solute called UVCat-1 have been used as UV-absorbing agent. As counter mostly α- hydroxyisobutyric acid were used due to its complexion ability with the inorganic cations increasing the separation selectivity [32]. Crown ether, 18-crown-6, was also ingredients of BGE in order to distinguish NH_4^+ and K^+ from each other since they have identical size and charge/mass ratio. Direct UV detection applying non-selective detection wavelength (200 nm) have been also used for the determination of Ca^{2+} and Mg^{2+} as EDTA complex from extracts of different vegetables [33]. The complexes were separated in a polyimide coated capillary with buffer containing 20 mmol/L borate at pH 9.2 and 2 mmol/L EDTA. Methyl mercury, which is a possible contamination factor due to its highly toxic property, was also determined from fish samples applying borate based separation electrolyte [34]. They were also separated as UV-active complex with agent of dithiozone sulphonate under acidic pH (pH 4.5) [35].

Several CZE methods have been developed for the determination of inorganic anions and LMW acids from different matrixes including food and have been reviewed elsewhere [32; 36; 37]. Inorganic anions including sulphite and sulphate used as antimicrobial agent and antioxidants; nitrate and nitrites added into the meat products as preservatives; halides which presence at high concentration are toxic; phosphate and carbonate are in the interest of food analysis. Organic acids are usually used for study of food quality and adulteration.

For the determination of inorganic anions and LMW acids, the EOF is generally reserved to achieve a suitable separation time. The most common method to control and reserve the EOF for the analysis of anionic species is the addition of surfactant into the separation electrolyte. The use of long-chain ternary alkylammonium cationic surfactants such as cetyltrimethylammonium bromide (CTAB), tetradecyltrimethylammonium bromide (TTAB) and dodecyltrimethylammonium bromide (DTAB) has been reported in applications requiring suppression or reversal of EOF [38]. In addition, amines like diethylenetriamine or tettraethylenepentaamine and alkylammonium hydroxide such as octadecyl-trimethylammonium hydroxide and tetradecyl-trimethylammonium hydroxide have been also used to reserve the EOF.

Since the LMW acids are lack of chromophor groups, the most used detection technique based on indirect-UV. The most common UV-absorbing anions within the range of mobilities of analyte anions of interest for food analysis are chromate, 1,3,5- benzenetricarboxylic acid and phthalate. Chromate based electrolyte was used for the determination of sulphite from different vegetables and wines [39] and for the determination of phosphate from cola as beverage matrix. Phthalic acid with combination of commercially available EOF-suppressor (OFM-Anion BT) has been applied for the determination of nine inorganic anions (halides, nitrate, nitrite, sulphate, sulphite, phosphate and carbonate). EDTA have been also added into the sample solution and into the separation buffer for the determination of LMW acids to avoid the mobility differences of the analytes caused by the high content of alkaline-earth ions [40]. The method applying chromate as UV-absorbing ion, TTAB as EOF reserve agent and EDTA as complexing agent was used for the determination of fluoride and LMW acids like oxalic, citrate, malic and glutamic acids from different beverages like wine and orange juice [40]. Organic acids were determined with direct UV detection too with use of non-absorbing electrolytes since the maximum absorbance of the analytes was observed at 200 nm. For the determination of citric, isocitric, malic and tartaric acid phosphate based buffer was used as a BGE at pH 7.5 where peaks causing interference were separated from the targets [41].

3.1.5. Amines and amino acids

Biogenic amines are organic bases of low-molecular mass comprising aliphatic, mono-, di- and poly-amines. Other amines like cathecolamines registered as functional food components, indoyl- and heterocyclic amines present as contaminants in different food products. Low-molecular weight amines like histamine, putrescine, cadaverine, tyramine and ethylamine are

forming during microbial process and their presence in alcoholic beverages (wine and beer) reflect unsanitary condition. They are analyzed in different foods like fish, cheese and sea food used as quality indicators for raw food materials. Since they are weak bases they can be separated with CE under acidic condition according to their charge and size. Their detection is mostly based on indirect-UV detection due to the absence of chromophores. The advantages of this approach are that primary amines through quaternary amines can rapidly be separated. LMW amines have been mostly determined from wine and fish applying formate based electrolyte (pH 3-4) containing cupper-sulphate function as UV-absorbing ion and 18-crown-6 to increase the selectivity of the separated targets [42]. The signal sensitivity can be improved two or three order of magnitude when the amines are derivatised before their capillary electrophoretic determination. Moreover, the interferences of other present and non-derivatised cations with similar mobilities might be eliminated. However, the separation of the derivatised products is more difficult than of the non-derivatised amines and application of MEKC is required.

Free amino acids have been determined by CZE from different matrix like beverages or soy sauce. Due to their zwitter ionic nature they can be separated in cationic mode using low pH carrier electrolytes [43] or in an anionic mode using separation buffer with pH above 10 [44]. The latter approach provides faster separations due to the high EOF but up to now the resolution of all 20 common amino acids without derivatisation has not been achieved. Using highly acidic carrier electrolytes, the selectivity differs from those obtained with high pH buffers and the 20 amino acids were successfully separated from each other applying phosphate based separation electrolyte (pH 2.4) with presence of octanesulphonic acid [43]. For the detection of the amino acids, spectrophotometry was applied at detection wavelength of 185 nm, since some amino acids do not possess a strong chromophore making their sensitive detection difficult. Thus other detection techniques like indirect UV detection [44], conductivity or mass spectrometry (MS) [45] have been used for the analysis of non-derivatised amines. CE coupled to MS gives a selective determination; it does not require baseline separation of all solutes, however, it is limited by the applicable electrolyte system which has to be volatile. Ammonia or short-chain alkyl amines can be used as high-pH carrier electrolyte and low-pH electrolytes contain mostly formic-, acetic- or trifluoroacetic acid can be regarded as suitable BGE for the purpose of separation the non-derivatised amino acids. For the determination of amino acids from infant food, triethylamine was used because of its lower volatility and an increased stability of the electrolyte concentration than applying ammonia as separation buffer [45].

Heterocyclic amines have been found in cooked food and due to their mutagenic activity their determination is an important task [46]. They contain strong chromophor thus can be detected with spectrophotometry after their CZE separation as cations present in the solution with pH of 2-3. A method was developed for the determination of 13 heterocyclic amines from salmon and meat extracts applying sodium phosphate buffer containing methanol and

sodium chloride [47]. Before the MS detection of the heterocyclic amines were also separated with buffer containing ammonium acetate at pH 3 in polyvinyl alcohol-coated capillary to prevent the adsorption of the target onto the capillary surface.

3.1.6. Phenolic compouns and vitamins

Different derivatives of phenols present naturally mostly associated to flavour and colour of the foods or as additive due to their antioxidant property. Phenolic compounds are divided into catechins, isoflavons and antocyanins [48] that are generally contains benzol ring allowing their direct spectrophotometric detection. They are mostly weak acids containing more than one hydroxyl group with pK_a value between 8 and 10 thus they can be separated at highly alkaline condition (pH 8-9) applying CZE. The developed and validated methods have been summarized in several reviews [48-52]. However, some derivatives of catechin present in food products are neutral at basic pH, therefore MEKC is preferred for their determination. Mostly borate based buffer at concentration of 20-150 mmol/L have been used as separation electrolyte [48] since borate have complex ability to α-hydroxy groups increasing the selectivity of the analytes. Concerning the analysis of polyphenol derivatives, tea, soy sauce and wine has been the most studied food samples; tea leaves may contain catechins up to 30% of dry mass and resveratrol is the most investigated derivative of polyphenols in wine and grape. Eleven catechins including epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, catechin, epicatechin and quercetin were separated with borate buffer at pH 8.5 applying an on-line coupled flow injection system used for microwave treatment [53]. Isoflavons have been determined from soy sauce with separation of borate based electrolyte. Antocyanins, a group of flavonoids, are the largest group of water-soluble natural pigments and they were also separated at basic pH, however, the sensitivity of the method was not sufficient and the analytes found to be unstable [54]. Thus anthocyanins present in berries have been separated at highly acidic (pH 1.5-2.1) condition applying phosphate based buffer [55].

Vitamins such as ascorbic acid have also antioxidant activity and they have been determined applying CE. Methods for the determination of water-soluble vitamins like riboflavin and niacin have been also developed and applied for different food samples like beverages (juice, beer, wine) or extracts of fruits and vegetables. Their capillary electrophoretic separation can be achieved at alkaline condition due to their ability of deprotionation and mainly borate or phosphate buffer have been used as BGE. Riboflavin and its related flavins, flavin mononucleotide, and flavin adenine dinucleotide are natively fluorescent which enables their detection by fluorescence overcoming the issues of selectivity and sensitivity that is an obstacle to the determination of other vitamins at trace levels in complex food matrices. The flavins are separable in CZE using a 30 mmol/L phosphate buffer with pH 9.8 and were detected by laser induced fluorescence detector [56-58]. This basic CZE method has been demonstrated as effective for determination of riboflavin in a number of

foodstuffs, including wines, vegetables, wheat flour, and tomatoes. However, other vitamins like ascorbic acid and niacin have been frequently determined by CE and reviewed elsewhere [59].

3.1.7. Carbohydrates

The main problem encountered in carbohydrate determination by CE is the neutral nature of these compounds and the absence of chromophores for the direct application of spectrophotometric detection. To overcome the problem of the absence of charge, they have been ionised by complexing with certain ions like borate or metals; ionization by increasing the pH of BGE to the strongly alkaline pH range or by chemical derivatisation. The presence of complexation agent in the BGE improving the UV-absorbance of the carbohydrates too, thus mostly borate based buffer have been applied for their determination. The pK_a values of the sugars are in the range of 12-14 thus their deprotonation take place at pH above 12. Having a separation electrolyte at this pH range with buffer capacity the choice of the buffer system is limited; phosphate is the frequently used buffer. Improving the resolution of carbohydrates and reducing the migration time, the EOF was reserved by inverting the polarity of the applied potential or by covering the negatively charged silica capillary surface with cationic surfactants as described for the determination of the LMW acids. Derivatisation is an approach to overcome the issue of absence of UV-absorbance too. Several derivatisation agents have been used like 4-aminobenzoic acid ethyl ester, 6-aminoquilonine and phenylethylamine. However, indirect UV detection was used for the determination of the sugars from beverages like orange juice. Potassium sorbate, 2,6-pyridinedicarboxylic acid, p-nitrophenol and naphthol blue-back have been used as absorbing ion in the separation electrolyte [60].

3.1.8. Proteins

Proteins extracted from milk, egg, cereal and meet can be separated by CZE based on the differences of their charge densities with use of separation electrolyte at broad pH range [61]. The most used buffer systems based on phosphate in the pH range of 1.5-8, borate at pH 8-10 and citrate at acidic pH (pH 3). Sarcoplasmic proteins from fish [62; 63] and whey proteins from milk were separated with phosphate buffer at pH 7.4 [62; 64] where the proteins are negatively charged and at pH 1.5-2.5 as cations. Whey proteins were also separated with borate based buffer at highly basic condition (pH 8-9) to differentiate the genetic variants of α-lactoglobulines [65-67]; high ionic strength at high pH increase the separation efficiency and prevent the formation of aggregates. Simple buffer system without additives is seldom used for the separation of protein extracts. The biggest problem in the capillary electrophoretic separation of the proteins is the interaction between the proteins and silanol groups present on the surface of capillary wall leading to distorted peak shapes and poor separation efficiency. However, working with highly acidic separation condition, the

electrostatic interaction is minimized because of the neutral form of the silanols; at high pH with combination of high-ionic strength may decrease the adsorption by the repulsion effect between the negatively charged proteins and silanol groups. Preventing the protein-wall interactions, polymer modifiers have been added into the BGE that are mostly cellulose based. For example natural and artificial shark fins were differentiated applying separation electrolyte containing 0.01% methylcellulose [68]. Other derivatives of cellulose like methylhydroxyethylcellulose [69; 70] and hydroxypropylcellulose [71] at volume ratio between 0.05% and 0.5% have generally been used. Recently, other type of polymers like poly(ethyleneoxide) and linear polyacrylamide were successfully applied as dynamic coating agents [72]. Both cationic and anionic polymeric additives have been assayed for the capillary electrophoretic separation of aqueous extracts of bovine and chicken muscles with BGE at pH 3-7. The protein-wall interactions were effectively minimized with polydiallyldimethylammonium chloride as cationic polyelectrolyte, but better resolution was achieved in the presence of co-additive sodium acetone sulphate. Anionic polymer, dextrane sulphate, provided high efficiencies in the separation of chicken muscle proteins that not only prevented the adsorption of the proteins but increased the selectivity of the separation. Other possibility to prevent the adsorption of proteins is the application of modified capillaries e.g. capillary masked by methylasition.

Other main task in the capillary electrophoretic separation of the proteins is the use of solubilization agents in the separation buffer. Mostly urea at high concentration (4-8 M) is used which additionally help in resolving the proteins like caseins from milk products. However organic solvents and detergents worked as well as urea in solubilizing the hydrophobic proteins [73]. Use of acetonitrile (ACN) at volume ratio of 20 % avoided the potential problems of crystallization and protein modification that are sometimes associated with urea and provide a buffer with good UV transmission properties and low viscosity.

Separation electrolyte uses sodium-based buffers generates substantial current due to the high conductivity of the sodium ions that turn limit the applied voltage. To apply higher voltage causing decreased separation time, glycine or β-alanine have been used in place of sodium that generated mostly half times less current. Wheat proteins and oat/rice prolamines have been successfully separated using these electrolytes containing 20% acetonitrile. Maize proteins have been also separated however the content of ACN had to be increased to maintain the solubility of the hydrophobic maize and sorghum proteins. Another approach to overcome the limitations of sodium based buffers is the use of isoelectric buffers. These unique compounds have an isoelectric point roughly equal to their pH in solution, thus they can buffer without the need for co-ion. Due to their amperometric nature, they produce low current even at high separation voltage favouring high resolution with short migration time. Iminodiacetic acid (IDA) and aspartic acid have been used to determine proteins from wheat, rice oat, maize and sorghum [74]. IDA based buffer containing urea, hydroxymethylethylcellulose and Tween 20 was used for separation of milk proteins to

determine the animal origin of globulines [69]. IDA has a lower pH in solution than aspartic acid that may provide fewer problems with the wall interactions and Tween 20 and other non-ionic surfactants have a discriminating effect on the retention behaviour of the bovine globulins related to the different strength of the protein-surfactant association complex.

Food samples often modify the wall of the capillary mostly by the presence proteins. The proteins might lead to fouling of the uncoated silica capillary causing increased migration times every injection of the sample. When the proteins migrate slower than the analytes, the capillary surface can be deprotonated during the rinsing to wash them away. However the determination of proteins by CE is an important task in food analysis and mostly applied for adulteration studies.

3.2. MEKC methods for food contaminants and components

MEKC is mostly applied capillary electrophoretic technique when one or more target components are neutral. Between the food components pesticides, toxins, polyphenols, derivatised amines, aminoalcohols and proteins have been determined from different food extracts. Methods developed for their determination with use of MEKC are summarized in Table 3.

3.2.1. Pesticides and toxins

Among the pesticides fungicides, triazines, urea derived and multiple pesticides have been analysed by MEKC coupled with spectrophotometric detection mostly from soft beverages. Eight widely used fungicides namely carbendazim, imazalil, methylthiophanate, o-phenylphenol, procymidone, prochloraz, triamidefon and thiabendazole were separated with BGE containing borate buffer at pH 9 and 75 mmol/L sodium cholate as surfactant [75]. The applicability have been shown through spiked food extracts from grape, tomato, lettuce and orange as shown in Figure 11 [75]. Sample stacking have been developed increasing the sensitivity of the MEKC method and successfully applied for non-spiked apple juice [76]. The separation electrolyte contained 10 mmol/L phosphate as buffer, 30 mmol/L SDS and isopropanol / isobutylalcohol function as organic modifier enhancing the selectivity of the separation. The sample stacking was achieved by salting the injected sample. Pre-concentration strategies like sweeping, stacking and modified staking with combination of MEKC have been studied for nine multiple pesticides determined from spiked carrot samples because the most problematic task in the CE analysis of food contaminant is the sensitivity of a method [14; 77]. Atrazine, a triazine type pesticide, have been determined by MEKC from juices with combination of supported liquid membrane and solid-phase extraction used for sample preparation to increase the selectivity and sensitivity of the analytical process [78].

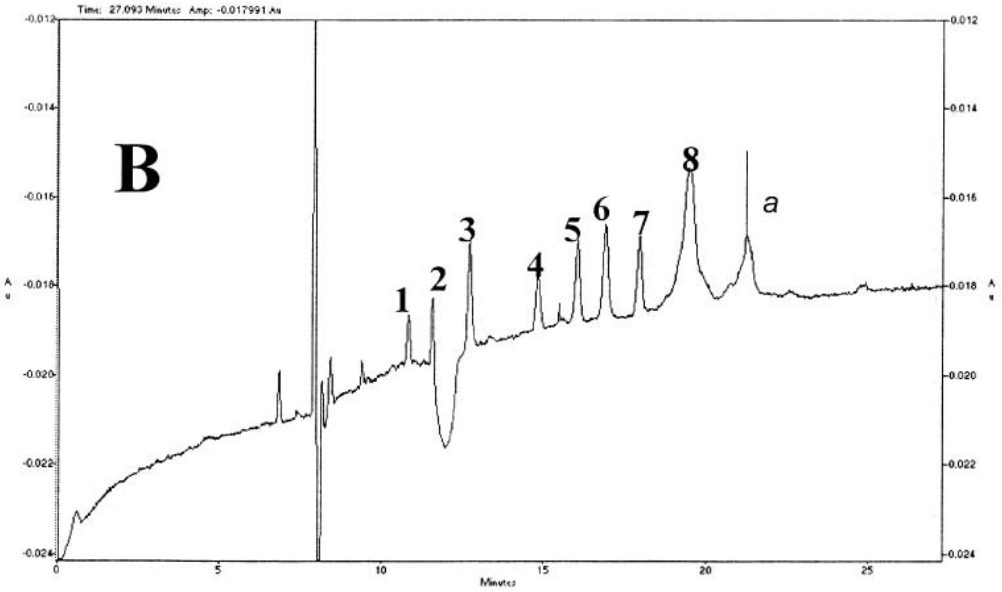


Figure 11: Electropherogram of spiked grape at 1 mg kg. Conditions: buffer: 4 mmol/L borate buffer, pH 9.2, 75 mmol/L sodium cholate; detection: 210 nm; separation voltage: 15 kV; capillary: 50 cm effective length, 75 µm I.D. Peak identification: (1) Methylthiophanate; (2) carbendazim; (3) thiabendazole; (4) procymidone; (5) *O*-phenylphenol; (6) imazalil; (7) triadimefon; (8) prochloraz. (Reproduced from [75] with permission from Elsevier© 2001).

Toxins have been determined mostly from spiked food samples or standard solutions of the targets with MEKC. A method was developed and applied for apple cider for the determination of patulin a type of mycotoxin [79]. After a single liquid-liquid extraction, the patulin were separated from the matrices applying a borate buffer with presence of SDS at pH 9. α-toxins an highly toxic metabolites of fungi like *Aspergillus* were also separated with application of SDS and cholate micelles in the separation electrolyte at highly alkaline condition from extracts of feeds and corn [80; 81].

3.2.2. Amino acids, additives and proteins

As mentioned above, amines and amino acids can be determined after their derivatisation with e.g. dansyl chloride, fluorescein isothiocyanate or 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde for improving the method sensitivity. However, the separation of the products is difficult task thus MEKC have to be used for achieving baseline separation of the targets. The derivatised amino acids are generally separated applying separation buffer with SDS or polyoxyethylene 23 lauryl ether. Seven fluorescein derivatives of amino acids were analysed from different orange juices applying borate based electrolyte containing 30 mmol/L SDS and

20 mmol/L β-CD at pH 9.4 allowing to distinguish the chiral form of the targets [82]. Method for the determination of dansyl derivatised amino acids were also developed applying similar separation electrolyte as describe above [83].

MEKC have been also frequently applied method for the determination of polyphenols from different food samples. Derivatives of catechins are neutral even at high pH and the selectivity of the method can improve by addition of SDS into the separation electrolyte as reviewed elsewhere [48; 49; 84]. Mostly, borate/phosphate buffer has been used as BGE; phosphate were used to assure the eligible current for the electrophoretic separation [85; 86].

Proteins have been also separated with MEKC, however, applying CZE result in a determination method with better performances. Major milk proteins were separated with BGE containing 3 mmol/L sodium borate buffer (pH 9.5) with SDS added at its critical micelle concentration (8.2 mmol/L). This buffer allowed rapid separation of the milk proteins with low separation time and the proteins were reported to be separated by their hydrophobicity [86].

3.3. Chiral CZE and chiral MEKC methods for food contaminants and components

Use of CE has emerged as a good alternative for enantiomer separation since several chiral selectors are available and their consumption for one separation is low. An enantiomer separation in food chemistry is useful for study of adulteration, monitoring of fermentation processes and analysis of chiral metabolites and prochiral constituents in food and beverages. Methods applying CE for chiral separation of food components are reviewed elsewhere and selected methods are shown in Table 3 [87].

3.3.1. Pesticides

Chiral determination of pesticides is an important task since organism degrade or produce chiral compounds by stereospecific enzymatic processes, thus the ratio of the chiral solutes that accumulate in food might be different as the used one. A comprehensive review of CE determination of pesticides have been published but no food as matrix was mentioned [88]. The developed methods for the chiral separation of pesticides were mostly done with BGE containing CD as chiral selector. A method was developed for the enantiomeric separation of vincozoline in wine. This fungicide was determined by MEKC applying BGE with content of SDS and γ-CD after extraction of the sample by SPE [89].

Table 3
Selected MEKC methods and chiral separation of food component

Detector	Analyte	Goals, cmment	Matrix	Ref
DAD	acrinathrin, bitertanol, cyproconazole, fludioxonil, flutriafol, myclobutanil, pyriproxyfen, tebuconazole	-pesticide analytic -comparison of SPE and SBSE	Lettuce, tomato, grape, strawberry	[135]
UV	theanin, caffeine, ascorbic acid, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate	-quality estimation of tea - characterization of fresh tea leaves according to plucking dates	green tea, oolong tea, black tea	[86]
UV	procyanidin B1, procyanidin B2, procyanidin B3 (+)-catechin, (-)-epicatechin, p- coumaric acid	-analytic of compounds with antibacterial activity	white and black beans	[85]
UV	(+)-catechin, catechin gallate, (-)-epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, theaflavin, theaflavin-3-monogallate, theaflavin-3`-monogallate, theaflavin-3,3`-gallate, caffeine, adenine, theophylline, quercetin, gallic acid, caffeic acid	-determination of tea polyphenols and tea ingredients -comparison of HPLC and CE	green and black tea	[136]
Conduct.	chloride, nitrite, nitrate, sulfate, fluoride, oxalate, malonate, pyruvate, tartrate,citrate malate, succinate, acetate, lactate, aspartate, glutamate, salycilate, gluconate, benzoate	-PMMA-microchip -determination of organoleptic characteristics of wine	Wine	[137]
ESI-MS	D- and L- amino acid	-chiral separation -derivatisation of amino acids with dansyl chloride and fluorescein isothiocyanate	Orange juices	[115]

Detector	Analyte	Goals, cmment	Matrix	Ref
UV	D,L-tartaric acid	-chiral separation -use of Cu(II)-D quinic acid as chiral selector	grape juice, red wine	[91]
DAD	naringin, pruning, narirutin, hesperidin, neohesperidin, eriocitrin	-chiral separation -analytic of flavanone-7-*O*-glycosides	lemon juice	[138]

3.3.2. Low molecular weight acids

Chiral analysis of LMW organic acids is complicated due to its short chain that can hardly makes the three-point interaction with the selector generally necessary for recognition. Target substances among the acids are mostly tartaric, malic and lactic acids applying the method mostly for food quality control. The chiral separation of these acids mostly based on ligand exchange process with chiral solutes. For the separation of D,L-tartaric acids a separation electrolyte containing cupper(II) ion and D-quinic acid at pH 5 and was applied for the determination of the targets in juices, jam and candies [90]. Direct spectrophotometric detection at selective wavelength could be used due to the light absorbing property of the complex. D,L-lactic acid have been separated from each other in yoghurt and beverages applying phosphate based separation electrolyte containing 2-hydroxypropyl-β-CD as chiral selector in coated capillary to decrease the analysis time [91].

3.4. CGE methods for food contaminants and components

Accurate quantitative determination of whey proteins was developed using cross-linked polyacrylamide gel-filled capillaries under denaturing and non-denaturing conditions, that allowed the separation of the major whey proteins with high efficiency in less than 30 min [92]. CGE using gel-filled capillaries still presents some limitations, such as time-consuming capillary preparation, poor reproducibility and short capillary lifetime. Moreover, due to the high viscosity of the gel, the injection of the sample must be performed by electromigration, which is strongly influenced by the ion concentration of the sample. To avoid these limitations, CGE using replaceable gels of polyethylene glycol (PEG) and a cross-linked polyacrylamide coated capillary was employed for the separation of whey proteins, yielding good separation between β-lactoglobulin and α-lactalbumin. In order to decrease the absorbance of the polymer, the use of polyethyleneglycol and dextran as sieving mediums has been purposed. Ready-to-use polymer solutions have recently become commercially available and have been successfully employed [93]; e.g. commercial kit for SDS-CGE (Applied Biosystems) has been applied to the analysis of whey proteins achieving baseline separations of the globulins [94].

CGE combined with PCR have been used for separation of DNA fragments to detect e.g. generally modified microorganism, food borne pathogens or toxin-producing microorganism [95]. The higher sensitivity and the potential to distinguish similar strains of the same species make PCR-CGE suitable for fast studies.

3.5. CEC methods for food contaminants and components

In the past 10 years increase amount of papers deal with CEC, although its application for determination purpose in food analytic is still rare [96]. Reversed phase CEC have been developed for the determination of 4 derivatives of phenylurea herbicides from vegetables and vegetable posessed food samples [97]. The method used capillary packed with non-end-capped C18 silica particles with average particle diameter of 5 μm at 8 cm long and a reduced amount of organic solvent in the mobile phase to increase the selectivity of the separation thus reduce the possibility of interference. Athough, matrix effect and high baseline noise was observed when spiked vegetable extracts were measured with the optimised process. Pressurized CEC with conventionally available capillary column packed with octadecylsilica particles at length of 20 cm were used for the separation of fluoroquinolones used as antimicrobial agent from extracts of spiked fish muscle [98]. To decrease the bubble formation that may generally occure during the CEC separation SDS was added into the mobile phase that reduce the surface tension at the solid-liquid interface. The mobile phase contained also triethylamine to mask the free silanol groups since at the separation pH they are deprotonised allowing ionic interaction with the analytes causing peak boardening [98]. Other solid phases have been also applied; methacrylate based monolith were used for the separation of acids from different foods like sirup or wine [99] and carotenoids were separated on silicagel with C_{30} functional group applying gradient CEC from algal extracts [100].

4.Conclusion and future trends

Capillary electrophoretic determination of the contaminants a in food became more popular because of the advantages of the technique comparing to the chromatographic techniques. However, the most of the papers describe methodological developments that is negligible to can use the method as a tool to solve specific problems. The poorer reliability and lower sensitivity could be one of the reason, although they loose their importance by the application of the “CE-way thinking”. Since the CE performances highly dependant on the physical-chemical properties of the solutes, small changes in the content of the BGE affect the separation eliminateing the possible interferences with the konwledge of the possible matrices determinable with the method optimised with standard solution of the target compounds. Thus methods applying for the determination of other food components such as LMW amines and phenols at trace level were also shortly discussed in this review. To increase the roboustness of the capillary electrophoretic separation, the separation electryole are aimed to dispose buffer capacity and phosphate have been generally used due to its low UV cut-off value and wide pH range with capacity. Changing the BGE from phosphate to borate, the separation could be highly affected if it composes complex with the analytes. However, not only the type of the electrolyte but other ingredients like organic modifier, surfactants or complax agents

have also big influence on the effective mobility of the solutes. Other possible way to increase the roubustness, is the application of mobility value not only for identification but also for quantification purpose if CZE is applied for the separation of food extracts. Increasing the method sensitivity, different concentration techniques such as sample stacking, electrokinetic injection or on-line SPE have been successfully applied for different analytes in different food matrix. Although UV-Vis detection is generally used, applying flourescence detection or coupling the CE to MS may impose more selective and sensitve method. With the help of the use of the concentration step, application of more roubust separation parameters and more selective detection systmes, the scale of the capillary electrophoretic separation could be decreased thus application of microchip in food analytic might be a promising thechnique allowing a low amount of required sample, highest determination speed and in-situ measurements.

References

[1] M.J. Desai, D.W. Armstrong, Microbiol. Mol. Biol. Rev. 67 (2003) 38.
[2] S.P. Radko, M. Stastna, A. Chrambach, Electrophoresis 21 (2000) 3583.
[3] U. Schnabel, C.H. Fischer, E. Kenndler, J. Micro. Sep. 9 (1997) 529.
[4] R.M. Fitch, Pollymer colloids: a comprehensive introduction, San Diego, CA, Academic Press, 1997.
[5] P. Schmitt-Kopplin, Capillary electrophoresis; from small ions to macromolecules, Humana Press, 2005.
[6] P. Schmitt, T. Poiger, R. Simon, A.W. Garrison, D. Freitag, A. Kettrup, Anal. Chem. 69 (1997) 2559.
[7] G. Bianco, P. Schmitt-Kopplin, G. De Benedetto, A. Kettrup, T.R. Cataldi, Electrophoresis 23 (2002) 2904..
[8] P. Schmitt-Kopplin, A.V. Garmash, A.V. Kudryavtsev, F. Menzinger, I.V. Perminova, N. Hertkorn, D. Freitag, V.S. Petrosyan, A. Kettrup, Electrophoresis 22 (2001) 77.
[9] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchija, T. Ando, Anal. Chem. 56 (1984) 111.
[10] G.C. Ping, W.B. Zhang, L.H. Zhang, P. Schmitt-Kopplin, Y.K. Zhang, A. Kettrup, Chromatographia 57 (2003) 629.
[11] G.C. Ping, Y.K. Zhang, W.B. Zhang, L. Zhang, L.H. Zhang, P. Schmitt-Kopplin, A. Kettrup, Electrophoresis 25 (2004) 421.
[12] G.C. Ping, L.H. Zhang, L. Zhang, W.B. Zhang, P. Schmitt-Kopplin, A. Kettrup, Y.K. Zhang, J. Chromatogr. A 1035 (2004) 265.
[13] G. Ping, P. Schmitt-Kopplin, N. Hertkorn, W.B. Zhang, Y.K. Zhang, A. Kettrup, Electrophoresis 24 (2003) 958.
[14] A. Juan-Garcia, G. Font, Y. Pico, J. Sep. Sci. 28 (2005) 793.
[15] D. Barron, E. Jimenez-Lozano, S. Bailac, J. Barbosa, Anal. Chim. Acta 477 (2003) 21.
[16] B. Saad, R. Mohamad, N. Mohamed, G.D. Lawrence, M.S. Jab, M.I. Saleh, Food Chem. 78 (2002) 383.
[17] D. Barron, E. Jimenez-Lozano, J. Cano, J. Barbosa, J. Chromatogr. B 759 (2001) 73.
[18] J.L. Beltran, E. Jimenez-Lozano, D. Barron, J. Barbosa, Anal. Chim. Acta 501 (2004) 137.
[19] M. Hernandez, F. Borrull, M. Calull, Electrophoresis 23 (2002) 506.

[20] T.S. Huang, W.X. Du, M.R. Marshall, C.I. Wei, J. Agric. Food Chem. 45 (1997) 2602.
[21] H. Oka, Y. Ito, H. Matsumoto, J. Chromatogr. A 882 (2000) 109.
[22] M.R.S. Fuh, S.Y. Chu, Anal. Chim. Acta 499 (2003) 215.
[23] B. Santos, A. Lista, B.M. Simonet, A. Rios, M. Valcarcel, Electrophoresis 26 (2005) 1567.
[24] R. Rodriguez, J. Manes, Y. Pico, Anal. Chem. 75 (2003) 452.
[25] A. Juan-Garcia, G. Font, Y. Pico, Electrophoresis 26 (2005) 1550.
[26] A.K. Malik, W. Faubel, Fresenius J. Anal. Chem. 367 (2000) 211.
[27] G. Bianco, P. Schmitt-Kopplin, A. Crescenzi, S. Comes, A. Kettrup, T.R.I. Cataldi, Anal. BioAnal. Chem. 375 (2003) 799.
[28] N. Pineiro, J.M. Leao, A.G. Martinez, J.A.R. Vazquez, J. Chromatogr. A 847 (1999) 223..
[29] P. Venter, J.F.R. Lues, Int. J. Food Microbiol. 84 (2003) 245.
[30] I.V. Kourkine, M. Ristic-Petrovic, E. Davis, C.G. Ruffolo, A. Kapsalis, A.E. Barron, Electrophoresis 24 (2003) 655.
[31] B. Palenzuela, B.M. Simonett, R.M. Garcia, A. Rios, M. Valcarcel, Anal. Chem. 76 (2004) 3012-3017.
[32] J. Sadecka, J. Polonsky, J. Chromatogr. A 834 (1999) 401.
[33] K. Fukushi, S. Takeda, S. Wakida, K. Higashi, K. Hiiro, J. Chromatogr. A 759 (1997) 211.
[34] A.M. Carro-Diaz, R.A. Lorenzo-Ferreria, R. Cela-Torrijos, J. Chromatogr. A 730 (1996) 345.
[35] P. Jones, S. Hardy, J. Chromatogr. A 765 (1997) 345-352.
[36] V. Galli, A. Garcia, L. Saavedra, C. Barbas, Electrophoresis 24 (2003) 1951-1981.
[37] D. Kaniansky, M. Masar, J. Marak, R. Bodor, J. Chromatogr. A 834 (1999) 133-178.
[38] Y.S. Fung, K.M. Lau, Electrophoresis 24 (2003) 3224-3232.
[39] V.C. Trenerry, Food Chem. 55 (1996) 299-303.
[40] H. Horie, Y. Yamauchi, K. Kohata, J. Chromatogr. A 817 (1998) 139.
[41] L. Saavedra, A. Garcia, C. Barbas, J. Chromatogr. A 881 (2000) 395.
[42] M. Timm, B.M. Jorgensen, Food Chem. 76 (2002) 509.
[43] C.W. Klampfl, W. Buchberger, M. Turner, G.S. Fritz, J. Chromatogr. A 804 (1998) 349.
[44] T. Soga, G.A. Ross, J. Chromatogr. A 837 (1999) 231.
[45] C.W. Klampfl, W. Ahrer, Electrophoresis 22 (2001) 1579.
[46] P. Pais, M.G. Knize, J. Chromatogr. B-Anal. Technol. Biomedi. Life Sc. 747 (2000) 139.
[47] J. Wu, M.K. Wong, H.K. Lee, B.L. Lee, C.Y. Shi, C.N. Ong, Food Add. Contam. 13 (1996) 851.
[48] M. Herrero, E. Ibanez, A. Cifuentes, J. Sep. Sci. 28 (2005) 883.
[49] J.J. Dalluge, B.C. Nelson, J. Chromatogr. A 881 (2000) 411.
[50] X.L. Gu, Q.Y. Chub, M. O'Dwyer, M. Zeece, J. Chromatogr. A 881 (2000) 471.
[51] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403.
[52] M.C. Boyce, Electrophoresis 22 (2001) 1447.
[53] L. Arce, A. Rios, M. Valcarcel, J. Chromatogr. A 827 (1998) 113.
[54] P. Bridle, C. GarciaViguera, Food Chem. 59 (1997) 299.
[55] C.T. da Costa, B.C. Nelson, S.A. Margolis, D. Horton, J. Chromatogr. A 799 (1998) 321.
[56] T.R.I. Cataldi, D. Nardiello, V. Carrara, R. Ciriello, G.E. De Benedetto, Food Chem. 82 (2003) 309.
[57] T.R.I. Cataldi, D. Nardiello, G.E. De Benedetto, S.A. Bufo, J. Chromatogr. A 968 (2002) 229.
[58] T.R.I. Cataldi, D. Nardiello, L. Scrano, A. Scopa, J. Agric. Food Chem. 50 (2002) 6643.
[59] P.F. Cancalon, Lc Gc Europe 16 (2003) 148.
[60] J. McCourt, J. Stroka, E. Anklam, Analytical and Bioanal. Chem. 382 (2005) 1269-1278.

[61] S.R. Bean, G.L. Lookhart, Electrophoresis 22 (2001) 4207-4215.
[62] E.L. LeBlanc, S. Singh, R.J. LeBlanc, J. Food Sci. 59 (1994) 1267.
[63] J.M. Gallardo, C.G. Sotelo, C. Pinerio, R.I. Perez-Martin, J. Agric. Food Chem. 43 (1995) 1238.
[64] C. Stathakis, E.A. Arriaga, N.J. Dovichi, J. Chromatogr. A 817 (1998) 233.
[65] N.M. Kinghorn, G.R. Paterson, D.E. Otter, J. Chromatogr. A 723 (1996) 371.
[66] M. deFrutos, E. Molina, L. Amigo, Milchw-Milk Sci. Int. 51 (1996) 374.
[67] J. Kim, M. Braunschweig, Z. Puhan, Milchw-Milk Sci. Int. 51 (1996) 435.
[68] S.S. Chou, S.C. Su, H.W. Shiau, D.F. Hwang, P.C. Yu, S.C. Lee, J. Food Sci. 63 (1998) 782.
[69] J.M. Herrero-Martinez, E.F. Simo-Alfonso, G. Ramis-Ramos, C. Gelfi, P.G. Righetti, J. Chromatogr. A 878 (2000) 261.
[70] A. Irigoyen, J.M. Izco, F.C. Ibanez, P. Torre, J. Chromatogr. A 881 (2000) 59.
[71] S.R. Bean, M. Tilley, Cereal Chem. 80 (2003) 505.
[72] S.R. Bean, J.A. Bietz, G.L. Lookhart, J. Chromatogr. A 814 (1998) 25.
[73] S.R. Bean, G.L. Lookhart, J.A. Bietz, J. Agric. Food Chem. 48 (2000) 318.
[74] P.G. Righetti, C. Gelfi, A. Bossi, E. Olivieri, L. Castelletti, B. Verzola, A.V. Stoyanov, Electrophoresis 21 (2000) 4046.
[75] R. Rodriguez, Y. Pico, G. Font, J. Manes, J. Chromatogr. A 924 (2001) 387.
[76] M. Molina, M. Silva, Electrophoresis 21 (2000) 3625.
[77] C.L. da Silva, E.C. de Lima, M.F.M. Tavares, J. Chromatogr. A 1014 (2003) 109.
[78] M. Khrolenko, P. Dzygiel, P. Wieczorek, J. Chromatogr. A 975 (2002) 219.
[79] R. Tsao, T. Zhou, J. Agric. Food Chem. 48 (2000) 5231.
[80] R. Pena, M.C. Alcaraz, L. Arce, A. Rios, M. Valcarcel, J. Chromatogr. A 967 (2002) 303.
[81] C.M. Maragos, J.I. Greer, J. Agric. Food Chem. 45 (1997) 4337.
[82] C. Simo, C. Barbas, A. Cifuentes, J. Agric. Food Chem. 50 (2002) 5288.
[83] C.F. Tsai, C.F. Li, H.M. Chang, J. Agric. Food Chem. 46 (1998) 979.
[84] H. Horie, K. Kohata, J. Chromatogr. A 881 (2000) 425.
[85] A. Cifuentes, B. Bartolome, C. Gomez-Cordoves, Electrophoresis 22 (2001) 1561.
[86] H. Horie, K. Kohata, J. Chromatogr. A 802 (1998) 219.
[87] C. Simo, C. Barbas, A. Cifuentes, Electrophoresis 24 (2003) 2431.
[88] Z. El Rassi, Electrophoresis 18 (1997) 2465.
[89] S. Kodama, A. Yamamoto, Y. Saitoh, A. Matsunaga, K. Okamura, R. Kizu, K. Hayakawa, J. Agric. Food Chem. 50 (2002) 1312.
[90] S. Kodama, A. Yamamoto, A. Matsunaga, K. Hayakawa, J. Chromatogr. A 932 (2001) 139.
[91] S. Kodama, A. Yamamoto, A. Matsunaga, T. Soga, K. Minoura, J. Chromatogr. A 875 (2000) 371.
[92] A. Cifuentes, M. de Frutos, J.C. Diez-Masa, Am. Lab. august (1994) 46.
[93] D.B.L. Gomis, S. Junco, Y. Exposito, D. Gutierrez, Electrophoresis 24 (2003) 1391.
[94] N.M. Kinghorn, C.S. Norris, G.R. Paterson, D.E. Otter, J. Chromatogr. A 700 (1995) 111.
[95] V. Garcia-Canas, R. Gonzalez, A. Cifuentes, Trac-Trends Anal. Chem. 23 (2004) 637.
[96] S. Eeltink, W.T. Kok, Electrophoresis 27 (2006) 84.
[97] A. De Rossi, C. Desiderio, Chromatographia 61 (2005) 271.
[98] H.X. Lu, X.P. Wu, Z.H. Xie, X.C. Lin, L.Q. Guo, C. Yan, G.N. Chen, J. Sep. Sci. 28 (2005) 2210.
[99] H.Y. Huang, C.W. Chiu, I.Y. Huang, J.M. Yeh, Electrophoresis 25 (2004) 3237.
[100] L.C. Sander, M. Pursch, B. Marker, S.A. Wise, Anal. Chem. 71 (1999) 3477.
[101] F. Kvasnicka, J. Sep. Sci. 28 (2005) 813.

[102] R.A. Frazier, A. Papadopoulou, Electrophoresis 24 (2003) 4095.
[103] Y.Y. Dong, Trends in Food Sci, Technol.10 (1999) 87.
[104] R.A. Frazier, Electrophoresis 22 (2001) 4197.
[105] S. Cortacero-Ramirez, M.H.B. de Castro, A. Segura-Carretero, C. Cruces-Blanco, A. Fernandez-Gutierrez, Trac-Trends Anal. Chem. 22 (2003) 440.
[106] G. Castaneda, J. Rodriguez-Flores, A. Rios, J. Sep. Sci. 28 (2005) 915.
[107] T. Watanabe, S. Terabe, J. Chromatogr. A 880 (2000) 295.
[108] Y. Pico, G. Font, J.C. Molto, J. Manes, J. Chromatogr. A 885 (2000) 251.
[109] F.E. Ahmed, Trac-Trends Anal. Chem. 20 (2001) 649.
[110] F.E. Ahmed, Trac-Trends Anal. Chem. 22 (2003) 170.
[111] C.M. Montero, M.C.R. Dodero, D.A.G. Sanchez, C.G. Barroso, Chromatographia 59 (2004) 15.
[112] T. Watanabe, S. Terabe, J. Chromatogr. A 880 (2000) 311.
[113] V.C. Trenerry, Electrophoresis 22 (2001) 1468.
[114] I. Molnar-Perl, J. Chromatogr. A 891 (2000) 1.
[115] C. Simo, A. Rizzi, C. Barbas, A. Cifuentes, Electrophoresis 26 (2005) 1432.
[116] I. Recio, M. Ramos, R. Lopez-Fandino, Electrophoresis 22 (2001) 1489.
[117] R. Rodriguez, Y. Pico, G. Font, J. Manes, J. Chromatogr. A 949 (2002) 359.
[118] J. Hernandez-Borges, M.A. Rodriguez-Delgado, F.J. Garcia-Montelongo, A. Cifuentes, Electrophoresis 25 (2004) 2065.
[119] M. Masar, M. Dankova, E. Olvecka, A. Stachurova, D. Kaniansky, B. Stanislawski, J. Chromatogr. A 1026 (2004) 31.
[120] N. Oztekin, M.S. Nutku, F.B. Erim, Food Chem. 76 (2002) 103.
[121] A.L. Cinquina, F. Longo, A. Cali, L. De Santis, R. Baccelliere, R. Cozzani, J. Chromatogr. A 1032 (2004) 79.
[122] J. Lange, K. Thomas, C. Wittmann, J. Chromatogr. B-Anal. Technol. Biomed. Life Sci. 779 (2002) 229.
[123] L. Arce, A. Rios, M. Valcarcel, J. Chromatogr. A 803 (1998) 249.
[124] S.P. Li, Z.H. Song, T.T.X. Dong, Z.N. Ji, C.K. Lo, S.Q. Zhu, K.W.K. Tsim, Phytomedicine 11 (2004) 684.
[125] D.H. Craston, P. Farnell, J.M. Francis, S. Gabriac, W. Matthews, M. Saeed, I.W. Sutherland, Food Chem. 73 (2001) 103.
[126] S.P. Wang, K.J. Huang, J. Chromatogr. A 1032 (2004) 273-279.
[127] F. Regan, Y. Shakalisava, Anal. Chim. Acta 540 (2005) 103.
[128] L.Y. Gao, Q.C. Chu, J.N. Ye, Food Chem. 78 (2002) 255.
[129] M. Scampicchio, J. Wang, S. Mannino, M.P. Chatrathi, J. Chromatogr. A 1049 (2004) 189.
[130] C. Simo, C. Elvira, N. Gonzalez, J.S. Roman, C. Barbas, A. Cifuentes, Electrophoresis 25 (2004) 2056.
[131] M.T. Veledo, M. de Frutos, J.C. Diez-Masa, J. Sep. Sci. 28 (2005) 935.
[132] G. Cartoni, F. Coccioli, R. Jasionowska, M. Masci, J. Chromatogr. A 846 (1999) 135.
[133] B. Miralles, V. Rothbauer, M.A. Manso, L. Amigo, I. Krause, M. Ramos, J. Chromatogr. A 915 (2001) 225.
[134] J.J. Dooley, H.D. Sage, M.A.L. Clarke, H.M. Brown, S.D. Garrett, J. Agric. Food Chem. 53 (2005) 3348.
[135] A. Juan-Garcia, Y. Pico, G. Font, J. Chromatogr. A 1073 (2005) 229.
[136] B.L. Lee, C.N. Ong, J. Chromatogr. A 881 (2000) 439.

[137] M. Masar, K. Poliakova, M. Dankova, D. Kaniansky, B. Stanislawski, J. Sep. Sci. 28 (2005) 905.
[138] N. Gel-Moreto, R. Streich, R. Galensa, J. Chromatogr. A 925 (2001) 279.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 16

Sensor, biosensors and MIP based sensors

Marinel·la Farré, Damià Barceló

Environmental Chemistry, IIQAB-CSIC, Jordi Girona, 18, S-08034 Barcelona

1. Introduction

Quality control analysis is an essential tool in food industry. Consumers expect adequate quality at a given price, good shelf-life and high safety, while food regulations require good manufacturing practices, safety and labeling. Food producers are increasingly asking for efficient control methods, in particular through on-line analytical devices, firstly to satisfy the consumer and regulatory requirements, and secondly to improve the production processes and reduction of the costs and production time.

Additionally, many new food safety concepts and key quality parameters have arisen during the last decade:

- Hazard Analysis Critical Control Points (HACCP)
- Total Quality Management (TQM)
- ISO 9000 Certifications, traceability, and authentication all require improved control methods.

In order to satisfy consumers, authorities, and food producers, analyses have to be carried out upon delivery of raw material to the manufacturers, during the food production, and prior to deliver the product to the consumers. Because of these reasons, there is a need for better diagnostic and real-time analytical methods beyond the existing on-line technologies, like control of weight, volume, temperature, pH, viscosity, color and appearance.

Pathogenic microorganisms, which can produce diverse diseases such as the Bovine Spongiform Encephalopathy (BSE); genetically modified organisms (GMOs), and many food toxicants including pesticides and mycotoxins require an intensive control.

For all these reasons, a significant number of rapid analytical methods have been described for food toxicants analysis: immunoassays, polymerase chain reaction (PCR) techniques, nucleic acid based assays, sensors and biosensors. Sensors technology is a powerful alternative to conventional analytical techniques.

Chemical sensors are based on the interaction of the analyte with a chemically sensitive layer immobilized on a base device. The main chemical sensors classes are: chromatographic, spectroscopic, electrochemical, mass, and optical.

Biosensors are analytical devices composed by a biological recognition element coupled to a chemical or physical transducer. The basic principle is to convert a biologically induced recognition event into a usable signal. In order to achieve this, a transducer is used to convert

the (bio) chemical signal into an electronic one, which can be processed in some way, usually with a microprocessor.

The biological recognition element can be enzymes, antibodies, whole cells including microbial, plant and animal cells, sub-cellular organelles, tissue slices, several plant glycoproteins that act like antibodies, and lately biomimics (synthetic molecules with similar affinity to biological ones). These biological recognition systems have been linked to electrochemical, optical-electronic, optical, and acoustic transducers.

Numerous components and technologies are required to obtain functional biosensors, which can be manufactured within the necessary performance and cost constraints. The advances in microelectronics, which have allowed the miniaturization of equipments; the development of semi-permeable membranes; the screen-printing processes that makes possible to produce a large number of reproducible devices; the development of photolithography, and the progress on liquid handling techniques, must be stood out.

One key step in the development of biosensors is the immobilization of the biological component at the transducer surface. The immobilization should reach the stabilization of the biomaterial, and the optimal proximity between the biomaterial and the transducer. The immobilization methods most generally employed are: physical adsorption at a solid surface, cross-linking between molecules, covalent binding to a surface, and entrapment within a membrane, surfactant matrix, polymer or microcapsule. In addition to these conventional methods, sol–gel entrapment, Langmuir–Blodgett (LB) deposition, electro polymerization, self-assembled biomembranes and bulk modification have been recently used.

Another important factor for the commercial successful development of biosensors is the possibility of the large-scale production of reproducible sensors at cost-effective prices. In this sense, screen-printing processes have permitted the mass production of inexpensive disposable sensors. In general a screen printed electrode is composed by a conductive silver strip printed onto a PVC or glass substrate covered by a carbon electrode layer, which is covered by a polymer dielectric layer which masks the conductive strip and exposes a defined area of the carbon layer. Using screen-printing technology the *memory effect* between one sample to another is avoided, and, the phenomenon referred to as *electrode fouling*, which is one of the main drawbacks of the electrochemical sensors, is overcome. Furthermore, these disposable sensors are characterized by high reproducibility and do not require calibration. For example the Abbott pocketsize glucose-sensing device relies on such methodology.

Sensor and biosensor applications cover a wide area of diverse techniques according to the most important food production quality parameters such as:

- Sensory quality: appearance, flavor, taste, texture, stability etc.
- Nutritional quality (percentage of fiber, cholesterol, etc)
- Health implications, such as GMOs or food allergens
- Pollutants
- Veterinary drugs

- Agricultural chemicals
- BSE-prions and mycotoxins
- Foreign body detection, such as glass or metal
- Microbial safety: *Listeria, Salmonella*, *Campylobacter*, *E. Coli* and *Yersinia*
- HACCP, traceability and authentication (from 2005)
- Process parameters: machine settings, temperature, pressure, flow, aseptic conditions and many physical parameters
- Package control: integrity, pin holes, gas permeability, migration control

The potentially huge of biosensors market can be attributed to the interest for the reliable detection of trace quantities of additives, contaminants and other chemicals. One of the main advantages of the biosensors over other kinds of sensors is their specificity of response and, in some cases, their ability to work in very complex matrices, such as food samples. Biosensors specific for food testing are now available on the market. However, many devices are still in development. In particular, the commercial success of biosensors is limited to a small number of applications, in which the potential market size justified the research and development investment. Immunosensors and other affinity sensors offer an ultra-low detection limit and electrochemical detection may be readily integrated with chromatographic techniques to yield user-friendly devices. However, at present less than 10% of this market has been exploited with two-thirds of sales accounted by glucose and BOD sensors.

On the other hand, in spite of the high specificity of biological materials, they have some limitation such as poor stability in front aggressive conditions, matrix effects, and sometimes lack of reproducibility between different batches of production, due to a wide labor of development have been done during recent years looking for new sensing materials. This is the case on molecularly imprinted polymers (MIPs), one of the biomimetic materials lately developed, which can overcome these limitations, offering a very good specificity without limitation of molecular weight or size.

This chapter deals with sensors and biosensors recently developed for determining analytes implicated in food safety. The chapter covers the fundamental principles and types of sensor formats, describes food specific applications in chemical and microbiological contaminants analysis, includes examples of commercial instrumentation and kits available, and indicates future development in the sensor field

2. Chemosensor and Biosensors: Basis and fundamentals

A chemosensor is composed by two main parts, the first is where the selective chemistry occurs and the second is the transducer. The chemical reaction produces a signal such as color change, fluorescence, o change in the oscillation frequency of a crystal, and the transducer translates the physico-chemical event into a recognizable physical signal. The majors regions of a typical chemosensor are shown in Figure 1.

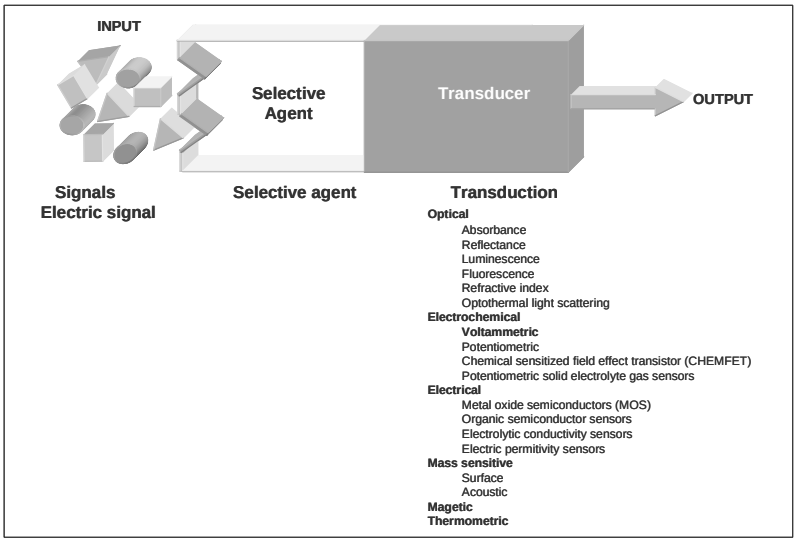


Figure 1. Principle of a sensor

Biosensors can be defined as an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element. A biosensor is converts a biological event into a detectable signal by the action of a transducer and a processor.

Biosensors use biologically-derived components integrated with a suitable transducer. Due to the development and implementation of biosensors has been narrowly related to the sensors technology advances. A general scheme of a biosensor device is shown in figure 2.

Biosensors are usually classified into various basic groups according either to the method of signal transduction, or to the biorecognition principle.

The main transduction elements involved in chemical sensors and biosensors are coincident, and it will be summarized in this section. Whereas, the basis of recognition elements, classification and food application of biosensors specifically will be developed in section 4.

Chemical sensors and biosensors can be classified according to the physicochemical transduction:

Optical sensors are based on various technologies of optical phenomena, which are the result of an interaction of an analyte with the receptor part. This group may be further subdivided according to the type of optical properties which have been applied in chemical sensors:

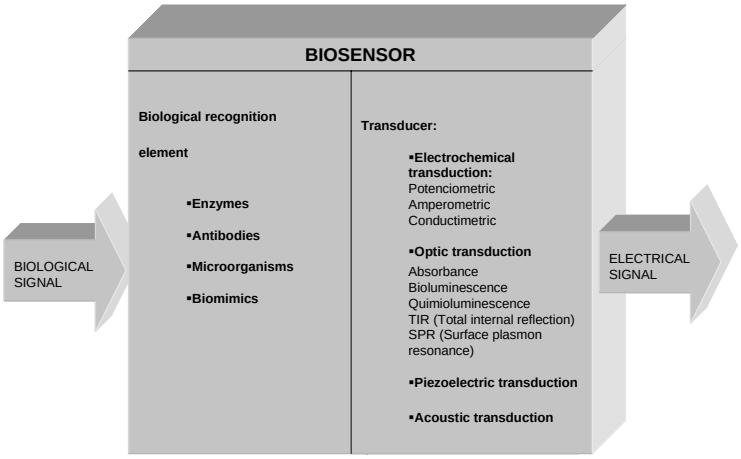


Figure 2. A general scheme of a biosensor device.

- Absorbance, measured in a transparent medium, caused by the absorptivity of the analyte itself or by a reaction with some suitable indicator.
- Reflectance is measured in non-transparent media, usually using an immobilized indicator.
- Luminescence, based on the measurement of the intensity of light emitted by a chemical reaction in the receptor system.
- Fluorescence, measured as the positive emission effect caused by irradiation. Also, selective quenching of fluorescence may be the basis of such devices.
- Refractive index, measured as the result of a change in solution composition. This may include also a surface plasmon resonance effect (SPR).
- Optothermal effect, based on a measurement of the thermal effect caused by light absorption.
- Light scattering, based on effects caused by particles of definite size present in the sample.

The application of many of these phenomena in sensors became possible because of the use of optical fibers in various configurations. Such devices have also been called optodes.

Electrochemical devices transform the effect of the electrochemical interaction between an analyte and the electrode into a primary signal. Such effects may be stimulated electrically or may result in a spontaneous interaction at the zero-current condition. The following subgroups may be distinguished:

- Voltammetric sensors, including amperometric devices, in which current is measured in the d/c or a/c mode. This subgroup include sensors based on

chemically inert electrodes, chemically active electrodes and modified electrodes, which in turn can be with or without (galvanic sensors) external current source.

- Potentiometric sensors, in which the potential of the indicator electrode (ion-selective electrode, redox electrode, metaVmeta1 oxide electrode) is measured against a reference electrode.
- Chemically sensitized field effect transistor (CHEMFET) in which the effect of the interaction between the analyte and the active coating is transformed into a change of the source-drain current.
- Potentiometric solid electrolyte gas sensors, differing from potentiometric sensors because they work at high temperature solid electrolytes and are usually applied for gas sensing measurements.

Electrical sensors are based on measurements, where no electrochemical processes take place, but the signal arises from the change of electrical properties caused by the interaction of the analyte.

- Metal oxide semiconductor (MOS) sensors used principally as gas phase detectors, based on reversible redox processes of analyte gas components.
- Organic semiconductor sensors, based on the formation of charge transfer complexes, which modify the charge carrier density.
- Electrolytic conductivity sensors.
- Electric permittivity sensors.

Mass sensitive sensors transform the mass change at a specially modified surface into a change of a property of the support material. The mass change is caused by accumulation of the analyte.

- Piezoelectric devices used mainly in gaseous phase, but also in solutions, are based on the measurement the frequency change of the quartz oscillator plate caused by adsorption of a mass of the analyte at the oscillator.
- Surface acoustic wave devices depend on the modification of the propagation velocity of a generated acoustical wave affected by the deposition of a definite mass of the analyte.

Magnetic devices based on the change of paramagnetic properties of a gas being analyzed. These are represented by certain types of oxygen monitors.

Thermometric sensors based on the measurement of the heat effects of a specific chemical reaction or adsorption which involve the analyte. In this group, the heat effects may be measured in various ways, for example in the catalytic sensors the heat of a combustion reaction or an enzymatic reaction is measured by use of a thermistor.

Other physical properties as for example X-, β- or Γ- radiation may form the basis for a chemical sensor in case they are used for determination of chemical composition.

The main transduction elements in food analysis sensors are electrochemical devices, such as ion-selective electrodes (ISE), ion-selective field effect transistors (FET), solid electrolyte

gas sensors and semiconductor based gas sensors. Piezoelectric transduction based on surface acoustic waves (SAW) are the next option, being quartz the most widely used piezoelectric material, followed by optical sensors, such as optical fibers, as well as the more traditional absorbance, luminescence and surface plasmon resonance (SPR) techniques and finally thermal systems.

3. Chemosensors

3.1. pH sensors

pH is an important control parameter as one of the factors controlling both, food processing operations and product stability. pH monitoring in food production let know whether the conditions are favorable for the growth of pathogens or for obtaining safe food. This is valuable information, because some of the stronger toxins in food (Aflatoxins, Ochratoxin A, Zearalenone, etc...) are produced by pathogen microorganisms.

Traditional potentiometric pH glass electrodes have a number of disadvantages, particularly for food industry applications, such as the fragility of the liquid filled glass membrane, because of the possibility of any glass fragments entering to the product in the case of electrode breakage, toxicity of filling solutions, laborious cleaning and recalibration, disability to dectect pH gradients or high pH pockets (assessment of microbial hazard), incompatibility for work at elevated temperatures, and slow response times. Alternative pH sensors, such as the electrodes based on electronically conducting metal oxides, microelectronics semiconductor devices, fiber-optic chemical sensors and magneto elastic chemical sensors, are being developed to overcoming these problems.

Metal oxide could be used to develop a pH electrode with wide application at high temperature and pressure [1]. This type of electrodes are based on the use of films of conducting oxides such as PtO_2, IrO_2, W_2O_3, Pb/PbO_2, Ru/RuO_2, Ir/IrO_2 or Sb_2O_3 produced either on their native metal or on another metal. The pH sensitivity of these oxides has been attributed to hydroxyl groups at the surface. Most of the work in this area has been focused on the Ir/IrO2 system because its good stability over a wide pH range and environmental conditions (high temperature and/or high pressure, aggressive environment, etc..) and exhibit fast response even in non-aqueous solutions [2]. Using film deposition techniques these sensors can be miniaturized and integrate with other sensors. However, these metal oxides are also sensitive to reducing or oxidizing agents, which constitutes a serious drawback in many applications [3].

Another important class of pH sensors is based on semiconductor materials. Different types of these devices have been developed such as field effect transistor (FET) and ion-sensitive field effect transistor (ISFET).

FETs are pre-amplifier devices. These pH sensors act converting a high-impedance signal into a low-impedance signal, which can then be processed conveniently. In a first step FET are incorporated into ion selective electrodes to amplify the signal at source. Next step is the modification of the FET to act as a sensor using the ion sensitive oxides such as SiO_2, Ta_2O_5 or Al_2O_3. An ISFET probe can be configured with a micro-tip to identify small regions of pH values, in order to check if there are favorable conditions for the pathogen growth or not. ISFET probes are constructed with strong materials, can be make in very small sizes, and it have low impedances and fast response, being one an important advantage for their implementation in flow injection analysis (FIA) systems. A number of ion sensitive oxides and films have been developed for pH sensing [4 , 5].

One of the last improvement introduced has been focused to the FIA systems [6]. Some of them use sophisticated technology, such as a time-of-flight-type flow-velocity sensor employing the in-situ electrochemically generation of ion-tracers [7]. This sensor consists of an ion generator and two downstream-placed pH-sensitive Ta_2O_5-gate ISFETs (ion-sensitive field-effect transistor) that detect generated H^+ or OH^- ions.

The potential advantages of the ISFET for food applications, especially for meat industry, have been well known during the last 25 years. Different configurations and applications are nowadays commercially available. ISFETs are characterized for the fast response time, specific multi-ion sensing and compact devices. Different configurations have been developed, as a Complementary Metal Oxide Semiconductor (CMOS) coupled to an ISFET has been used to create a complete system-on-chip digital pH meter, capable of real-time drift compensation [8].

Fiber-optic probes are another relevant group of pH sensors. This kind of devices is based on a fiber tip, which carries a reagent that exhibits a reversible change in an optical property such as color, absorbance, reflectance, etc.., when a change in pH is produced [9, 10]. One of the main advantages of fiber-optic pH sensing is the immunity to electromagnetic noise.

The last group of pH sensors is composed by the magneto elastic pH sensor [11, 12]. These devices consist of a magneto elastic thick film coated with a mass-changing pH-responsive polymer. In response to a magnetic query field, the magneto elastic sensor mechanically vibrates at a characteristic frequency that is inversely dependent on the mass of the attached polymer layer. The vibrations of the magneto elastic sensor launch a magnetic flux that can be detected remotely using a pickup coil. Similar remote devices have been developed for viscosity [13] and pressure [14] measurements.

3.2. Electronic noses

This group of techniques was originally developed for quality control analysis and quality classification, nevertheless during the last years there has been an extensive development and different devices has been constructed for the detection and quantification of toxins

originating in pathogens, rancidity substances, and migration products from packaging materials.

Electronic noses are inspired by mammalian olfactory system. Figure 3 illustrates the basic components of the human system in comparison with the electronic noses.

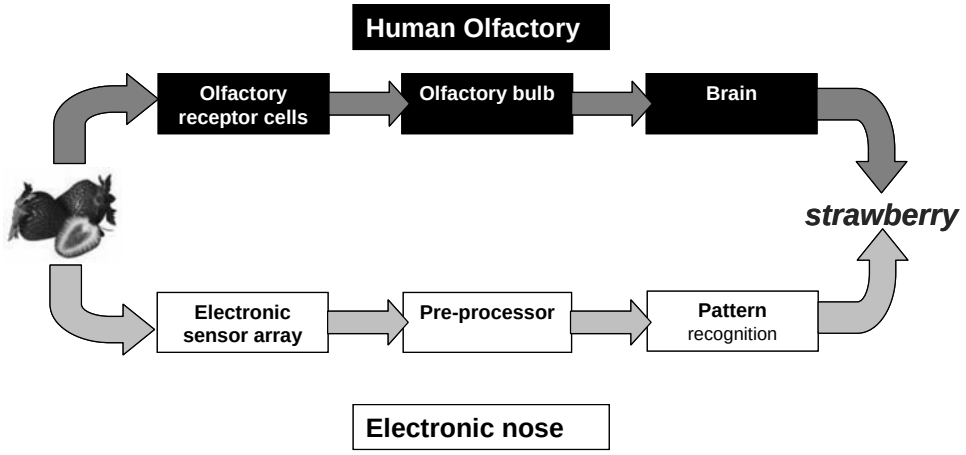


Figure 3: Basic components of the human olfactory system in comparison with the electronic noses.

Current electronic noses systems employ fewer than fifty sensors and are still far removed from the full complexity of the human nose with its 10^7 to 10^8 receptors and apart of the central nervous system for signal processing. However, the application-specific-electronic-nose (ASEN) with sensors and algorithms specialized for a specific application offers a cost-effective option for the on-line monitoring of flavor. Figure 4 shows a scheme of the main components of the ASEN system of odor detection.

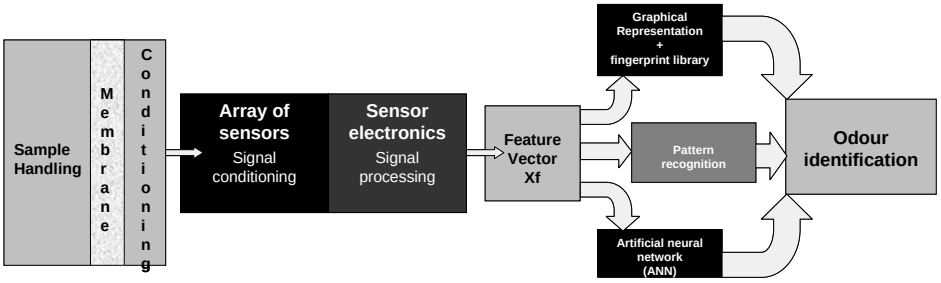


Figure 4: General scheme of the main components of the application-specific-electronic-nose (ASEN) system of odor detection.

A basic system comprises the delivery system which provides the sample to the sensor array. The array of sensors includes various sensors using one or more types of transduction.

The electronic noses employ sensor arrays where each sensor is non-specific, similarly to olfactory neurons in human system.

The sensors most frequently employed are semi conducting metal oxides (for example, SnO_2 or Ga_2O_3), and electronically conducting polymers (such as polypyrroles, polysiloxanes and polythiophenes). Both have sensitivities in the mg/L range, and both can be configured as simple resistor structures, or even they can be incorporated into electronic devices such as chemically sensitive field effect transistors (CHEMFETs) with the advantage of a low-impedance pre-amplified output.

The sensing materials nowadays in development include the adsorbent polymers, lipid membranes, zeolites and perovskites [15]. These materials are deposited on acoustic devices to provide the detection of the interaction with the adsorbed or absorbed volatile substances. The main acoustic devices used are quartz microbalance (QMB) and the surface acoustic wave (SAW).

A combination of different transducers in a hybrid sensor can be employed within an application-specific electronic nose (ASEN). Thereby the molecular receptive range (MRR) can be widened, and different aspects of the same compound can evaluated. In some applications, it can also be included biosensors.

Finally, the signal is processed and evaluated and an in general a vectorial response obtained. This vector is compared to a fingerprint library of odors. Moreover, characterized vectors for a population of odor samples can be assessed using pattern recognition techniques (PARC). Each sampled odor can be described by its principal and then can be identified.

The pattern interpretation can be carried out using an artificial neural network (ANN) [16]. Neural networks can be combined with fuzzy logic [17, 18] into neuro-fuzzy systems [19]

During last years, an intense development has been noted. Different advances have been done, for example in the field of CHEMFET new systems based on GaAs substrate instead of Si [20] have been developed. Different works have also been addressed to optimize sensing materials [20, 21]. The latest improvements have been based on nano-materials [22] and a wide range of applications have been published. Table 1 summarizes some of the latest application to electronic nose to food analysis.

3.3. Electronic tongues

The concept of electronic tongues has developed rapidly during recent years due to their potential screening analysis in on-line monitoring of cleaning industrial processes, or microorganisms contamination.

Table 1. Applications of electronic noses to food analysis.

Aplication of electronic nose	Ref.
Characteristics of gamma-irradiated salted and fermented anchovy sauce	[23]
Ripening of Danish blue cheese	[24]
Ripening of cheese	[25]
Freshness evaluation of cod fish	[26]
Microbial quality classification of grains	[27]
Quality assessment of modified atmosphere packaged poultry meat	[28]
Quality control of medicinal plants with an electronic nose	[29]
Monitoring of milk rancidity	[30]
Detection of mastitis milk	[31]
Evaluation of oranges and apples	[32]
Evaluation of interaction of active packaging materials	[33]
Analysis of volatiles from printing inks on assorted plastic films	[34]
Fast detection of rancidity in potato crisps	[35]
Detection and quantification of ochratoxin A and deoxynivalenol in barley grains	[36]
Characterization of vegetable oils	[37]
Classification of white wine aromas	[38]
Characterization of olive oil	[39]

The human tongue responds to five kinds of basic tastes: sour, bitter, salty, sweet and umami. The tastes are received by the biological membrane of gustatory cells in taste buds on the tongue. The tongue responds to a conjunct of substances without differentiation between the different compounds.

A sensor responding to the taste qualities was developed by Toko [40] who designed a multi-channel electronic taste-sensing system with devices carrying lipid-polymer membranes. The system was tested in standard solutions and in foods obtained good correlation with the human sense.

After the first achievements [23, 41] feasible efforts have been made for the development of electronic tongues. The first group of devices was based in lipids dispersed in PVC matrix [23, 42]. The second taste sensors group was based on chalcogenides glasses and metalloporphyrins films as transducers through potentimetry [24], a third type was based on

pulsed cyclic voltammetry using six working electrodes (Au, Ir, Pd, Pt, Rh, Re), and a silver counter electrode [43].

More recently, an integrated taste sensor based on the light-addressable potentiometric sensing (LAPS) principle has been used. The LAPS principle has already been used to produce gas sensors, pH sensors, etc.. The LAPS technique is a contact less method leading to a simple structure of multiple sensor systems on a single semiconductor chip. The LAPS method allows a patterning flexibility for the chemically sensitive layers since any position on the chip is accessible by scanning the interrogating light beam with high spatial resolution. The reference electrode and chemically sensitive films on the chip are in contact with the sample solution.

Due to the interaction between the sample solution and the sensor surface, the semiconductor surface potential changes. A modulated photon beam irradiating the silicon surface generates a photocurrent in the surface depletion layer. This photocurrent yields a photo voltage corresponding to the depletion layer impedance. The photo voltage is picked up by a lock-in amplifier. The surface potential map is obtained by scanning the light beam along the semiconductor surface. The potential distribution caused by the interaction between the sample and the sensitive films on the chip provides a two dimensional chemical information map corresponding to the distribution of the chemically sensitive films on the chip. In table 2, some applications of the electronic tongues are summarized.

Table 2. Applications of electronic tongues to food analysis.

Aplication of electronic tongues	**Ref.**
Classification of beverages	[44]
Rapid off-line monitoring of batch *Escherichia coli* fermentations	[45]
Differentiation of four *Aspergillus* species and one *Zygosaccharomyces*	[46]
Recognition of six microbial species	[47]
Classification of wine	[48]

4. Biosensors

A general introduction to the main transduction elements have been treated in section 17.2, therefore in the present section we will just short discuss the main transduction approaches for food biosensors development.

4.1. Transduction elements in food biosensors

The main transduction elements in biosensors developments have been:

- Electrochemical
- Calorimetric
- Optical
- Acoustic

Electrochemical biosensors are based on monitoring electro active species that are produced or consumed by the action of the biological elements (e.g., enzymes), and can be performed basically under potentiometric and amperometric measurements.

Amperometric based devices have been widely reported coupled to enzymes for the detection and quantification of pesticides residues in food and water, see Table 3.

The principle operation of amperometric biosensors is defined by a constant potential applied between a working and a reference electrode. The imposed potential promotes a redox reaction, which produces a current. The magnitude of this current is proportional to the concentration of electro active species present in solution.

Oxidase enzymes have been the most frequently investigated and applied. A number of amperometric biosensors are based on the monitoring of oxygen consumption, or the hydrogen peroxide generation. Both are electrochemically active; oxygen can be electrochemically reduced, and hydrogen peroxide can be oxidized. The current generated is proportional to the concentration of the enzyme substrate present in a sample.

The use of mediators should permit to replace oxygen as an electron acceptor and to operate at a much lower potential, reducing the effects of other electrochemically active species found in many food matrices [49].

Potentiometric biosensors are based on the monitoring of the potential at a working electrode, with respect to a reference electrode. The potentiometric measurements are related to the analyte activity. Some examples of potentiometric biosensors applied to food analysis have been reported, such as the quantification of sucrose in soft drinks [50], urea levels in milk [51], and pesticides analysis (table 3). A potentiometric enzyme biosensor for the direct measurement of organophosphate pesticides was described by Mulchandani et al. [52], based on a pH electrode modified with an immobilized organophosphorus hydrolase.

Calorimetric Biosensors. These biosensors detect variations of heat during a biological reaction. In the field of food analysis. Several examples of it application in biosensors for food analysis have been reported. Thavarungkul et al. [53] described a thermometric biosensor for the determination of sucrose in sugar cane using invertase immobilized on a thermistor.

Optical Biosensors. A high number of optical transduction techniques can be used for biosensor development. These may employ linear optical phenomenon, including adsorption, fluorescence, phosphorescence, polarization, rotation, interference, etc. or non-linear phenomena, such as second harmonic generation.

Total internal reflection fluorescence (TIRF) has been used with planar and fiber optic waveguides as signal transducers in a number of reported biosensors. In these transducers, light is propagated down a waveguide which generates an electromagnetic wave (evanescent wave) at the surface of the optically denser medium of the waveguide and the adjacent less optically dense medium. The amplitude of the standing wave decreases exponentially with distance into the lower refractive index material. The fluorescence of a fluorophore excited within the evanescent field can be collected.

Surface plasmon resonance (SPR) biosensors are optical sensors exploiting special electromagnetic waves-surface plasmon-polaritons-to probe interactions between an analyte in solution and a biomolecular recognition element immobilized on the SPR sensor surface. A surface plasmon wave can be described as a light-induced collective oscillation in electron density at the interface between a metal and a dielectric. At SPR, most incident photons are either absorbed or scattered at the metal/dielectric interface and, consequently, reflected light is greatly attenuated. The resonance wavelength and angle of incidence depend upon the permittivity of the metal and dielectric.

The general advantages of optical techniques involve the speed and reproducibility of the measurement, and the main drawback is the high cost of the apparatus.

Acoustic Biosensors. The vibration of piezoelectric crystals produces an oscillating electric field in which the resonant frequency of the crystal depends on its chemical nature, size, shape and mass. By placing the crystal in an oscillating circuit, the frequency can be measured as a function of the mass. When the change in mass (m) is very small compared to the total mass of the crystal, the change in frequency (f) relates to m as follows:

$$\text{delta } f = Cf^2 \text{ delta } m / A$$

where f is the vibration frequency of the crystal in the circuit, A is the area of the electrode and C is a constant determined in part by the crystal material and thickness. Piezoelectric crystals, sometimes referred to as a quartz crystal microbalances (QCM), are typically made of quartz and operate at frequencies between 1 and 10 MHz. These devices can operate in liquids with a frequency determination limit of 0.1 Hz, the detection limit of mass bound to the electrode surface is about 10^{-10} to 10^{-11} g. Limitations for this transduction method involve format and calibration requirements.

Biosensors based on acoustic transduction have been mainly used for the detection of pathogen microorganisms such as *Escherechia coli* [54], other examples of applications describes the use of an acoustic sensor to detect genetically modified organisms (GMO) [55].

Table 3
Example of enzyme based biosensors for food toxicant analysis

Analyte	Biosensor: type and basis	Matrix	Sensitivity	Ref
2,4-Dichlorophenoxyacetic Acid	AchE inhibition amperometric detection	Milk	1.10-11– 5.10-7 M	[56]
Carbamates	Choline oxidase, AChE and acetylcholine. Amperometric detection	Fruit and vegetables	1.10-8- 4.10-7 M	[57]
Carbamates	AchE, BChE. Potentiometric detection	Synthetic samples	1.5.10-5– 2.5.10-3 M	[58]
Carbaryl	AchE inhibition amperometric detection	vegetables	5_10_5– 50mg/kg	[37]
Carbaryl	AchE and BchE. Amperometric transduction	Fruit and vegetables juices	0.5– 2500mg/L (carbaryl)	[59]
Carbofuran	Butyryl cholinestearase BuChE Photothermal transduction	Lettuce, onion	<0.02 mg/Kg	[49]
Diazinon	Tyrosinase-mediated inhibition with amperometric detection	Aqueous solution	5 μM	[60]
Diazinon	Tyrosinase-mediated inhibition with amperometric detection	Aqueous solution	5 μM	[40]
Dichlovos	Tyrosinase-mediated inhibition with amperometric detection	Aqueous Solution	75 nM	[40]
Dichlovos	AchE inhibition amperometric detection	River water	7.10-10 M	[61]
Dichlovos	Tyrosinase-mediated inhibition with amperometric detection	Aqueous Solution	75 μM	[122]

Analyte	Biosensor: type and basis	Matrix	Sensitivity	Ref
Dimethyl-and diethyldithiocarbamates	Tyrosinase Amperometric detection	Spiked apple samples	0.2– 2.2 m mol/l	[62]
Methyl paraxon	Alkaline phosohatase inhibition with chemiluminescence detection	Aqueous solution	80 ppb	[63]
Organophosphate Pesticide	AchE inhibition on a piezoelectric QCM with acoustic detection	Aqueous solution	<100 µg/L	[64]
Organophosphorus pesticides	AchE inhibition optical transduction		2 mg/L	[43]
Parathion	Parathion hidrolase enzyme action with amperometric detection	River water	10 ng/ml	[65]
Paraxon	AchE inhibition optical transduction	Aqueous solution	152 µg/L	[44]
Paraxon	BchE inhibition with potentiometric detection	Aqueous solution	<10-8	[66]
Paraxon	AchE inhibition photothermal detection	Drinking water, fruit juices	1.5 ng/ml tap water, 2.8 ng/ml orange juice 4µg/ml apple juice	[67]
Paraxon	Alkaline phosohatase inhibition with chemiluminescence detection	Aqueous solution	50 ppb	[125]
Propoxur	AchE inhibition and pH detection	Letuce Onion	Proporxur: 0.4 ng	[45]

QCM: quartz crystal microbalances; Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)

4.2. Recognition element classification and biosensors for food toxicant analysis

The main classes of biological elements used in recognition for the development of food analysis devices are:

- Enzymes
- Antibodies
- Microorganisms
- Biomimetics

4.2.1. Enzyme biosensors

The first biosensor described by Clarck and Lyons in 1962 was based on the use of glucose oxidase with electrochemical detection [68]. Since then, this principle has been widely extended in biosensors development, especially oxido-reductases (as tyrosinase, peroxidase and lacase) [69, 70] and hydrolases (choline estearases) [71, 72].

Enzyme biosensors can be distinguished into (i) those that measure the inhibition of a specific enzyme due to the presence of target analytes, and (ii) those that measure the catalytic transformation of target analytes by a specific enzyme. Enzyme biosensors have been described using a range of transduction elements (amperometry, potentiometry, optical and photo-thermal). The principal combinations reported for food toxicant analysis are described in Figure 5.

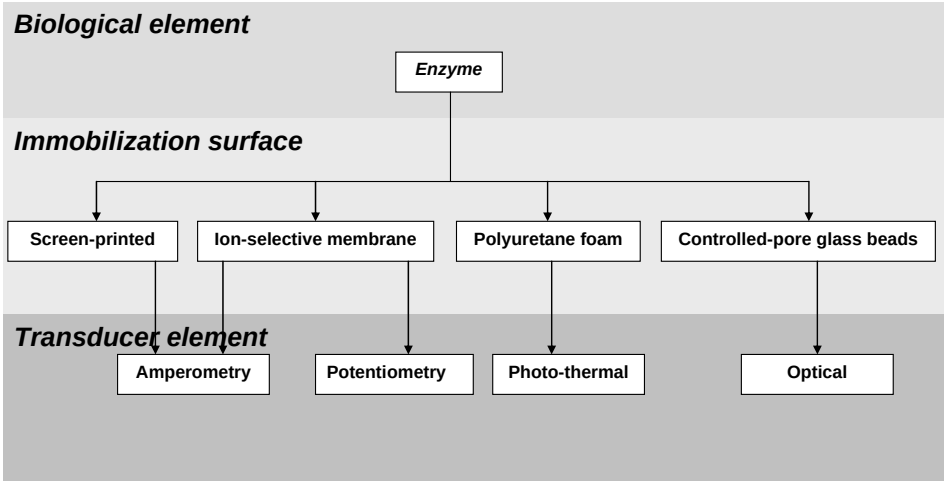


Figure 5. Main combinations of enzyme biosensors -biological recognition element *vs*. transduction- reported for food toxicant analysis.

The main application of enzyme based biosensors in food toxicants analysis have been the determination of pesticides and herbicides, but also some contributions have been done for antibiotics analysis, or heavy metals.

Examples of enzyme based biosensors reported for food toxicant compounds are summarized in Table 3.

The selective inhibition of cholin estearases, principally acetylcholine estearase (AchE) [73] and butylcholine estearase (BchE) [74], have been used for the detection of organophosphorous and carbamate insecticides, both widely used in agriculture and horticulture. However, there are several inherent limitations for biosensors based on enzyme inhibition. Sometimes is necessary the use of different substrates, cofactors and mediators, and the oxidation of analytes to metabolic intermediates could be necessary in order to increase sensitivities. The irreversible nature of many analyte-enzyme interactions, that result in increased sensitivity also renders the biosensor inactive after a single measurement. In those cases the sensing elements must be reactivated or should be employed disposable elements. An example is the determination of diazinon and dichlorvos, using tyrosinase immobilized on screen printed electrodes using a redox mediator (1,2-naphtaquinone-4-sulfonate) [75].

Another potential limitation to this type of biosensor is the lack of selectivity of some specific enzyme, because samples with complex matrices such food matrices may lead to unexpected results.

The catalytic transformation of target analytes is the second mechanism used for enzyme based biosensors. They are simple in design and operation. These biosensors can be configured to operate continuously and reversibly. They can also be configured such that the only required reagent is the analyte of interest.

Using potentiometric transduction phosphoric and carbamic pesticides were evaluated through the use of a pH electrode that measures the activity of acetyl cholinesterase [76]. Potentiometry has been used coupled to urease using ammonium ion sensors for the determination of heavy metals [77].

Most of transduction elements associated to enzyme based biosensors are electrochemical: amperometric or potentiometric. Enzyme based sensors with electrochemical transduction have been reported for about 140 different analytes including low molecular-weight substances (metabolites, drugs, nutrients, gases, metal ions, coenzymes, enzyme activators and vitamins) as well as macromolecules (enzymes, lectins, nucleic acids, polymeric carbohydrates like starch and cellulose), viruses and microorganisms.

Several examples of enzyme biosensors have been reported using optical transduction [78, 79]. Xavier et al. [80], developed an acetyl cholinesterase biosensors for the determination of proporxur and carbaryl in lettuce and onion, based on a reflectance measurement. During last years, optodes, such fiber optic biosensors have demonstrated to be of great interest because they provide some advantages such as no direct electric connection, ease of miniaturization,

possibility of remote sensing, and in situ monitoring, due to different examples have been reported such as a sol-gel acetyl cholinesterase fiber optic biosensor for organophosphorus neurotoxins [45], or an acetyl cholinesterase viologen hetero Langmuir-Blodgett film also for the analysis of organophosphorus compounds [44].

In order to improve the storage stability on enzyme based biosensors, different immobilizations and electrodes have been assayed, carbon paste electrodes (CPEs), solid graphite electrodes [81], surface modified electrodes [82], ion-sensitive field effect transistors (ISFETs) and pH-sensitive field-effect transistors (pH-FETs)) [83].

More recently, other types of enzymatic biosensors have developed based on termistor or opto-electronic sensors. Pogacnik and Franko [84] presented a photothermal biosensor for the determination of low concentration of organophosphate and carbamates pesticides in vegetables without sample treatment.

In addition to the extensive work for pesticides analysis other analytes of interest have been antibiotics, specially for penicillin V and G, using penicillase [85].

4.2.2. Immunosensors

The recognition elements are immunochemical interactions antibody-antigen. This type of devices combines the principles of solid-phase immunoassay with physicochemical transduction elements (electrochemical, optical, piezoelectric, evanescent wave and surface plasmon resonance).

Small molecular weight organic residues in food (such as pesticides or their metabolites) have few distinguishing optical or electrochemical characteristics, the detection of stoichiometric binding of these compounds to antibodies is typically accomplished using competitive binding assay formats. The main limitation of these techniques is the electrochemical detection of the immunoreaction, due to it is necessary to use enzymes that will generate electrochemical active compounds.

Electrochemical immunosensors have been widely use for food analysis in amperometric, potentiometric and conductimetric configurations. Some examples of new developments are the disposable screen-printed electrodes for the detection of polycyclic aromatic hydrocarbons (PAHs) [86] and the use of recombinant single-chain antibody (scAb) fragments [87] for atrazine determinations.

Acoustic transducers have been also applied in immunosensors for food or water analysis [88]. The resonant frequency of an oscillating piezoelectric crystal can be affected by a change in mass at the crystal surface. Piezoelectric immunosensors are able to measure a small change in mass. Last publications have been based on immunosensors using a quartz crystal microbalance (QCM) to the detection of trace amounts of chemical compounds, such as dioxins [89].

A novel contribution to this field is the use of magneto elastic transduction. An example is a mass-sensitive magneto elastic immunosensor for detection of *Escherichia coli* [90].

Many optical devices, based on solid-phase fluoroimmunoassays combined with an optical transducer chip chemically modified with an analyte derivate, have been developed for the measurement of different pesticides in the environment, and for water control analysis [91, 92]. These biosensors allow even the multidetection of pollutants at low nanogram per liter level in a single few-minutes analysis [93]. Different optical immunosensors have been constructed for the control of bacterial growth [94].

A variety of devices have been developed for bacterial detection, using a range of transduction elements. Seo et al. [95], described an immunosensor for *Salmonella typhimurium* detection in chicken carcass wash fluid, using a flow-through cell that comprised two channels, one immobilized with anti-*Salmonella* antibodies through a silane-derivatised surface, and a reference channel immobilized with human IgG. An integrated optic interferometer allowed the specific measurement of Salmonella by comparing the phase shift generated by the refractive index variation with the reference channel. Although the assay time was 10 min, 12-h pre incubation time is needed in order to allow a cultural growth level detectable by the sensor. DeMarco et al. [96] developed a portable polystyrene fiber optic device for *Escherechia coli* O157:H7 and staphylococcal enterotoxin B analysis in 20 minutes. This rapid and sensitive device is now commercially available from Research International.

Several approaches are based on the principle of total internal reflection fluorescence (TIRF), a recent work reports a sandwich immunoassay coupled to TIRF for the determination *Campylobacter* and *Shigella* bacteria with limit of detection for *Shigella dysenteriae of* 4.9- 104 cfu mL^{-1}, and low than 9.7-102 cfu mL^{-1} was achieved for *Campylobacter jejuni*, [97].

Surface plasmon resonance (SPR) has been used to measure the binding of antibodies to antigens immobilized at the sensor surface because the critical angle depends of the refractive index of metal surface materials. Some applications of SPR-immunosensors have been developed for the food quality control, such as the caseine content in milk products [98], for the detection of genetically modified organisms (GMO)[99], and for the detection of pathogens in food and water [100, 101].

Examples of immunosensors reported for the analysis food toxicant compounds are summarized in table 4.

Table 4

Examples of immunosensors for food toxicants analysis

Analyte	Type and basis	Matrix	Sensitivity	Ref.
Pesticides and Herbicides				
Atrazine	Inhibition assay electrochemical detection	Water	0.03μg/L	[102]
Atrazine	Inhibition assay Optical detection	Water	1-10μg/L	[103]
Atrazine	Indirect assay and TIR-fluorescence detection	Water	0.06-0.2μg/L	[104]
Chlorsulfuron	Competitive assay amperometric detection	Aqueous solution water	0.01 ng/mL	[105]
Isoproturon	Indirect assay and TIR-fluorescence detection	Water	0.01-0.14μg/L	[132]
Simazine	Competitive immunoreaction potentiometric detection	Meat extract, milk, tomatoes, cucumbers, potatoes	3ng/mL	[106]
Triazines	Inhibition assay SPR detection	Water	0.16μg/L	[107]
Triazines	Inhibition assay surface refractive index change detection	Water	15 μg/mL	[108]
Triazines	Flow-through amperometric immunosensor based on peroxidase chip and enzyme channeling system	Aqueous solution	1 ng/L	[109]
Pesticides	Competitive immunoassays fluorescence detection	Aqueous solution		[110]
Mycotoxins				
Aflatoxins	Direct immunoassay fluorometric detection	Aqueous solution	0.01 μg/L	[111]
Fumonisin B1	Direct assay SPR detection	Aqueous solution	15μg/mL	[112]
Ochratoxin A	Inhibition assay SPR detection	Liquid food samples	5 ng/mL	[113]

Analyte	Type and basis	Matrix	Sensitivity	Ref.
Food borne microorganisms and bacterial toxins				
Cyanobacter hepatotoxins microcystins	Recombinant antibodies	Food and drinking water		[114]
E coli enterotoxin	QCM	Food	35 pM	[115]
E. coli enterotoxin	SPR	Food	70 pM	[143]
E. coli O157:H7	Anti *E.coli* covalently immobilized to Biodyne membrane Amperometric detection	Aqueous suspension	100 cells/mL	[116]
E.coli O157:H7	Anti *E.coli* immobilized in a fiber optic and fluorescence detection	Beef	3-30 cfu/mL	[117]
Enterotoxin B	SPR sensor chip-MALDI-TOF detection	Milk, mushrooms	1 ng/ml	[118]
Pathogens (*Campylobacter*, C. *jejuni*) and micotoxins	Array biosensor	Food		[119]
S. typhimurium	Anti.*Salmonella* antibody covalently bound to sensor surface refractive index change detection	Chicken carcass wash fluid	1.10^4 cfu/mL	[141]
S. typhimurium	Anti.Salmonella antibody adsorbed to gold electrode QCR detection	Aqueous suspension	1.10^6 cfu/mL	[120]
Staphylococcal enterotoxin A	Sandwich assay evanescent wave detection	Food	10 ng/g	[121]
Staphylococcal enterotoxin B	Sandwich assay Surface plasmon resonance	Milk	0.5 ng/mL	[122]
Staphylococcal enterotoxin B	Automated fiber optic	Food		[123]

Analyte	Type and basis	Matrix	Sensitivity	Ref.
Antibiotics				
Chloramphenicol	Inhibition assay SPR detection	Milk	1 ng/mL	[154]
Chloramphenicol	Label free detection, quartz crystal microbalance measurement		5. 10-6 M	[124]
Ciprofloxacine	Inhibition assay SPR detection	Milk	1.5 µg/Kg	[154]
Enterofloxacine	Inhibition assay SPR detection	Milk	1.5µg/Kg	[154]
Sulphadiazine	Inhibition assay based on indirect immobilization of sulphamethazine to a sensor chip and SPR detection	Pig bile	0.02 µg/mL	[125]
Sulphamethazine	Inhibition assay SPR detection	Milk	1 µg/L	[126]
Sulphamethazine	Inhibition assay SPR detection	Milk	1.7µg/Kg	[127]
Sulphamethazine	Inhibition assay SPR detection	Milk	1µg/Kg	[128]
Sulphamethazine	Inhibition assay based on indirect immobilization of Sulphamethazine to a sensor chip and SPR detection	Milk	0.5 µg/Kg	[129]

SPR: Surface plasmon resonance; QCM: quartz crystal microbalances

4.2.3. Microbial Biosensors

The major application of microbial biosensors is in the environmental field. The use of immobilized whole cells (usually bacteria) as the recognition element for biosensor has been described for chemical residues, such as phenols, pesticides, benzene, toluene, xilene, endocrine disruptors and the water biological oxygen demand (BOD). A large number publications have been appeared describing BOD microbial biosensors, and different devices are commercial available. However, a fewer number of applications have been developed for food toxicant analysis. Typically, electrochemical transduction methods have been used, particularly, the Clark oxygen electrode. These sensor systems rely on the interaction of a particular microorganism in the presence of a target analyte. By monitoring the respiratory activity of the microorganism, it has proved possible to detect and quantify the target analyte in a range of food matrices.

Recombinant organisms are an exploited alternative, that exhibits a number of important traits, such as the expression of cellular degradative enzymes, specific binding proteins against a target analyte, and reporter enzymes, for example bacterial luciferasa which is induced by target analytes with light production. Examples of microbial based biosensors application is the detection of antibiotic residues in milk [130-131], residues of pesticides in fish meat [132], or in aqueous solutions [133].

4.3. Commercial instrumentation and future perspectives

During the last years biosensors are increasingly becoming an analytical tool for real-time measurements in routine analysis. A number of instruments for food analysis are already commercially available. However, these commercial available devices are focused on few applications, such as the determination of saccharides (YSI), or the detection of for bacterial toxins (Research International) and pathogens. Many devices are still in a prototype step, and the commercial success of biosensors is limited to a small number of applications, where the market size justified more research, validation and development investment. Tables 5 shows some examples of commercial biosensor instrumentation available, for toxicant compounds and pathogens analysis in food. The future commercial status and general acceptance of this technology will depend on the performance characteristics, sample throughput, associated costs, validation and acceptance by regulatory authorities.

Some of important keys in the future of biosensors development is allow more stability of biological components; more robustness assays; more repeatability between different batch of production when disposable elements are involved, and the integration of new technologies coupled to biosensors, such as the polymerase chain reaction (PCR). Finally, there are at least two other developments that are expected to have significant impact, the namely laboratory on a chip (LOC) [134], and nanotechnology [135]. The concept of LOC entails miniaturization of all the essential components of analytical instrumentation (e.g. sample preparation,

components, reaction with appropriate reagents and detection) by micro fabrication on a chip. Some of the components in LOC technology have already been released on the market (GeneChip® from Affimatrix). Nanotechnology refers to the exploitation of processes to generate and utilize structures, components and devices with a size range 0.1 nm (atomic and molecular scale) to 100 nm or larger in some cases, by control at atomic, molecular and macromolecular levels. It has been suggested that nanoscale sensors and ultra miniaturized sensors could lead to the next generation of biotechnology-based industries. A recent contribution to nano-biosensors development has been a nanoparticle-based DNA biosensor for visual detection of genetically modified organisms in soy samples [136].

5. Biomimics: Molecularly imprinted polymers (MIPs)

5.1. Development and application of MIP-based sensors

Due to the frequently poor stability (thermal, pH..) and short life times of biological components, synthetic molecules with high affinity properties, similar to biological ones, are been introduced. One of the most promising groups of biomimetic material are molecularly imprinted polymers (MIPs).

MIPs are becoming an important class of synthetic materials mimicking molecular recognition by natural receptors, like as antibodies. Non-covalent imprinting, in particular, has a great range of applications because of the theoretical lack of restrictions on size, shape or chemical character of the imprinted molecule. The possibility of tailor-made, highly selective receptors at low cost, with good mechanical, thermal and chemical properties makes these polymers appear ideal chemo receptors. There are great hopes for development of a new generation of chemical sensors using these novel synthetic materials as recognition elements [137-138].

The selected ligand or *print molecule* is first allowed to establish bond formations with polymerizable functionalities, and the resulting complexes or adducts are subsequently copolymerized with cross-linkers into a rigid polymer. Following the extraction of the print molecule, specific recognition sites are left in the polymer, where the spatial arrangement of the complementary functional entities of the polymer network together with the shape image correspond to the imprinted molecule.

Molecular imprinted polymers can be prepared by self-assembly, where the prearrangement between the ligand and the functional monomers is formed by noncovalent bond, or metal coordination interactions; or by a preorganized approach, where the aggregates in solution prior to polymerization are maintained by reversible covalent bonds. By use of a high percentage of cross linker, polymers of substantial rigidity and complete insolubility are obtained

Table 5

Commercial devices

Name	Analyte	Description	Company
Analyte 2000™	Staphylocaccal enterotoxin B, *Escherechia coli* O157:H7, spores...	4-channel, single wavelength fluorometer optimized for performing evanescent-wave fluoroimmunoassays	Research International, 18706 142bd Ave, N.E., Woodinville, WA, 98072 USA www.resrchintl.com
ANDREA	Direct Measurement of Molecular Locking and DNA-Hybridization	The measurement of antibody-antigen reactions is done by a new technique on basis of ellipsometry. Ellipsometry is an optical method, which simply measures the state of polarization of light that is reflected from a substrate.	DRE - Dr.Riss Ellipsometerbau GmbH Feldstr. 14 D-23909 Ratzeburg, Germany www.dre.de
BIAcore (1000, 2000, 3000, Q, A100, Flexchip)	Hormons, vitamins, mycotoxins, antibiotics, low weight molecules	Surface plasmon resonance affinity based biosensor	BIAcore AB, Rapsgatan 7, Uppsala, Swden www.biacore.com
IBIS	Generic sensor for studding binding interactions with low molecular weigh molecules.	Surface plasmon resonance affinity based biosensor	Windsor Scientific Limited 264 Argyll Avenue Slough Trading Estate Slough Berkshire SL1 4HE United Kingdom. www.windsor-ltd.co.uk

Name	Analyte	Description	Company
RAPTOR™	Staphylococcal enterotoxin B, *Bacillus anthracis, Yersinia pesti,* micotoxins, spores...	Portable fluoroimmuno biosensor	Research International, 18706 142bd Ave, N.E., Woodinville, WA, 98072 USA www.resrchintl.com
SPREETA™	Generic sensor for studding binding interactions with low molecular weigh molecules.	Miniaturized surface plasmon resonance affinity based biosensor	Texas Instruments Inc., 12500 TI Boulevard Dallas, TX 75243-4136, USA. www.ti.com

The main characteristics of these materials is their resistant in front physical and chemical environments, mechanical stress, high temperatures and pressures, resistant against treatment with acid, base, or metal ions, stables in a wide range of solvents, and can be used repeatedly-

5.2. MIPs in preparation/preconcentration and separative applications

MIPs are recognition elements that have been successfully applied in analytical chemistry as an alternative to immunoaffinity systems. The have been applied in affinity chromatography, binding assays, and sensor developments.

The past decade was characterized by the transition of molecular imprinting techniques from a predominantly theoretical research to practical analytical applications. The first application of MIPs was as stationary phases in affinity chromatography, in particular for the enantio separation of racemic mixtures of chiral compounds, and much of the early work on MIPs was devoted to this aspect.

Capillary Electrochromatography (CEC) is a hybrid separation method that couples the high separation efficiency of Capillary Zone Electrophoresis (CZE) with liquid chromatography, and uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. Since there is no back pressure it is possible to use small diameter packings and thereby achieve very high efficiencies. CEC might be one of the more promising chromatographic techniques to be used in combination with MIPs, in particular for chiral separations. MIP-based CEC profits from the inherent separation power of this method; compared with MIP-based HPLC, appreciable more resolution can be achieved.

One of the most studied areas of application was preparation/preconcentration techniques. Sellergren in 1994 [139] published one of the first relevant works on MIP-based solid phase extraction (SPE), since then a new window was open, and intensive labour of development have been carried out on molecularly imprinted solid-phase extraction (MISPE). The potential advantages of this technique compared to conventional solid phase extraction sorbents are the specificity, batch reproducibility, can be regenerated and cost-effective synthesis. Due to these reasons, during last years the potential of MIPs application for SPE materials has been thoroughly investigated culminating in the commercial availability of MIP-based SPE cartridges by companies, such as ELIPSA (Germany) [140] and MIP Technologies [141] (Sweden). However, some limitations of these new SPE material have also been reported [142] such as diminution of recovery percentage after repetitive regenerations.

For food analysis some relevant contributions have been done using MISPE in different areas, such as the analysis of flavones in wine [[143], [144]], or in food toxicant analysis. Main applications in food toxicants analysis have been carried out in three specific areas:

- Residues of pharmaceutical compounds (antibiotics, β-agonists)
- Microbial pathogens and mycotoxins
- Residues of pesticides

The extraction of mycotoxins demonstrates the versatility of MIPs even for more complex natural compounds such as deoxynivalenol and zearelenone [145], or ochratoxin A [146]. On the other hand, a high sensitivity (0.05-0.2 μg/L) method was developed for the analysis of chlorotriazine in environmental samples by Ferrer et al. [147]. A clenbuterol-selective MISPE cartridge has been released by MIP Technologies, for detection of traze concentrations of this illegal growth promoter in meat products. Recently, membranes become increasingly attractive materials for efficient affinity separations [148]. Some examples of reported applications of MIPs for SPE and packing column materials are shown in table 6.

5.3. Development and application of MIP-based sensors

During last decade the number of application of MIP-based sensors has increased drastically. The high selectivity and affinity properties of MIP for target analytes make them ideal recognition elements in sensors development [59]. Capacitive [149], conductimetric [150], field effect [151], amperometric [152], and voltammetric [153] electrochemical transduction systems have been used. Applications of MIP based sensors for contaminants analysis are summarized in Table 7.

Based on conductimetric transduction, Piletsky *et al*., [88] have been developed sensors for herbicides analysis with a linear range of 0.01–0.50 mg/l for atrazine, without interference of simazine. Chloroaromatic acids were determined by Lahav et al. [89], using a TiO_2 sol-gel system. Using a voltammetric transduction, Pizzariello et al. [91] developed a sensor for clembuterol analysis.

MIP-based sensors coupled to piezoelectric transducers is one of the most promising areas. Different devices have been developed for their use in food industry, such as a supported-piezoelectric detection based on MIP for the quantification of caffeine content in coffee and tea samples[154], the detection of sorbitol [155], antibiotics in milk [176], and the detection of toxicant compound such as polycyclic aromatic hydrocarbons (PAHs) [156].

Other optical tranducers reported is a chemiluminescent sensor for clembuterol determination [157].

5.4. Future trends

Current research demonstrates the progression of MIP chemistry and the potential of these materials to solving a wide variety of analytical problems. However, MIPs still have a series of significant limitations:

- Unless the print-material is inexpensive, their preparation use to be very costly
- There is a lack of robustness and sensitivity, due to a inefficient removal of the print molecules during MIP preparation
- Several times there is a lack of reproducibility.

Table 6

Applications of MIPs for SPE and packing column materials for food analysis.

Analyte	Analysis format	Functional monomer	Ref
(+)-Catechin	MIP-based-SPE	EDMA	[158]
Flavonoids	MIP-based SPE	4-VP + EDMA	[159]
ß-blockers	MIP-based SPE	MAA+EDMA	[160]
Clembuterol	MIP-packed HPLC column	MAA	[161]
Micotoxins	MIP Based SPE		[162]
Microsystin-LR	MIP-based SPE		[163]
Ochratoxin A	MIP-based SPE	PAM	[164]
Listeria monocytogenes	Polymeric microcapsules	Poly(allilamine)	[165]
Penicillin V, Penicillin G, Oxacillin	MIP-packed HPLC column	MAA+4-VP	[166]
Chloramfenicol	MIP-based SPE	NS	[167]
4-nitrophenol	MIP-based SPE	4-VP	[168]
Ametryn/Triazines	MIP-based SPE	DEAEM	[85]
Chloro triazines	MIP-based SPE		[169]
Simazine	MIP-Based SPE	MAA	[170]
Triazines	RAM-MIP-SPE		[171]
Triazines	MIP Based SPE		[172]

4-vinylpyridine (4-VP); Ethylene glycol dimethacrylate (EDMA); Methacrylic acid (MAA), Ethylene glycol dimethacrylate (EDMA); *N*-phenylacrylamide (PAM); Diethylamino ethyl methacrylate (DEAEM)

Table 7

MIP-based sensors for food toxicant analysis

Analyte	Functional monomer	Transduction	Ref.
o-Xilene	NS	QCM	[173]
PAHs	Phloroglucinol and tiisocyanate	QCM, SAW and optical transduction	[94]
2,4-dichlorophenoxy-acetic-acid	4-VP	Electrochemical detection using screen printed electrodes	[174]
Atrazine	MAA	Conductimetric sensor	[88]
Atrazine	MAA+EDMA	Amperometric detection	[175]
Trichloroacetic acid and haloacetic acids	4-VP+EDMA	Conductimetric sensor	[176]
Domoic acid	2-(diethylamino) ethyl methacrylate	SPR-QCM	[177]
Ochratoxin A	Polypyrrole film	SPR (SPREETA)	[178]
Sulphamethazine	MAA	Voltammetric	[179]
Clembuterol		Chemiluminescence	[95]
Caffeine	MAA+EDMA	acoustic sensor, PQC	[92]
Caffeine	MAA+EDMA	BAW	[180}

Quartz crystal microbalance (QCM); Surface plasmon resonance (SPR); Piezoelectric quartz crystal (PQC); Bulk acoustic wave (BAW); surface acoustic wave (SAW); 4-vinylpyridine (4-VP); Ethylene glycol dimethacrylate (EDMA); Methacrylic acid (MAA), Ethylene glycol dimethacrylate (EDMA); *N*-phenylacrylamide (PAM); Diethylamino ethyl methacrylate (DEAEM).

General MIPs preparation procedures are adequate at the laboratory level, and now these preparation procedures need to be developed for scaling up to commercial production.

Due to these reason MIPs are not yet used for routine applications.

The combination of MIPs with a range of tranducers to produce on line and real time sensors is expected, and their potential has been demonstrated, the but limitations described previously should be addressed.

The majority of applications of MIPs are directed to low molecular weight, organic soluble analytes of interest in the pharmaceutical industry (e.g. chiral drug separation), but new applications have been developed in environment and food industry. Several new approaches to MIP production (e.g. surface imprinting) are expected to allow MIPs to be prepared in aqueous media. Other new approaches in development are Magnetic MIPs, and Catalytic MIPs (also referred to as enzyme mimics or plastizyme).

References

[1] P. Shuk, K.V. Ramanujachary M. Greenblatt *Solid State Ionic*, 86-88 (1996) 1115.

[2] W. Olthius, M. A. M. Robben, P. Bergveld, M. Boss and W. E. Van Der Linden, *Sens. Actuators B-Chem. 2* (1990) 247.

[3] P Bergveld Development and application of chemical sensors in liquids. In *Sensors and Sensory Systems for Advanced Robots*, NATO ASI Series (1998) F43, ed. P.Dario, Springer, Berlin, 397.

[4] M.J. Schöning, D. Tsarouchas, L. Beckers, J. Schubert, W. Zander, P. Kordo, H. Lüth, *Sens. Actuators B-Chem.-Phys.*, 35 (1996) 228.

[5] J.C. Chou, J.L.Chiang, *Sens. Actuators B-Chem.* 62 (2000) 81.

[6] A Poghossian, L. Berndsen, J.W. Schultze, H. Lüth, M.J. Schöning. In: M. Butler, P. Vanysek, N. Yamazoe (Eds), *Chemical and Biological Sensors and Analytical Methods,* The Electrochemical Society Inc., Pennington, USA, 2001, pp. 143-152.

[7] A. Poghossian, T. Yoshinobu, M.J. Schöning, *Sensors* 3 (2003) 202-212

[8] Hammond P.A., Cumming, D.R.S. *Sens. Actuators B-Chem.* 111 (2005) 254.

[9] Z. Jin Y Duan *Sens. Actuators B-Chem.* 71 (2000) 118.

[10] P.K. Choudhury, T. Yoshino, *Optik* 114 (2003) 13-18

[11] Q.Y. Cai C.A. Grimes, *Sens. Actuators B-Chem.* 71 (2000) 112.

[12] S. Voliotis, A. Hristoforou, G. Christodoulou *Sens. Actuators A- Phys.* 106 (2003) 137.

[13] K Loiselle, C.A. Grimes. *Rev. Sci. Instum.* 71 (2000) 1141– 1146.

[14] W.J. Karl, A.L. Powell, R. Watts, M.R.J. Gibbs, C.R. Whitehouse. *Sens. Actuators* A-Phys. 81 (2000) 137.

[15] C.L. Bohnke,H. Duroy, J.L. Fourquet, *Sens. Actuators B-Chem.* 89(2003) 240.

[16] J.W. Gardner E.L. Hines. (1997) Pattern Analysis Techniques,. 633– 52 in *Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment*, Kress-Rogers E. (ed.), Boca Raton, New York, London, Tokyo, CRC Press Inc.

[17] P.G. Berrie (1997) Fuzzy Logic in the Evaluation of Sensor Data, pp. 469–500 in *Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment*, Kress-Rogers E. (ed.), Boca Raton, New York, London, Tokyo, CRC Press Inc.
[18] B. Lazzerini, A. Maggiore, F. Marcelloni, *Pattern Recognit.* 34 (2001) 2215.
[19] M. Theisen A. Steudel, M. Rychetsky, M. Glesner (1998) Fuzzy Logic and Neuro-Systems Assisted Intelligent Sensors, pp. 29– 59 in *Sensors Update Volume 3*, H. Baltes, W. Göpel, J. (eds.) Wiley-VCH.
[20] J. Wöllenstein F. Ihlenfeld, M. Jaegle, G. Kühner, H. Böttner, W.J. Becker, *Sens. Actuators B-Chem.* 68 (2000) 22.
[21] A. Hierlemann, D. Lange, C. Hagleitner, N. Kerness, A. Koll, O. Brand, H. Baltes *Sens. Actuators B-Chem.* 70 (2000) 2.
[22] F. Quaranta, R. Rella, P. Siciliano, S. Capone, M. Epifani, A. Taurino, *Sens. Actuators B-Chem.* 84 (2002) 55.
[23] J. H. Kim, H. J. Ahn, H. S. Yook, K. S. Kim, M. S. Rhee, G. H. Ryu, M. W. Byun *Radiat. Phys. Chem.* 69 (2004) 179.
[24] J. Trihaas, L. Vognsen, P.V. Nielsen, *Int. Dairy J.* 15 (2005) 679.
[25] L. Marilley, M.G. Casey, *Int. J. Food Microbiol.* 90 (2004) 139.
[26] C. DiNatale, G.Olafsdottir, S.Einarsson, E. Martinelli, R. Paolesse, A. D'Amico, *Sens. Actuators B-Chem.* 77 (2001) 572.
[27] A. Jonsson, F. Winquist, J. Schnurer, H. Sundgren, I. Lundstrom *Int. J. Food Microbiol.* 35 (1997) 187.
[28] T. Rajamäki, H-L. Alakomi , T. Ritvanen , E. Skyttä, M. Smolander, R. Ahvenainen, *Food Control* 17 (2006) 5.
[29] R. Baby, M. Cabezas, E. Castro, R. Filip, N.E. Walsöe de Reca *Sens. Actuators B-Chem.* 106 (2005) 24.
[30] S. Capone, M. Epifani, F. Quaranta, P Siciliano, A. Taurino, L.Vasanelli, *Sens. Actuators B-Chem.* 78 (2001) 174.
[31] A.Eriksson, K.Persson Waller, K.Svennersten-Sjaunja, J-E.Haugen, F.Lundby, O. Lind, *Int. Dairy J.* 15 (2005) 1193.
[32] C.Di Natale, A. Macagno, E. Martinelli, R. Paolesse, E. Proietti, A D'Amico *Sens. Actuators B-Chem.* 78 (2001) 26.
[33] S. Strathmann, S. Pastorelli, C. Simoneau, *Sens. Actuators B-Chem.* 106 (2005) 83.
[34] D. Van Deventer, P. Mallikarjunan, *Innovat. Food Sci. Emerg. Tech.* 93 (2002) 93.
[35] M. Vinaixa, A. Vergara, C. Duran, E. Llobet , C. Badia, J. Brezmes, X. Vilanova, X. Correig, *Sens. Actuators B-Chem.* 106 (2005) 67.
[36] J. Olsson, T. Börjesson, T. Lundstedt, J. Schnürer *Int. J. Food Microbiol.* 72 (2002) 203.
[37] Y. González Martín, M.C. Cerrato Oliveros, J. L. Pérez Pavón, C. García Pinto, B. Moreno Cordero, *Anal. Chim. Acta*, 449 (2001) 69.
[38] J. Lozano, J.P. Santos, M.C. Horrillo, *Talanta* 67 (2005) 610.
[39] R. Stella, J.N. Barisci, G. Serra, G.G. Wallace, D. De Rossi, *Sens. Actuators B-Chem.* 63 (2000) 1.
[40] K. Toko, *Meat Sci. Tech.,* 9 (1998) 1919.
[41] C. Di Natale, A. Macagnano, F. David, A. D'Amico, A. Legin, Y. Vlasov, A. Rudnitskaya, B. Selenev, *Sens. Actuators B-Chem.* 44 (1997) 423.
[42] S. Takagi, K. Toko, K. Wada, T. Ohki, *J. Pharm. Sci.* 90 (2001) 2042.

[43] F. Winquist, S. Holmim, C. Krants-Rülker, P. Wide, I. Lundstrom, *Anal. Chim Acta* 406 (2000) 147.
[44] P. Ciosek, Z. Brzózka, W. Wróblewski, *Sens. Actuators B-Chem.*, 103 (2004) 76.
[45] C. Turner, A. Rudnitskaya, A. Legin, *J. Biotechn.* 103 (2003) 87.
[46] C. Söderström, A. Rudnitskaya, A. Legin, C. Krantz-Rülcker, *J. Biotechn.* 119 (2005) 300.
[47] C. Söderström, F. Winquist, C. Krantz-Rülcker, *Sens. Actuators B-Chem.* , 89 (2003) 248.
[48] S. Buratti, S. Benedetti, M. Scampicchio, E.C. Pangerod, *Anal. Chim. Acta* 525 (2004) 133.
[49] L.A.Abayomi, L.A. Terry, S.F. White, P.J. Warner, Development of biosensor to determine pungency in onions. 8th World Congress on Biosensors, Granada, Spain, 24-26 May, 2004.
[50] L. Rotariu, C. Bala, V. Magearu, *Anal. Chim. Acta* 29 (2002) 215.
[51] N. Verma, M.A. Singh, *Biosens. Bioelectron.* 18 (2003) 1219.
[52] A. Mulchandani, P. Mulchandani, W. Chen, *Field Anal. Chem.Technol.* 2 (1998) 363.
[53] P. Thavarungkul, P. Suppapitnarm, P. Kanatharana, B. Mattiasson, *Biosens. Bioelectron.* 14 (1999) 19..
[54] J.C. Pyun, H. Beutel, J.U. Meyer, H.H. Ruf, *Biosens. Bioelectron.* 13 (1998) 839.
[55] I. Mannelli, M. Minunni, S. Tombelli, M. Mascini, M. *Biosens. Bioelectron.* 18 (2003)129.
[56] E.P. Medyantseva, M.G. Vertlib, G.K. Budnikov, M.P. Tyshlek, *Appl. Biochem.and Microbiol.* 34 (1998) 202.
[57] I. Palchetti, A. Cagnini, M. Del Carlo, C. Coppi, M. Mascini, A.P.F. Turner, *Anal. Chim. Acta* 337 (1997) 315.
[58] A.N. Ivanov, G.A. Evtugyn, R.E. Gyurcsanyi, K. Toth, H.C. Budnikov, *Anal.Chim. Acta* 404 (2000) 55.
[59] G.S. Nunes, D. Barceló , B.S. Grabaric, J.M. Diaz-Cruz, M.L. Ribeiro, *Anal. Chim. Acta* 399 (1999) 37.
[60] J.Q. Xu, X.Z.He, Y.X. Zhou, L.T. Liu, J. Cheng, Chin. *Sci. Bull*. 45 (2000) 101.
[61] J.J. Rippeth, T.D. Gibson, J.P. Hart, I.C. Hartley, G. Nelson, *Analyst*, 122 (1997) 1425.
[62] M.T.P. Pita, A.J. Reviejo, F.J.M. Villena, J.M. Pingarrón, *Anal. Chim. Acta* 340 (1997) 89.
[63] M.S. Ayyagari, S. Kamtekar, R. Pande, K.A. Marx, J. Kumar, S.K. Tripathy, J. Akkara, D.L. Kaplan, *Biotechnol. Progr*. 11 (1999) 699.
[64] P. Sritongkham, N. Taravanit, T. Suwannakum, M. Tanticharoen, K. Kirtikara, in A.P.F. Turner (Editor), Biosensors 94: the Third World Congress on Biosensors, New Orleans, June 1994: Abstracts, Elsevier Advanced Technology, Oxford, 1994.
[65] V. Sacks, I. Eshkenazi, T. Neufeld, C. Dosoretz, J. Rishpon, *Anal. Chem*. 72 (2000) 2055.
[66] Y.A. Cho, H.S. Lee, G.S. Cha, Y.T. Lee, Biosensors and Bioelectronics. Y.A. Cho, H.S. Lee, G.S. Cha, Y.T. Lee, *Biosensors Bioelectron*. 14 (1999) 435.
[67] L. Pogacnik, M. Franco, *Biosensors Bioelectron*. 14 (1999) 569.
[68] L.C. Clarck, C. Lyons, *Ann. N.Y. Acad. Sci.* 102 (1962) 29
[69] A.E.G Cass, D.G. Francis, H.A.O. Hill, W.J. Aston, I.J. Higgins, E.V. Plotkin, L.D.L. Scott, A.P.F. Turner, *Anal. Chem.* 56 (1984) 667.
[70] J. Kulis, R. Vidziunaite, *Biosens. Bioelectron.,* 18 (2003) 319.
[71] S. Andreescu, L. Barthelmebs, J-L. Marty, *Anal. Chim. Acta.* 464 (2002) 171.
[72] G. Nunes, P. Skládal, H. Yamanaka, D. Barceló *Anal. Chim. Acta,* 362 (1998) 59.
[73] G.G. Guilbault, J. Das. *Anal. Biochem*, 33 (1970) 341.
[74] P. Skládal *Anal. Chim. Acta* 269 (1992) 281.
[75] W.R. Everett, G.A. Rechnitz, *Anal. Chem*. 70 (1998) 807.

[76] M.C. Budnikov and G.A. Evtugyn, "Sensitivity and Selectivity of Electrochemical Biosensors for Inhibitor Determination" in *Biosensors for Direct Monitoring of Environmental Pollutants in Field*, ed. by D. Nikolelis, U. Krull, J. Wang and M. Mascini, Kluwer Academic Publishers, London, 1998, pp. 239-253.
[77] I. T. Show, M. B. Show, and L. R. Williams, "Modeling the Contributions of Cross-Reacting Chemicals in Immunoassays" in *Aquatic Toxicology and Hazard Assessment: 12th vol., ASTMSTP 1026*, ed. by U. M. Cowgill and L. R. Williams, American Society for Testing and Materials, Philadelphia, 1989, pp. 21-33.
[78] J.W. Choi, Y.K. Kim, I.H. Lee, J. Min, W.H. Lee, *Biosen. Bioelectron.* 16 (2001) 937.
[79] R.A. Doong, H-C. Tsai, *Anal. Chim. Acta.* 434 (2001) 239.
[80] M. P. Xavier, B. Vallejo, M.D. Marazuela, M.C. Moreno-Bondi, F. Baldini, A. Falai, *Biosen. Bioelectron.* 14 (2000) 894.
[81] K. Streffer, E Vijgenboom, A.WJW. Tepper, A Makower, F W. Séller, G. W. Canters, U. Wollenberger. *Anal. Chim. Acta* 427 (2001) 201.
[82] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu, *Anal. Chim. Acta.* 387 (1999) 309.
[83] T. Mai Anh, S. V. Dzyadevych, A. P. Soldatkin, N. Duc Chien, N. Jaffrezic-Renault, J.M. Chovelon, *Talanta* 56 (2002) 627.
[84] L. Pogacnik, M. Franko, *Biosensors Bioelectron.* 18 (2003) 1.
[85] M. Nishizawa, T. Matsue, I. Uchida, *Anal. Chem.* 64 (1992) 2642.
[86] K.A. Fahnrich, M. Pravda, G.G. Guilbault, *Biosens. Bioelectron.* 18 (2003) 73.
[87] K. Grennan, G. Strachan, A.J. Porter, A.J. Killard, M.R. Smyth, *Anal. Chim. Acta* 500 (2003) 287.
[88] G.G. Guilbault , *Biosensors Bioelectron.* 7 (1992) 411.
[89] S. Kurosawa *Meat. Sci. Tech.* 14 (2003) 1882.
[90] C. Ruan, K. Zeng, O.K. Varghese, C.A. Grimes, *Anal. Chem*. 75 (2003) 6494.
[91] E. Mallat, C. Barzen, A. Klotz, A. Brecha, G. Gauglitz, D. Barceló. *Environ. Sci. Technol.* 33 (1999) 965.
[92] S. Rodriguez-Mozaz , S. Reder , M. Lopez de Alda , G. Gauglitz and D. Barceló *Biosensors Bioelectron.* 19 (2004) 633.
[93] J. Tschmelak, G. Proll, J. Riedt, J. Kaiser, P. Kraemmer, L. Bárzaga, J. S. Wilkinson, P. Hua, J. P. Hole, R. Nudd, M. Jackson, R. Abukneshaf, D. Barceló, S. Rodriguez-Mozaz, M-J. López de Alda, F. Sacher, J. Stienh, J. Slobodnýk, P. Oswald, H. Kozmenko, E. Korenkov, L. Tothov, Z. Krascsenits, G. Gauglitz, *Biosenensors. Bioelectron.* 20 (2005) 1509.
[94] H. Chuang, P. Macuch, M.- B. Tabacco, *Anal. Chem*. 73 (2001)462.
[95] K.H. Seo, R.E. Brackett, N.F. Hartmann, D. P. Capbell. *J. Food Prot.* 62 (1999) 431.
[96] D.R. DeMarco, E.W. Saaski, D.A. McCrae, D.V. Lim, . *J. Food Prot.* 62 (1999) 711.
[97] K. E. Sapsford,A. Rasooly,Chris R. Taitt,Frances S. Ligler, *Anal. Chem*. 76 (2004) 43.
[98] S. Muller-Renaud, D. Dupond, P. Dulieur, *J. Agric. Food. Chem*. 52 (2004) 659.
[99] G. Ferriotto, M. Borgatti, C, Mischiati, N. Bianchi, R. Gambari *J. Agric. Food Chem.,* 50 (2002) 955.
[100] E. Kaclikova, T. Kuchta, H. Kay, D. Gray, *J Immunol. Met.* 46 (2001) 63.
[101] J. Haines, P.D. Patel BIAJ 2 (1995) 31.
[102] R.J.M. Fernandez, M. Stiene, R. Kast, M.D. Luque de Castro, U. Bilitewski, *Biosensors Bioelectron.* 13 (1998) 1107.
[103] P. Skladal, *Biosensors Bioelectron.* 14 (1999) 257.

[104] E. Mallat, C. Barzen, R. Abuknesha, G. Gauglitz, D. Barceló, *Anal. Chim. Acta*, 426 (2001) 209.
[105] B.B. Dzantiev, E.V. Yazynena, A.V. Zherdev, Y.V. Plekhanova, A.N. Reshetilov, S.-C.Chang, C.J. MxNeil, *Sens. Actuators B-Chem.*, 98 (2004) 254.
[106] M.F. Yulaev, R.A. Sitdikov, N.M. Dmitrieva, E.V. Yazynina, V. Zherdev, B.B. Dzantiev, *Sens. Actuators B-Chem.*, 75 (2001) 129.
[107] R.D. Harris, B.J. Luff, J.S. Wilkinson, J. Piehler, A. Brecht, G. Gauglitz, R.A. Abuknesha, *Biosensors Bioelectron.* 14 (1999) 377.
[108] F.F. Bier, R. Jockers, R.D. Schmid, *Analyst* 119 (1994) 437.
[109] J. Zeravik, T. Ruzgas, M. Franek, *Biosen. Biolelectron*. 18 (2003) 1321.
[110] J. Tschmelak, G. Proll, J. Riedt, J. Kaiser, P. Kraemmer, L. Barzaga, J.S. Wilkinson, P. Hua, J.P. Hole, R. Nudd, M. Jackson, R. Abuknesha, D. Barceló, S. Rodríguez-Mozaz, MJ Lopez de Alda, F. Sacher, J. Stien, J. Slobodnik, P. Oswald, H. Kozmenko, E. Korenkova, L. Tothova, Z. Krascsenits, G. Gauglitz, *Biosensors Bioelectron*. 20 (2004) 1509.
[111] M.A. Carlson, C.B. Bargeron, R.C. Benson, A.B. Fraser, T.E. Phillips, J.T. Velky, J.D. Groopman, P.T. Strickland, H.W. Ko, *Biosensors Bioelectron*., 14 (2000) 841.
[112] W. Mullett, E.P.C. Lai, J.M. Yeung, *Anal. Chem*., 258 (1998) 161.
[113] S. Hauck, C. Kosslinger, S. Drost, H. Wolf, *Lebensmittelchemie* 52 (1998) 158.
[114] J.McElhiney, L. Lawton, *Toxicol. Applied Pharmacology*, 203 (2005) 219.
[115] B.D. Spangles, E.A. Wilkinson, J. T. Murphy, B.J. Tyler, *Anal. Chim. Acta*, 12 (2001) 149.
[116] I. Abdel-Hamid, D. Ivnitski, P. Atanasov, E. Wilkins, *Biosensors Bioelectron*. 14 (1999) 309.
[117] M.A. Carlson, C.B. Bargeron, R.C. Benson, A.B. Fraser, T.E. Phillips, J.T. Velky, J.D. Groopman, P.T. Strickland, H.W. Ko, *Biosensors Bioelectron*. 14 (2000) 841.
[118] D. Nedelkov, A. Rasooly, R.W. Nelson, *Int. J. Food Microbiol*. 60 (2000) 1.
[119] K.E. Sapsford, M.N. Ngundi, M.H. Moore, M.E. Lassman, L.C. Shiver-Lake, C.R. Taitt, F.S. Ligler. *Sens. Actuators B-Chem.*. 113 (2006) 599.
[120] I.S. Park, N. Kim, *Biosensors Bioelectron.* 13 (1998) 1091.
[121] L. Rasooly, A. Rasooly, *Int. J. Food Microbiol*. 49 (1999) 119.
[122] J.Homola, J. Dostálek, S. Chen, A. Rasooly, S. Jiang S.S. Yee, *Int. J. Food Microbiol*. 75 (2002) 61.
[123] K.D. King, G.P. Anderson, K. E. Bullock, M. J. Regina, E. W. Saaski, F. S. Ligler, *Biosen. Bioelectron*. 14 (1999) 163.
[124] N. Adányi, M. Varadi, N. Kim, I. Szendrö, *Current Appl. Phys.* 6 (2006) 279.
[125] C.T. Elliot, G.A. Baxter, S.R.H. Crooks, W.J. McCaughey, *Food Agric. Immunol*. 11 (1999) 19.
[126] L. Wahlstrom, in Proceedings of the 25th International Dairy Congress, Aarhus, September 1998. Volume 2: Dairy Science and Technology, Danish National Committee of the IDF, Aarhus, 1999, p. 344.
[127] V. Gaudin, M.L. Pavy, *J. AOAC Int.* 82 (1999) 1316.
[128] Stanker (Editors), Immunoassys for Residue Analysis, Food Safety, American Chemical Society, Washington, DC, 1996, p. 463.
[129] M.R.S. Fuh, L.W. Burgess, G.D. Christian, *Anal. Chem.* 60 (1988) 433.
[130] G. E. Pellegrini, G. Carpico, E. Coni, *Anal. Chim. Acta 520* (2004) 18.
[131] J.L. García, C.J. Nuñez, E.G. Gonzalez, J. Osuna, X. Soberón, E. Galindo, *Appl. Biochem. Biotechnol.* 73 (1998) 243.

[132] M. Hishi, Y. Sasamoto, M. Nonaka, K. Toyama, E. Watanabe, *Biosensors Bioelectron.* 6 (1991) 15.
[133] A. Mulchandani, P. Mulchandani, I Kaneva, W. Chen, *Anal Chem*. 70 (1998) 4140
[134] D.Figeys, D. Pinto, *Anal. Chem*. 72 (2000) 330.
[135] J.M.Laval, P.E. Mazeran, D. Thomas, *Analyst* 125 (2000) 29.
[136] D. P. Kalogianni, T. Koraki,T. K. Christopoulos, P.C. Ioannou, *Biosensors Biolectron.* 21 (2006) 1069
[137] K. Haupt, K. Mosbach, *Chem. Rev*. 100 (2000) 2495.
[138] K. Haupt, *Analyst* 126 (2001) 747.
[139] B. Sellergren, *Anal. Chem*. 66 (1994) 1578.
[140] www.elipsa.de.
[141] www.miptechnologies.se.
[142] G. Theodoridis, P. Manesiotis, *J. Chromatogr.* A 948 (2002) 163.
[143] R. Weiss, A. Molinelli, M. Jakusch, B. Mizaikoff, *Biosep.* 10 (2001) 379.
[144] A. Molinelli, R. Weiss, B. Mizaikoff, *J. Agric. Food Chem.* 50 (2002) 1804.
[145] R. Weiss, M. Freundenschuss, R. Krska, B. Mizaikoff, *Food Add. Contam*. 20 (2003) 386.
[146] J. Jodlbauer, N.M. Maier, W. Lindner, *J. Chromatogr. A* 945 (2002) 45.
[147] I. Ferrer, D. Barceló, *Trends Anal. Chem*., 18 (1999) 180.
[148] T.A. Sergeyava, H. Matuschewski, S. A. Piletsky, J. Bendig, U. Schedler, *J. Chromatogr. A.* 907 (2001) 89.
[149] T.L. Panasyuk, V.M. Mirsky, S.A. Piletsky, O.S. Wolfbeis, *Anal. Chem*., 71 (2001), 720.
[150] S.A.Piletsky, E.V. Piletskaya, A.V. Elgersma, K. Yano, I. Karube, , *Biosens Bioelectro*, 10 (1995) 959.
[151] M. Lahav, A.B. Kharitonov, O Katz, T. Kunitake, I. Willner, *Anal. Chem*. 73 (1994) 1578.
[152] D. Kritz, K. Mosbach Chen *Anal. Chim. Acta* 300 (1995) 71.
[153] A. Pizzariello, M. Stredansky, S. Stredanska, S. Miertus, Sens. Actuators B-Chem., 76 (2001) 286.
[154] M. Zougagh, A. Rios, M. Valcarcel *Anal. Chim. Acta* 539 (2005) 117.
[155] L. Feng, Y. Liu, Y. Tan, J. Hu, *Biosensors Bioelectron*. 19 (2004) 1513.
[156] F.L. Dickert, M. Tortshanoff, W.E. Bulst, G. Ficherauer *Anal. Chem.* 71(1999) 4559.
[157] H. Zhou, Z. Zhang, D. He, Y, Hu, Y, Huang, D. Chen, *Anal. Chim. Acta* 523 (2004) 237.
[158] E. Blahová, J. Lehotay, Ivan Skačáni, *J. Liq. Chromatogr. Relat.Technol.*, 27 (2004) 2715.
[159] R. Weiss, A. Molinelli, M. Jakusch, B. Mizaikoff, *Biosep.* 6 (2002) 379.
[160] P. D. Martin, G.R. Jones, F. Stringer, I. D. Wilson, *Analyst* 128 (2003) 345.
[161] V. Crescenzi, G. Masci, M. Fonsi, G. Casati, American Chemical Society meeting, held in Boston, USA (August 1998).
[162] R. Krska, E. Welzig , F. Berthiller, A. Molinelli, B. Mizaikoff, *Food Add.Contam.* 22 (2005) 345.
[163] I. Chianella, S.A. Piletsky, I.E. Tothill, A.P. Turner, *Biosensors Bioelectron*. 18 (2003) 119.
[164] SN Zhou, EPC Lai, JD Miller, *Anal.Bioanal. Chem*. 378 (2004) 1903.
[165] J. Whitcombe, C. Alexander, E.N. Vulfson, *Trends Food Sci. Tech.* 8 (1997) 140.
[166] K. Skudar O. Brüggemann, A. Wittlesberger, O. Ramström, *Anal. Commun.* 36 (1999) 327.
[167] M. Matsui, M. Okada, T. Tsuruoka, T. Takeuchi, *Anal. Commun.* 34 (1997) 85.

[168] N. Masque, R.M. Marce, F. Borull, P.A.G. Cormack, D.C. Sherrington, *Anal. Chem*. 72 (2000) 4122.
[169] I. Ferrer, A. Tolokan, V. Horvath, B. Sellergren, G. Horvai, D. Barceló, *Anal. Chem*. 72 (2000) 3934.
[170] K. Haupt, A. Dzgoev, K. Masbach, *Anal. Chem*. 70 (1998) 628.
[171] R. Koeber, C. Fleischer, F. Lanza, K.S. Boos, B. Sellergren, D. Barcelo, *Anal. Chem.* 73 (2001) 2437.
[172] F. Chapuis, V. Pichon, F. Lanza, S. Sellergren, M.C. Hennion, *J. Chromatogr. A* .30 (2003) 2.3
[173] F.L. Dickert, O. Hayden, *Trends Anal. Chem*,.18 (1999) 192.
[174] S. Kröger, A.P. Turner, K. Mosbach, K. Haupt, *Anal. Chem*. 71 (1999) 3698.
[175] R. Shoji, T. Takeuchi, I. Kubo, *Anal. Chem.* 75(2003):4882.
[176] R. Suedee, T. Srichana, C. Sangpagai, C. Tunthana, P. Vanichapichat, *Anal. Chim. Acta*. 504 (2004) 89.
[177] M. Lotierzo, O. Y. F. Henry, S. Piletsky, I. Tothill, D. Cullen, M. Kania, B. Hock, A. .P. F. Turner, *Biosensors Bioelectron.*, 20(2004) 75.
[178] J.C.C. Yu, E.P.C. Lai, *Reac. Func. Polm.* 63 (2005) 171.
[179] A. Guzmán-Vázquez de Prada, P. Martínez-Ruiz, A.J. Reviejo, J.M. Pingarrón. *Anal. Chim. Acta* 539 (2005) 125–132
[180] C. Liang, H. Peng, X. Bao, L. Nie, S. Yao, *Analyst* 124 (1999) 1781.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 17

Atomic Absorption Spectroscopy

Suleyman Akman, Birsen Demirata-Ozturk and Nilgun Tokman

Istanbul Technical University, Turkey

1. Introduction

The main source of elements (toxic and non-toxic; trace and major) in food samples (vegetables, animals, food products originated from plants or animals, water, drinks, beverages) is environment. Elements and other components are included into food materials during nutritional chain, production, storage, etc. Therefore, analyses of environmental sources, which may be directly or indirectly connected with the sample, e.g. soil, fertilizer, irrigation water, pesticides and waste of nearby plants for drinking water, vegetable and fruit analysis; animal feed and water for animal products (meat, dairy products etc) analysis; sea-water, sludge and waste of plants for fish farming, etc are highly important to find the origin of analytes in food samples.

In food industry trace element analysis is significant. Thus, in recent years widespread research is being carried out in that field. Analytical methods and their implementation procedures (limit of detection, precision, accuracy, speed, etc.) depend on the analyte and its concentration in the sample.

Atomic absorption spectrometry (AAS) is one of the most common methods for the determination of trace elements in all matrices. Therefore standard methods for the analysis of trace elements are mostly based on AAS. In this chapter, the general principles of AAS are explained by stressing food analysis specially.

2. Theory of atomic absorption spectroscopy

AAS is principally used for the trace quantitative analysis of almost seventy metals and metalloids. It is based on the absorption of UV-visible electromagnetic radiation by gaseous atoms. AAS is not suitable for (i) analysis of ametals because of their resonance absorbance in vacuum UV (<200 nm) where the components of air (N_2, O_2) absorbs the radiation, and (ii) qualitative analysis because the method requires the change of radiation source for each analyte element and adjustment of instrumental conditions every time so that any qualitative analysis would be a series of time consuming step-wise processes limited with the lamps in the laboratory. This is the most important disadvantage of the method against emission techniques. Nowadays, a continuum source AAS (CS AAS) instrument has been developed so that a genuine ultra-fast sequential multielement analysis is implemented without the need for a multitude of different light sources.

3. Atomic absorption spectrophotometers

Simple block diagrams of a single beam alternative current (a.c) and a double beam alternative current atomic absorption spectrophotometers are shown in Figure1. The radiation emitted from a spectral line source (mostly hollow cathode lamps or less frequently electrodeless discharge lamps) is modulated electronically (pulsing of lamp current) or using a chopper (rotating metal disc) and intermittently passed through the atomizer (flame or graphite furnace) where gaseous analyte atoms are obtained thermally and absorption occurs.

The resonance line (or the line to be used for analysis) is selected by a monochromator (grating) and converted to electrical signal by the detector (e.g. photomultiplier). In addition, there are many optical parts (lenses, mirrors etc) to direct the radiation in the instrument and many electronic circuits for signal processing (controlling the instrument, graphite furnace, autosampler, transformation of signals to absorbance, concentrations etc).

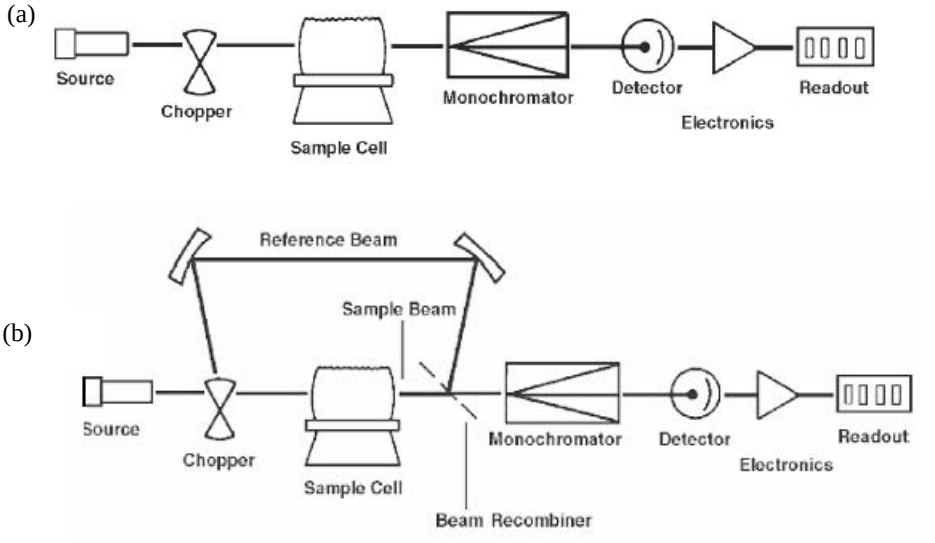


Figure 1. Block diagrams of a single beam (a) and double beam (b) atomic absorption spectrophotometers

Hydride Generation and Cold Vapor Techniques: As, Sb, Se, Te, Bi, Sn, Tl, Pb and Cd can be converted to their volatile hydrides in a reaction vessel upon reduction reaction of analyte element with metallic zinc or sodium tetrahydroborate ($NaBH_4$) in dilute acidic solution [1,2].

The hydrides formed are transported into flame (very seldom), graphite tube or most frequently to a heated quartz cell placed on the optical pathway with the flow of inert gas (N_2, Ar). This technique is known as hydride generation AAS (HG-AAS). Mercury is determined using $NaBH_4$ or $SnCl_2$ for the reduction of analyte to its volatile metallic form in the same reaction vessel as that used for hydride generation. The only difference is that no heating is applied to obtain gaseous mercury atoms because it is already in elemental form with high vapour pressure after reduction reaction. Thus the method is called as cold vapor AAS (CV-AAS).

Both methods are mostly free of interferences because analyte elements in the atomic form (Hg) or in hydride from are separated from the sample solution and matrix is not present in the absorption cell. However, matrix may influence the hydride formation. In order to further preconcentrate the analyte elements, gaseous hydrides or elemental Hg are collected by different traps before reaching atomizer unit. Mercury is collected on a gold sponge as amalgamate. The trap is then heated to vaporize mercury [3]. Hydrides are passed from a liquid nitrogen trap and condensed by freezing. During the trapping step, the analyte is concentrated and excess hydrogen formed in the reaction vessel is removed [3]. The hydrides trapped (frozen) are then released by heating and conducted to the heated atomizer where they are thermally decomposed. Another possibility for the collection and preconcentration of analyte is to heat the gaseous hydride when it passes through the atomizer i.e. graphite tube or heated quartz cell. The temperature is chosen so that hydride is decomposed thermally whereas the analyte element is not vaporized. The analyte is then vaporized upon heating the atomizer to higher temperatures [3].

Different instrument manufacturers produce various cold vapor or hydride generation units. The details of those generators are out of scope of this chapter.

4. Interferences

Quantification with AAS is based on the comparison of absorbance for analyte in standard (mostly matrix-free analyte solution) with that in sample matrix assuming that their sensitivies are the same, i.e. the same concentrations of analyte in standard and sample cause the same absorbances. This is a mandatory condition for accuracy but mostly cannot be provided due to interferences. Interference is the depressive or increasing effects of matrix on the sensitivity of analyte. Interferences are divided into two main groups namely spectral and non-spectral interferences [1,4]

4.1. Spectral Interferences (Background absorption)

Spectral interferences are originated from the attenuation of analytical line of primary source by matrix contaminants at the wavelength to be used for quantification. The causes of spectral interferences are the absorption of analytical line (analyte wavelength) by gaseous atoms of other elements, gaseous matrix molecules or their decomposition products as well as scattering of analytical line of analyte by solid or liquid microparticles, which are neither vaporized nor dissociated in flame or atomization stage of graphite furnace.

Spectral interferences are principally eliminated or corrected chemically by pretreatment of sample prior to determination or instrumentally during the measurement of absorbance step. By using an appropriate flame type, e.g. high temperature flames for stable compounds, adjusting the most suitable fuel to oxidant ratio and burner height, the concentration of molecular species and/or radiation scattering particles causing spectral interferences are reduced so that the background attenuation is negligible. Formation of refracting metal oxide particles can be prevented by working with fuel rich flames (i.e. lowering oxygen concentration in flame) [5]. In graphite furnace, some molecular species which are more volatile than analyte species are removed thermally by vaporizing during the pyrolysis step as well as some interfering molecules e.g. sulphates or hydrated chlorides are converted to their oxides upon thermal decomposition or hydrolysis, respectively which do not cause molecular absorption [6]. Some chlorides can be converted to their nitrates, which further decompose to oxide.

4.1.1. Instrumental Background Correction

If the composition of blank is exactly known, molecular absorption value is measured and subtracted from total absorption for sample (background attenuation). However, the most practical and versatile technique for the elimination of molecular absorption is the use of instrumental background correction systems. The principle of all background correction systems is based on measurement of total absorbance (atomic and background) and the background attenuation, and finally, subtraction the latter from the former. The frequency of measurements is synchronized with that of modulation. Three background correction techniques are available in AAS instruments:

- Continuum sources
- Zeeman effect
- Self reversal (Smith-Hieftje)

The details of those techniques can be found in AAS textbooks [1-3]

4.2. Non-Spectral Interferences

Non spectral interferences are the effects causing the change of the sensitivity of the analyte for sample (i.e. the number of gaseous analyte atoms per its unit concentration in sample) with respect to that for standards. In this case, the same concentrations of analyte in

sample and standard would be different resulting in wrong results. Non spectral interferences are originated from reducing or enhancing effects of matrix concomitants and/or chemical form and/or oxidation state of analyte on the number of gaseous analyte atoms which are responsible from atomic absorption. If the analytes in matrix-free standard and in sample are influenced equally e.g. due to oxide or carbide formation, incomplete atomization then it is not described as interference because the analytes in standards and/or sample have still the same sensitivies resulting in accurate results.

The origins of interferences have been extensively studied by many workers and literature is full of different interpretations for the effects of various interferents on the determination of analyte elements [1,7-9].

4.2.1. Nonspectral Interferences in Flame

The most common interference in flame is the formation of a thermally stable compound between the analyte and the matrix constituents. A typical example is the determination of calcium or any alkaline-earth element in the presence of phosphate in air-acetylene flame. Calcium phosphate is formed upon drying of sample droplets which is further converted to thermally stable pyrophosphate resulting in lower atomization efficiency and thus lower absorbance compared to calcium nitrate or chloride used as a standard. These kinds of interferences are reduced or completely eliminated using a high temperature flame, e.g. acetylene-nitrous oxide flame in order to decompose the stable compound effectively or adding a releasing agent into the sample and standard solutions. Releasing reagent reacts preferentially with interfering ion forming a stable compound so that less amount of (or practically no) interferent remains to interact with analyte. For example La or Sr, which are known to form very stable compound with phosphate, are used as releasing agents for phosphate interferences. Another possibility is to add a reagent, which preferentially reacts with analyte forming an easily decomposing compound. By this way, analyte cannot react with the interfering ion. Especially, complexing agents such as EDTA (ethylene diamine tetra acetytic acid) or APDC (ammonium pyrolidine dithio carbamate) are suitable for this purpose. Another interference in the flame is gas-phase combination reaction between analyte atoms and decomposition products of matrix. For example, in the presence of excess chloride, gaseous analyte atoms react with free chlorine atoms in the gas-phase decreasing the free analyte atoms. If there are other metals in the gas phase originating from the sample, they may react with interfering species reducing the effect of interference.

The differences between some physical properties of sample and standard solutions may cause interferences as well. If the viscosity, surface tension and density of sample are different from those of standards, solution uptake rate as well as average droplet size of aerosol may change. In this case, the amount of solution reaching to flame per unit time is not the same for the sample and standards. Many organic solvents have lower surface tension and viscosity resulting in higher sample uptake rate, smaller aerosol droplets, better nebulization

efficiency (less solution to drain) and consequently higher absorbance with respect to aqueous solutions of the same analyte concentration. In addition, combustion of organic solvents is an exothermic reaction increasing the flame temperature whereas vaporization of water (drying of aqueous droplets) is endothermic lowering the flame temperature. If the sample is originally organic and/or dissolved in organic solvent while standard is prepared in water, the sensitivies of analyte in sample and standard would not be the same causing wrong (positive) results. The physical interferences can be solved by matrix matching of sample and standard, standard addition method and if possible diluting the sample with water.

4.2.2. Non-spectral Interferences in ETAAS

The origins of non-spectral interferences in ETAAS are (i) formation of a volatile compound of analyte with matrix constituents in the condensed phase before atomization step, e.g. volatile chlorides of analytes in the presence of chloride-containing matrices which may loss in the pyrolysis step at relatively low temperatures [6,7,10-14] ; (ii) combination reaction between analyte atoms and matrix decomposition products in the gas phase in the atomization step [13-16]; (iii) formation of a thermally stable compound between analyte and matrix constituents in the condensed phase during drying and pyrolysis steps [7,8,17,18]. (iv) Occlusion of analyte in microcrystal or agglomerates of matrix which are thrown out of tube without decomposing in the atomization step and lower sensitivities are the result [19-21]. (v) Expulsion of analyte atoms out of tube by rapidly expanding matrix gases due to ultra fast heating rate in the atomization step [6,8,11,21].

The differences between physical properties (viscosity, surface tensions, density) of standards and samples are less troublesome in ETAAS compared to flames. Generally the use of matrix-matching standards is not solution to all kinds of interferences because (i) the exact qualitative composition of sample is not known (ii) it is nearly impossible to find analyte-free reagents to prepare matrix matching standards and blanks (iii) the interferences depend on the analyte as well as analyte to matrix ratio which is already unknown.

Standard addition technique has some limited benefits especially for physical interferences.

The processes for the separation of matrix such as extraction, ion-exchange, co-precipitation prior to analysis are tedious, time consuming and unpractical. In addition, the risk of contamination and loss of analyte is high. Recovery of separation process is not always complete and reproducible causing errors and low precision.

Finally, at routine analysis laboratories, the practical (simple, easy, fast etc) procedures with minimum efforts are always preferred by the analysts.

*Precautions for the elimination of interferences in ETAAS***:** The first step for a interference-free determination is to optimize the time–temperature program of graphite furnace for sample. The most suitable furnace program for sample may be different from that for standard. Even if an optimum furnace program for sample is fixed, the sensitivities for

analytes in standards and sample may not be the same due to interferences. The requirements to eliminate all possible interferences were stated by Slavin et al. [22] as Stabilized Temperature Platform Furnace (STPF) concept:

(i) Instead of pipetting the solution directly onto the tube wall, sample should be pipetted onto a platform, which is a graphite plate placed on the bottom of the tube. When the analyte is atomized from platform into a hotter gas- phase environment, recombination reactions between gaseous analyte atoms and matrix decomposition products are less likely.

(ii) In the atomization step, the maximum (fastest) heating rate should be applied to increase the temperature difference between platform and gas-phase. By this way, decomposing rate of molecules containing analyte, i.e. atomization rate, is increased whilst gas-phase combination reaction interferences are suppressed resulting in higher free analyte atom.

(iii) Peak area (integrated absorbance) rather than peak height should be used for quantification. The maximum peak height represents the absorbance when the atom formation rate is equal to dissipation rate both of which are directly influenced from matrix (i.e. interferences) and instrumental conditions. On the other hand, peak area is directly proportional to the number of absorbing atoms and independent from kinetics. Thus even if the atomization kinetics change, the sensitivity remains the same as long as the number of atoms does not change.

(iv) If the flow of purge gas is stopped during the atomization step, the residence time of atoms in the tube increase. In addition, the final temperature of gas-phase (thermal equilibrium) is more quickly reached, because it is not cooled by the inert gas resulting in less extent of gas phase combination reactions and higher decomposition efficiency of analyte-containing molecules. All those effects cause higher sensitivity for the analyte in the sample and standards.

(v) Detection systems should be fast enough to prevent signal distortion due to rapidly changing signals.

(vi) Atomization efficiency depends on the surface properties, which changes with the age of graphite tube. The surface of polycrystalline electrographite used for the manufacture of graphite tubes becomes more and more porous and reactive when subjected to high temperature. Thus, the sensitivity changes when the tube is used. In order to protect the surface, it is coated with pyrolytic graphite [23]. Pyrolytical coated or total pyrolytic graphite tubes and platforms are available commercially.

(vii) The Zeeman background system, which is the most powerful background correction method should be used for the compensation of spectral interferences. The most remarkable advantage of Zeeman system is that it eliminates the background attenuation up to a 2.0 A exactly at atomic absorption wavelength but other systems cannot do it. The other systems cannot make a correction at atomic absorption

wavelength (they measure the BG value in a band) and correction capabilities are limited to around 0.7 A.

In spite of all those precautions, STPF concept is meaningful only with the use of an appropriate modifier. Modifier is a chemical reagent added to standards and samples to match their physical and chemical properties as well as to separate the matrix from analyte before atomization step by decreasing the volatility of analyte (analyte modifier) and/or increasing the volatility of matrix (matrix modifier). Modifier converts the analyte into its a less volatile compound so that higher pyrolysis temperatures can be applied to remove the matrix before atomization step without loss of analyte. Another possibility is that modifier may occlude the analyte and thereby prevents its volatilization physically during the pyrolysis step. Alternatively, modifier reacts with matrix. The reaction products are volatile enough to be removed from the furnace at relatively lower pyrolysis temperatures without loss of analyte as well as some reaction products does not cause any interference. Even if the modifier changes the sensitivity of analyte, since the same sensitivies are obtained for analyte in sample and standard, the results would be accurate.

Numerous papers have been published in which different reagents were proposed as the appropriate modifiers for selected analyte elements in different samples. The mixture of palladium nitrate-magnesium nitrate, proposed by Schlemmer and Welz [24] has been accepted as the universal modifier being suitable for majority of elements determined in AAS and it is well-advised by all manufacturers' instrument cookbooks. The chemical producers sell this mixture as "modifier for atomic absorption spectrometry".

5. Miscellaneous Sample Introduction Systems

In order to avoid the above-mentioned errors, risks and drawbacks of digestion procedure, such as loss of analyte by volatilization or sorption vessels and laboratory atmosphere, incomplete dissolution, loss of time during digestion, consumption of expensive ultrapure reagents and pollution of laboratory atmosphere due to acid vapours, in some applications, samples are introduced into the graphite furnace either directly (solid sampling) or after being slurried. The both techniques are not convenient for FAAS.

5.1. Solid sampling

All the attempts for direct solid sampling in ETAAS have been made with modified furnaces, platforms and sampling tools for research purposes and not supported by AAS manufacturers except one, which developed a commercial solid-sampling AAS. This instrument is equipped with a fully automatic solid-sampling autosampler. Special platforms with and without samples and standards are transported to a built-in closed balance and into the furnace automatically by means of robotic arm of autosampler (Figure 2). Nevertheless,

most of the above-mentioned drawbacks except instrumental problems still cause problems in solid-sampling analysis.

Figure 2. Solid sampling autosampler (with kind permission of Analytik Jena SSA 61)

5.2. Slurry sampling

In order to eliminate some disadvantages of solid sampling and solution techniques whereas to make use of their many advantages, solid samples are first finely ground, slurried with an appropriate liquid mostly adding surfactants, e.g. Triton-X 100, homogenously mixed manually, ultrasonic agitation and finally pipetted into the furnace by means of a micropipette or autosampler. Slurry technique is between solution and solid sampling methods. The superiority of the method in comparison with solution technique is that no digestion is necessary.

The advantages of slurry technique in comparison with solid sampling can be summarized as follows: Samples can be homogenously and easily (but not unlimitedly) diluted. The amount of sample slurried is much greater than that introduced into the furnace in solid-sampling. Since a greater portion of sample is represented, heterogeneity of analyte distribution in micro scale range does not cause any precision problem. The last but not least, no any sample introduction system is necessary for slurry technique. Slurries are directly

pipetted into the furnace like solutions. The method has no additional cost with respect to instrumentation.

However the preparation of slurry is sometimes troublesome. Solid samples should be finely ground. The slurry prepared should stay homogenous during analysis, i.e. particles should not precipitate and the concentration of solid particles in slurry should be the same everywhere. This is provided using surfactants (e.g. Triton X-100), mixers, air bubbles, ultrasonic probe etc. This priority limits the concentration of slurry (solid to liquid ratio, total volume, particle size). All those parameters require extra effort and optimization of slurry parameters. In addition a blank control is necessary.

Commercially an autosampler modified for slurry pipetting is available. In this system, the slurry put into a special vial of the autosampler is continuously stirred with an ultrasonic probe in order to provide homogenous slurry.

6. Determination of Toxic Metals

At that moment, number of known elements is 106. In those, 84 of them are metals. Metals are spread in environment through geological and biological cycles. Metals, which may cause detrimental impacts when they are taken into live metabolism, are defined as “toxic metals” [27-29]. Toxicity depends on the kind of metal and living metabolism. Poisoning occurs even in low concentrations of highly toxic metals. When taken at high or low amounts, essential metals may create toxic effect by disrupting body homeostasis. Toxic metals are taken into living metabolism through air, water and especially from foods. The numbers of toxic metals are fewer than non-toxics. Some parts of them are found in ground in form of trace level and may have much more detrimental impacts. (Metals, which are found in ground lower than 1/1000 ratio, are called as trace metals). Majority of metallic contaminations are accumulated in waters. As accumulation in water supply can be in form of dissolve in water it can also be in form of torture in water. Metallic contaminations cannot be broken down by means of chemical and biological ways like organic contaminations. However a metal compound can be converted into another compound. Drinking waters may be polluted by underground waters containing toxic metals.

Toxic metals may accumulate in food structure intensively and poison humans and animals. During the year 1952, Hg toxication defined as “Minamata illness” was found in many individuals after consuming fish and living in waters which is contaminated by factory sewage materials from the factory using high amounts of mercury as catalizator during plastic production for Minamata bay. Similar Hg toxication was found in many other countries as a result of using fungicides containing mercury during grain production [30]. Many metals including toxics are accumulated in human body. For example; half life duration of Pb in human body is 1460 days, Cd’s is 200 days, Zn’s is 933 days so on. Despite those effects, metals have important effects in technological development and they are a part of our life. In

the solutions for important problems presently metals are used. Humans always deal with metals during collecting ores from ground, bringing them into possible form for implementation using and throwing them environment.

The elements which are present at concentrations lower than 100 μg/g in a sample are described as trace elements whereas ultra-trace elements are found in too much smaller amounts (lower than 10 ng/g) [31]. Despite their low concentrations, trace and ultra-trace elements play very important roles in many areas (including their impacts for our health). In terms of their impacts for living organisms, trace elements can be classified as essential and non-essential elements. If inadequate supply of an element causes physiological and metabolism illnesses repeatedly and if that is cured by using the same element it is classified as an essential element. In addition to 11 major elements defined as essential (H, C, N, O, Mg, Ca, P, S, Cl, Na and K), the trace elements F, Si, V, Cr, Mn, Fe, Co, Ni, Zn, Cu, Se, Mo, Sn and I are also termed as essential elements. Concentrations of these elements in body are 1/10000 to 2/10000 or below 50 mg per kg body**.** Like all chemicals or drugs, the excess of essential elements causes toxic effects and health problems. Therefore, not only the lack of essential elements but their excessive amounts above a certain limit cause problems for living bodies (human, animal and plants).

As it is known, iron is an essential element. Hemoglobin contains iron keeping oxygen thus providing oxygen release to tissues from lungs. Underlying factor for inadequate iron may be lack of iron supply in foods consumed or a high requirement for iron. In developing countries, since many individuals provide their nutrition almost by vegetables and grain consumption, inadequate iron intake may be seen and pregnant women and children of 4 and 24 months constitute the serious risky group. On the other hand, soluble iron salts, if taken over 0.5 g, may cause illness, which can lead to intense impairment in digestion system such as hepatitis. Excessively high iron intake cause cirrhosis [32].

Non-essential elements are present in living organisms but their essential amounts are not certainly known. Pertaining to advancements in analysis methods, apart from elements having vital importance, 40-50 elements are found to be present in human body. Their functions, intake ways to body, their benefits and harms are not entirely known yet.

Some toxic elements such as Cd, Hg and Pb have detrimental effects in living organisms even at their infinitesimal concentrations and classified as non-essential elements group. When these elements are found in foods they are called as “contaminant”.

Elements in foods are originated from different sources:

(i) They are varied naturally on the basis of the type of the soil. Plants grown in different districts have different minerals.

(ii) They are added into processed foods as additives.

(iii) They may be included from can, tin, metallic boxes or containers such as pipe, silo etc.

While daily dose for macro minerals (Ca, P, Na, K, Mg, Cl, S) is 100 mg, it is approximately 1 mg/day for micro elements (F, Cr, Mn, Fe, Co, Zn, Cu, Se and I).

6.1. Some Toxic Elements And Their Properties

Although all the elements (essential and nonessential) cause problems below and above a given range, a few toxic elements are described briefly in the following.

Arsenic (As) [33]

Arsenic is used in mine sewage process, glass manufacturing, pesticide production, in dyes, rat poisons, fungicides, etc. It is one of the most complicated elements. Especially in seafoods, it is found in 20 various chemical forms. Moreover, it has been found that sea and land organisms contain different arsenic species. Seafoods, especially mussel and shrimp, have the highest arsenic content.

Inorganic arsenic species –As(III)/As(V)- are much more toxic than organic arsenic species. In seafoods, organic-arsenic species are dominant.

Arsenic must be carefully treated for food safety. Its natural ratio in soil is 2.2 -25mg/kg. In England and USA, the permitted value in drinking water is below 0.05 mg/kg. In all materials including foods, As determinations (total or speciation) are carried out by GFAAS, HG-AAS, ICP-OES and ICP-MS. However, HG-AAS is, without doubt, the most preferred technique due to the reasons given previously.

Lead (Pb)

Lead is a grey colored soft metal. Although there are four electrons in valance shell, it can, generally, be ionized by releasing its only two electrons. Lead is a toxic metal having no function in body. Most significant lead sources are lead-based gasoline additives to improve the number of octane (tetra ethyl lead, TEL), lead-based dyes, lead-soldered canned boxes, ceramic glazes and industrial pollution. It is most frequently used in pesticides, pencils and batteries. In children it can cause decrease of cognitive ability and in adults it causes drop of blood pressure and increase of cardiovascular diseases. Lead influences bone mineralization because of its calcium-like behaviour in metabolism. In adults, 95% of lead is accumulated in skeleton. After investigations on children, in 1975, Centers for Disease Control (CDC) reduced the toxic lead level in blood from 40µg/dl to 30µg/dl; in 1985 to 25µg/dl and in 1991 down to 10µg/dl [34, 35]. Principally, lead is transferred to plants via soil, strictly speaking, the water from soil. Roots contain higher lead concentration than body and leaves whereas in seeds and fruits it is at lowest concentration. The lead in the air may be attached to leafy vegetables. In vegetables grown in or around city centers, lead accumulation increase [36]. Its natural level in soil is 10-84 mg/kg and permitted value in drinking water is <0.05 mg/kg in England and <0.005 mg/kg in USA. Daily oral lead intake in USA on an average is 100 µg while in some European countries it is below 30 µg [37-40].

Mercury (Hg)

Mercury is the only liquid metal at room temperature. It is estimated that annually 20.000 tons of mercury spread into environment from mine ore beds, volcanic activations, solid and liquid fossil based fuels. Metallic mercury and its inorganic and organic compounds are implemented at least in 80 industrial areas by over 300 different ways. In soil, its natural

abundance is 0.02-0.41 mg/kg; in drinking water its permitted ratio in England is <0.001 mg/kg [41-43]. It is used in pesticides and in catalyzers. Organometallic mercury compounds such as methyl-Hg are very toxic [44].

Generally, mercury effects central nerve system and kidney. As metallic mercury vaporizes easily, it may lead to fatal corrosive bronchitis and pneumonia. Even after recovery, long-term effects may appear in the body. Chronic exposure is frequently defined as mercuryalizm. Tremor, thyroid development, tachycardia, irregular pulse, gingivitis may occur. Coordination disruption of motor movements, behavioral corruption, nervousness, memory loose, depression and delirium are the neuropsychiatry symptoms as a result of influences over central nerve system [45].

Cadmium (Cd)

Cadmium is a silver color metal. In air, it is rapidly transformed into cadmium oxide. Inorganic salts such as cadmium sulphate, cadmium nitrate, and cadmium chloride are dissolved in water. In zinc mineral it is found in a very low amount in the form of $CdCO_3$ or CdS. Since cadmium is found nearly in all zinc ores, it is provided as side product during its zinc refining. Cadmium compounds are used in alloys, alkali batteries, plastics (as stabilizer or pigment) and metal plating. It is present in soil, fertilizers, mushroom, cigarette and pesticides [46].

Cadmium pollution is caused by cadmium containing goods and materials thrown into the environment or their burning in incinerators or by activations carried out during cadmium usage.

In soil, its natural ratio is 0.06-1.1 mg/kg; in drinking water maximum permissible concentrations are 0.005mg/kg in England and 0.01 mg/kg in USA. Cadmium and its compounds are highly toxic. Cadmium in the air is transferred to the soil as a result of dry and wet accumulating processes, and from here to food chain by means of plants. It was found that if concentration of cadmium in the air, its accumulation in the soil will also increase and thus the amount of cadmium taken by plants will rise up [47].

In drinking water cadmium concentration changing normally between 0.1-2 µg/L range may rise up to 10µg/L. By considering a 2 L of water consumption daily, the amount of cadmium taken into the body only by water may be estimated to be 1-10µg/day. According to nutrition habits, daily average intake of cadmium quantity from foods is 10-30µg [48-50].

Due to difficulty to remove cadmium from body, its detrimental impacts on health are observed by time. Kidneys are the most seriously and intensively influenced organs from long term exposure. If the concentration of cadmium accumulated in kidney is above 200 mg/kg (from wet weight), functional losses are observed. Cadmium in humans lead to digesting difficulty and genital inadequacy and it is suspected that it may give the effect of a cancerogenesis agent [51-53].

Selenium (Se)

Selenium is used in electronic, glass, ceramic, and steel and dye production industry as well as in medicine as an additive for diet and in anti-dandruff shampoos. Sodium selenide is a very significant essential trace element for warm blooded animals [54]. Selenium is one of the most powerful nutrition agents, which should be taken in sufficient quantity from foods and foodstuffs [55]. The effective dose of selenium in diet is 0.022 ppm (ED_{50}=2.2 g). It protects the cell from oxidative free radicals detrimental effects by diffusing into glutathione peroxides enzyme structure. Inadequate selenium intake leads to very different problems in humans and animals as well as in case of excessive intake, toxic effects are emerged [56--59]. In vegetables grown in selenium-rich soils, methyl selenosistein/selenosistation are accumulated and after those plants are eaten by beasts such as cow, sheep and goat, chronic selenium toxication is emerged and deaths are being occurred. One of toxic symptoms of selenium is respiration insufficiency [60,61].

6.2. Sample Mineralization and Mineral Analysis in Foods

Element analysis in all kinds of samples by AAS is carried out either directly i.e. without treating sample prior to measurement or after a series of pretreatment steps including sampling, homogenization, dissolution/digestion, separation of analyte from matrix, enrichment of analyte etc. As the the number of pre-treatment stages needed are reduced and/or simplified, errors are decreased. Therefore, an analysis method having the lowest number of pretreatment steps should be preferred [62].

In order to avoid the disadvantages of pretreatment procedures, different attempts have been made to analyse the solid samples directly (solid sampling technique) or with minimum treatment (slurry analysis technique). However, those techniques necessitate many requirements and have inavoidable drawbacks as mentioned previously. Some liquid food samples can be analysed by AAS after acidification, dilution and sometimes filtration [63].

On the other hand, in many cases, a multi-stage complicated analysis procedure may be necessary with the intention of providing accurate analytical result. If the interferences cannot be eliminated, elements, which are going to be analysed, should be separated from the matrix. Moreover, if the concentration of analyte is too low to be determined directly, then an enrichment procedure must be performed.

Determination of trace elements usually consists of 6 stages. Orders and numbers of those stages may change depending on the properties of samples:

i. Sampling, conservation, transportation and subdividing of sample
ii. Ashing (if necessary)
iii. Dissolution/digestion
iv. Qualitatitive analysis (if necessary and/or possible)
v. Separation/enrichment (if necessary)
vi. Quantitative analysis (AAS, ICP-OES,ICP-MS, Flame Emission)

The flowchart of all steps for the preparation and analysis of samples is depicted as a whole in Figure 3.

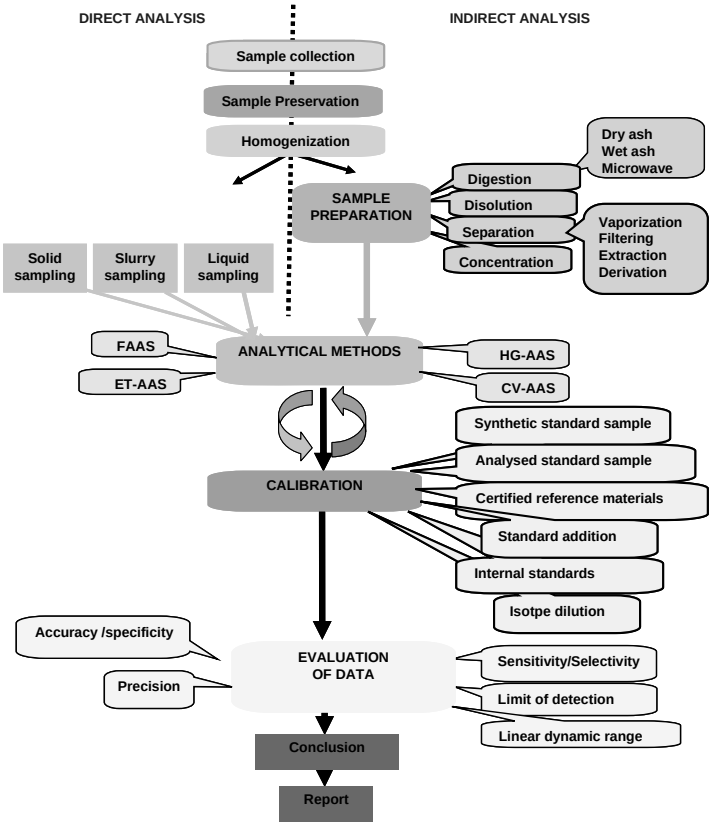


Figure 3. The flowchart of all steps for the preparation and analysis of samples

6.2.1. Ash, Decomposition/Digestion Procedures

Ash amount in a food material constitutes its total mineral content and varies roughly in a range of 0.5-15% depending on the sample and its humidity; for example it is around 0.3-0.8% in wet fruits and about 3% in dried fruits. While in milk it is 0.5-1% (mostly as calcium compounds), in fat-free milk powder that ratio is found around %8. In dried beef, ash amount may reach up to %15. The major mineral composition of a food material consists of such

elements as Ca, Na, K, Mg, P, Fe and Cl. However, trace elements such as Cd, Hg, Pb etc may be found as well because of contamination and environmental pollution. Mineral composition of plants is influenced from environment (soil, atmosphere and water) very much where they are grown. In other food materials, inorganic compounds may be found as additives (such as NaCl) or as contaminants in foods production processes.

Ashing procedure is performed by removing water and organic components in foods. Thus, the interference effect of organic matrix during measurement is eliminated. However, in dry ashing procedure the loss of some minerals such as As, Cd, Hg, Pb, Zn by volatilization should not be ignored and alternative procedures should be implemented. Following the ashing procedure, the analyte elements are transferred into solution.

Mineralization is defined as conversion organic compounds into inorganic compounds through various decomposition procedures. Mineralization is a crucial step in the determination of heavy metals in food products. During the analysis, most of the time is spent for this procedure. Therefore it is the most extensively studied subject in trace element investigations. The aim of those studies is to reduce the duration of procedure and solvent consumption as well as to simplify the procedure, to lower the blank values and to reach better precision and recovery parameters. There are three major procedures implemented during decomposition of foods as well as other samples [64, 65].

-Dry ashing (flame, muffle furnace, microwave)

-Wet ashing (with open or closed systems)

-Low temperature or plasma dry ashing

The decomposition procedure to be applied depends on the matrix of the food sample, instrumental method used for final measurement and the analyte element.

6.2.2. Dry Ashing

The homogenized solid samples are put in a crucible (steel, nickel, platinum or porcelain) and atmospheric oxygen is used as the oxidant. If the sample is liquid, it is dried keeping in an oven at 110ºC over night or longer. In some studies, pre-ashing is performed by adding ethyl alcohol to solid samples and keeping at 400ºC to accelerate the ashing procedure. Crucibles are then inserted into muffle furnace [66]. The sample is kept in the furnace at 500-700ºC until all material is ashed (approximately 2 hours**)**. Ashing is completed when ash color in crucible is light grey. Black color ash refers to incomplete ashing of the material and still organic parts remain. While organic materials are converted into CO_2, N_2 and water, minerals are converted into sulphate, phosphate, chloride and silicate. Crucibles are cooled to room temperature and nitric acid solution is added to dissolve the residue. The solution is taken into a volumetric flask after filtration. In order to prevent phosphate interference, a 3% of lanthanum chloride or strontium chloride solution is added and completed to the volume with distilled and/or deionized water or diluted acidic solution (mostly 0.2% HNO_3). Despite the advantages of using only a furnace and applicable to many elements and samples, dry ashing

method has some disadvantages such as contamination and/or vaporization losses of some elements. During dry ashing, the material of the crucible used is important. Quartz crucibles are particularly resistant to acids and halogens and not against bases. Porcelain crucibles have similar structure with quartz. However, they accumulate trace elements. For that reason, they must be washed with acids before every use. Although stainless steel crucibles are resistant to acids and bases, they are not used in the analysis of Ni and Cr elements. Platinum crucible should be preferred in all analyses owing to their inert structure. However, they are quite expensive compare to other crucibles.

Dry ash procedures can be modified depending on the sample and analyte. Different ash-aids are added to accelerate ashing and to prevent spattering. Solution of Mg acetate in alcohol, 1 to 2 drops of HNO_3, pure cotton or pure glycerine is some convenient ash-aid examples. Of course, in dry ash and all other decomposition techniques, a blank sample should be prepared to eliminate contamination and blank values originating from laboratory wares and reagents used [67, 68].

6.2.3. Wet Ashing

For oxidation of organic parts in food material, strong oxidants, usually oxidative mineral acids, are used. In wet ashing procedure, food samples are usually treated by acid in Kjeldahl flask. An aliquot of solid sample or solution, the amount of which depends on the approximate concentration of analyte, is put into a Kjeldahl. Usually concentrated nitric acid (HNO_3), sulphuric acid (H_2SO_4), and perchloric acid ($HClO_4$) at 7:1:1 (V/V/V) ratio is used. Apart from that, concentrated nitric acid (HNO_3), sulphuric acid (H_2SO_4) and (H_2O_2) mixture is also suitable [66]. The content of Kjeldahl flask is heated at relatively low temperatures as long as fuming of brown colored gaseous nitrogen oxides is completed and then kept at a higher temperature until no white fumes is appeared and the solution become clear. The content of the flask is cooled to room temperature, diluted with distilled/deionized water and filtered. The duration of procedure depends on the sample and solvent mixture, e.g. for a sample 8 hours is needed when (HNO_3+H_2SO_4) is used as the solvent whereas 10 minutes may be enough for the same sample in case of (HNO_3+$HClO_4$). After adding 3% lantanium chloride or strontium chloride, it is completed to a certain volume with distilled/deionized water [63, 64, 69]. The oily or fatty samples should be shaked with ether to remove the oil or fat before the above-mentioned process is applied.

6.2.4. Low Temperature Plasma Ashing

In low temperature plasma ashing procedure, special devices to which electromagnetic field are applied, are used. In those devices, oxidation is performed by oxygen under partial vacuum. This procedure is a special kind of dry ashing procedure but carried out at lower temperatures. Therefore it is suitable for the analysis of easily vaporized elements [70].

6.2.5. Microwave Digestion

Microwave procedure was used for the first time in 1975 for rapid heating in wet ashing open systems [64, 65, 71]. Biological samples were dissolved in 5-15 minutes by microwave technique, whereas they took 1-2 hours by conventional wet ashing techniques. In microwave systems, except a few special applications, the tightly closed vessels has been mostly replaced the open glass or Teflon containers. In that system pressure and temperature are controlled according to the program adjusted for each kind of sample. Finally, the amounts of solid samples and solvents used in microwave system are less than those used in alternative procedures (a few grams and less than 10 mL, respectively). On the basis of power applied; microwave systems can be classified as [72]:

1-single and
2-multimode

and depending on the vessels, the classification may be made as in the following:

1-Open vessel (under atmospheric pressure)
2-Closed vessel,
High pressure closed vessel (>80 atm)
Medium pressure closed (>10 atm, <80 atm)
Low pressure closed vessel (<10atm)
3-Flow-through (Flowing stream of reaction mixture passing through a microwave field).
4-Stopped flow (Flowing stream of reaction mixture is stopped within microwave field)

In microwave systems, microwave radiation produced by magnetron is absorbed, reflected or move in their ways without being exposed to any variation depending on the structure of material. Polar molecules in a food material (such as water) change their rotational directions together with the fluctuation of microwave radiation fields produced from magnetron. Owing to that movement occurring millions of times in a second, heat is released due to molecular friction and material is warmed up. As a result, electromagnetic energy is converted into heat energy. The depth effect of microwave radiation depends on its wavelength and dielectric properties of the sample. Since microwave radiation moves all the polar molecules in the sample at the same time, the heating is very fast (almost instantaneous) and homogenous. In traditional heating methods heat is transferred to the sample by conduction, convection and/or radiation whereas in microwave process, the heat directly transferred (enter) into (food) material. Heating rate varies depending on the power of microwave radiation, specific heat of the material, surface area of the samples and temperature adjusted.

In dissolution/digestion procedure carried out by microwave system, the selection of solvents is as important as the temperature /pressure/time program of the instrument.–Of course, the main criterion for the selection of the most suitable solvent(s) is the dissolution of the sample. However, formation of explosive compounds (e.g. formation of nitroglycerin derivatives upon reaction between nitric acid and oils) as well as excessive gaseous products upon reaction between sample components and solvents (which may cause explosion, too due

to uncontrollable high pressure) should also be considered. In addition, solvent should not cause high blank values and interferences during measurement step. Usually, nitric acid, hydrochloric acid, hydrofluoric acid, sulphuric acid, perchloric acid, hydrogen peroxide and their various combinations are used for dissolution. For each kind of sample the most convenient solvent(s), the temperature /pressure/time program and the amounts of sample and solvents to be put into vessels can be found in the software of the instrument.

In spite of their advantages, microwave digestion systems are not extensively used and traditional methods are still applied in many laboratories due to simplicity and easiness of the latter for many samples as well as high cost of microwave systems. Finally, microwave systems are quite delicate instruments and can be easily out of order upon improper use. For these reasons, in many publications on the analysis of trace elements in food samples, traditional methods are still used. The comparison of different ashing-digestion methods is given in Table 1 [73-77]. The working conditions of microwave systems (solvents, exposure time, power, vessel type) for the analysis of different elements in various samples are summarized in Table 2. Finally Table 3 presents some selected applications on the digestion of different kinds of samples for analysis of lead, cadmium, arsenic, selenium and mercury by AAS. As shown in the table nitric acid, alone or together with other reagents, is commonly used as the main solvent for most of the samples due to its both acidic and oxidative properties.

Table 1. Comparisons of ashing/digestion procedures

Dry ashing	Wet ashing	Microwave
Slow	Rapid	Rapid
Simple&easy	Complicated	Simple&easy
High Temperature (the risk of losses)	Low&moderate temperature (no losses)	Low&moderate temperature (no losses)
Less operator skills	Operator skills	Less operator skills
More scope of applicability	Large samples are not convenient	More scope of applicability
No chemical reagents	Corrosive-explosive reagents	Corrosive-explosive reagents
No expensive	No expensive	Expensive

Table 2. Working conditions of microwave systems applied for the digestion of various samples

Element	Sample	Solvent	Vessels	Digestion Procedure	Ref.
Cd, Cu, Pb	Rice	HNO_3	teflon vessels	350 W 10 min	[78]
Zn, Cd, Cu, Pb, Ni, Cr	wheat, potato, eggs, mussels, marine fish, river fish	HNO_3+HF	teflon vessels	1200 W 60 min	[79]
Se, Cd, Cu, As, Zn	Fish	HNO_3	Polytetrafluro acetate (PFA) closed digestion vessels	600 W 2 min 0 W 2 min 450 W 45 min	[80]
Se, Cu, As, Zn, Ni, Mn	Rice flour, wheat flour, apple leaves, tomato leaves, vegetable	HNO_3+H_2O_2	teflon vessels	600 W 60 min	[81]
Pb	Bone, milk, egg shell, chelated algae	HNO_3	teflon vessels	600 W 60 min	[82]
Fe, Mn, Zn, Cu	beans, rice, chickpeas, lentils	HNO_3+H_2O_2	closed vessels	600 W 15 min	[83]
Hg	Fish	HNO_3+H_2O_2	teflon vessels	300 W 4 min 600 W 2 min	[84]
Pb	Fruits	HNO_3+H_2O_2	-	-	[85]
Fe, Zn	Biscuits	HNO_3+HCl	teflon vessels	600 W 20 min	[86]
Hg, Cd, As	Bone, milk, eggshell, algae	HNO_3	teflon vessels	600 W 65 min	[87]
Ca, Cu, Cr, Fe, Co, Mg, Al, As, Be, Ba, Ca, Cd, Na, Zn, Mo, Ag, Hg, Ni, Pb, Sb, Sn, Sr, Ti, Tl, U, V	Milk	HNO_3	teflon vessels	1000 W 20 min	[88]
Al, B, Ba, Cu, Fe, K, Mg, Mn, Na, P, Sr, Zn	Seafood	HNO_3 HNO_3+H_2O_2 HNO_3+H_2O_2+THF HNO_3+H_2O_2+HF	teflon vessels	-	[89]
Al, Ca, Cu, Fe, Mg, Mn, Zn	Beer	HNO_3+H_2O_2	teflon vessels (perfluoroalkoxy teflon)	-	[90]

Table 2 continued

Element	Sample	Solvent	Vessels	Digestion Procedure	Ref.
Al, B, V, Cr, Co, Ni, As, Sr, Mo, Cd, Sn, Ba, Pb	mackerel, pig liver, and digestive biscuit digestates	HNO_3+H_2O_2	teflon vessel	-	[91]
Pb, Cd, Cu	wheat, wheat products, corn bran, rice flour	HNO_3+H_2O_2	closed vessel/ focused open-vessel	-	[92]
Cd	Plant	HNO_3	teflon vessels	-	[93]
Pb, Cu, Hg, Cd	Egg	HNO_3+H_2SO_4	teflon vessels	-	[94]
Cu, Zn	Plants	HNO_3+H_2O_2	teflon vessels	250 W 5 min 400 W 5 min 600 W 5 min	[95]
Al, As, B, Ba, Ca, Cd, Co, Cr, Fe, K, Mo, Mn, Ni, P, Na, , Zn Pb, Se	beef, beer, broccoli, corn, eggs, haddock, lemonade, white bread, mayonnaise, pancakes, peanut butter, pears, sweet potato	HNO_3	teflon-lined heavy duty vessels	300 W 13 min 400 W 13 min 600 W 13 min 600 W 13 min	[96]
Pb, Cd	Food products	HNO_3	teflon vessels	-	[97]

Table 3. Literature survey on the digestion of various food samples using different techniques for the determination of some selected toxic elements(lead,cadmium,arsenic,mercury and selenium) by AAS

Element	Sample	Procedure	Ref.
Pb	bovine liver, pig kidney	HNO_3; high-pressure digestion	[98]
	freshwater mussels	HNO_3 (350 W, 30s); microwave digestion	[99]
	soybean meal, fish-bone meal	HNO_3 (90^{o}C, 45 min; 150^{o}C, 8 h);wet digestion muffle furnace (200-250^{o}C, 30 min; 480^{o}C, 4 h), HNO_3 (400^{o}C, 15 min), HCl (Swirl); dry ashing HNO_3-$HClO_4$; wet digestion H_2SO_4 (room temperature, 30 min), H_2O_2 (250^{o}C, 30 min); wet digestion	[100]
	rice flour, pig kidney	HNO_3 (sonication in an ultrasonic batch, 5-10 min, dilution, centrifuge); ultrasonic-assisted extraction HNO_3-H_2O_2; microwave digestion	[101]
	Mussels	HNO_3-H_2O_2 (200^{o}C, 1 h); teflon bombs	[102]
	fish, shellfish	muffle furnace (450^{o}C, overnight), HNO_3; dry ashing	[103]
	beef, pork, chichken, fish, shellfish, vegetables, fresh fruits, eggs, milk, cheese, yogurt, cereals, pulses, fats, oils	HNO_3-H_2O_2; microwave-assisted digestion	[104]
	white wine, red wine	HNO_3-V_2O_5 (80^{o}C, 30 min; 120^{o}C, 90 min); wet ash digestion	[105]
	foodstuffs, animal organs, tissues, human food	-	[106]
	bovine liver, mussel tissue, milk, cheese, fish, eggs, yogurt, red meat, cereals, fats, oils, fruits, nuts, vegetables, drinks	muffle furnace (450^{o}C, 24-48 h); HNO_3; dry ashing	[107]
	milk, milk powder	wet digestion	[108]
	wine, beverages, fruits, beer	microwave digestion	[109]
	Honey	HNO_3-H_2O_2-Triton X-100; slurry technique	[110]
	mineral water	direct analysis	[111]
	food colourants	HNO_3-H_2O_2-Triton X-100; slurry technique	[112]
	baby food	HNO_3-H_2O_2-Triton X-100; slurry technique	[113]
	Honey	HNO_3-H_2O_2; slurry technique	[114]
	sweets, chewing gum	partial dry ashing, ethanol; slurry technique	[115]
	biscuits, bread, cereal-based products	Ethanol; slurry technique	[116]
	Vegetables	Ethanol, H_2O_2; slurry technique	[117]
	sweet fruit-flavored powder drinks, syrups, honey	direct analysis, wet digestion	[118]
	Milk	Triton X-100; slurry technique	[119]
	wine, alcoholic beverages	direct analysis	[120]
	Fruit	Triton X-100; slurry technique	[121]
	dairy products	Triton X-100; slurry technique HNO_3- V_2O_5; microwave acid digestion	[122]
	food, feed crops	HNO_3- V_2O_5; microwave acid digestion	[123]
	Fish	microwave acid digestion	[124]

Table 3 continued

Element	Sample	Procedure	Ref.
Cd	bovine liver, pig kidney	HNO_3; high-pressure digestion	[98]
	milk powder, rice flour, bovine muscular powder	ultrasonic probe extraction	[125]
	freshwater mussels	HNO_3 (350 W, 30s); microwave digestion	[99]
	soybean meal, fish-bone meal	HNO_3 (90°C, 45 min; 150°C, 8 h);wet digestion muffle furnace (200-250°C, 30 min; 480°C, 4 h), HNO_3 (400°C, 15 min), HCl (Swirl); dry ashing HNO_3-$HClO_4$; wet digestion H_2SO_4(room temperature, 30 min), H_2O_2 (250°C, 30 min); wet digestion	[100]
	rice flour, pig kidney	HNO_3 (Sonication in an ultrasonic batch, 5-10 min, dilution, centrifuge); ultrasonic-assisted extraction HNO_3-H_2O_2; microwave digestion	[101]
	Mussels	HNO_3-H_2O_2 (200°C, 1 h); teflon bombs	[102]
	fish, shellfish	muffle furnace (450°C, overnight), HNO_3; dry ashing	[103]
	cereals, patatoes, sugars, sweeteners, pulses, nuts, seeds, vegetables, mushrooms, olgae, fish, meats, eggs, milk, oils, beverages	-	[126]
	white wine, red wine	HNO_3-V_2O_5 (80°C, 30 min; 120°C, 90 min); wet ash digestion	[105]
	Foodstuffs, animal organs, tissues, human food	-	[106]
	milk, milk powder	wet digestion	[108]
	Honey	HNO_3-H_2O_2-Triton X-100; slurry technique	[127]
	mineral water	direct analysis	[111]
	beer, wort, brewer's yeast, malt, raw grain, hops	H_2O_2-HNO_3	[128]
	food colourants	HNO_3-H_2O_2-Triton X-100; slurry technique	[112]
	baby food	HNO_3-H_2O_2-Triton X-100; slurry technique	[113]
	sweets, chewing gum	partial dry ashing, ethanol; slurry technique	[115]
	biscuits, bread, cereal-based products	Ethanol; slurry technique	[116]
	Vegetables	Ethanol, H_2O_2; slurry technique	[117]
	Milk	Triton X-100; slurry technique	[119]
	Fruit	Triton X-100; slurry technique	[121]
	dairy products	Triton X-100; slurry technique HNO_3- V_2O_5; microwave acid digestion	[122]
	wine, beer, alcoholic, beverages	HNO_3-V_2O_5 (120°C, 90 min); microwave acid digestion	[129]
	seafood (fish, crustaceans, cephalopods)	HNO_3-V_2O_5 (120°C, 90 min); microwave acid digestion	[130]
	food, feed crops	microwave acid digestion	[131]

Table 3 continued

Element	Sample	Procedure	Ref.
As	bovine liver, pig kidney	HNO_3; high-pressure digestion	[98]
	fish, shellfish	HNO_3; wet digestion	[103]
	beef, pork, chichken, fish, shellfish, vegetables, fresh fruits, eggs, milk, cheese, yogurt, cereals, pulses, fats, oils	HNO_3-H_2O_2; microwave-assisted digestion	[104]
	milk, milk powder	wet digestion	[108]
	fish, coffee bears	HNO_3-HCl; wet digestion	[132]
	seafood, cereals, meat, corn, white rice, cheese	HNO_3 (90^oC, 45 min), HNO_3-$HClO_4$ (130^oC, 2 h); wet digestion	[133]
	baby food, raw fish	enzymatic digestion procedure	[134]
	white beet sugar	wet digestion	[135]
	Seafood	dry ashing	[136]
	Drinking water, mineral water	direct analysis	[137]
	food colourants	HNO_3-H_2O_2-Triton X-100; slurry technique	[112]
	Honey	HNO_3-H_2O_2-Triton X-100; slurry technique	[138]
	beer, food	HNO_3-H_2O_2-Triton X-100; slurry technique	[139]
	baby foods	HNO_3-H_2SO_4-$NaBH_4$; Kjeldahl digestion	[140]
Hg	bovine liver, pig kidney	HNO_3; high-pressure digestion	[98]
	Mussels	HNO_3-H_2O_2 (200^oC, 1 h); teflon bombs	[102]
	beef, pork, chichken, fish, shellfish, vegetables, fresh fruits, eggs, milk, cheese, yogurt, cereals, pulses, fats, oils	HNO_3-H_2O_2; microwave-assisted digestion	[104]
	fish, shellfish	HNO_3; wet digestion	[103]
	mussel, clams, fish	H_2SO_4-$K_2Cr_2O_7$; wet digestion	[141]
	Food	Wet Digestion	[142]
	Mushroom	HNO_3; microwave digestion	[143] [144]
	milk, fish, cereal	-	[145]
	food colourants	HNO_3-Triton X-100, $KMnO_4$, $AgNO_3$; slurry technique	[146]
	babyfood, seafood	HNO_3-Triton X-100, $KMnO_4$, $AgNO_3$; slurry technique	[147]
Se	fish, eggs, meet, fruits, vegetables	$HClO_4$-H_2SO_4-HNO_3; wet digestion	[148]
	dietary products	$HClO_4$-H_2O_2; microwave oven	[149]
	Drinking water, mineral water	direct analysis	[137]
	Babyfood	HNO_3-H_2O_2-Triton X-100; slurry technique	[113]
	Seafood	HNO_3-H_2O_2-Ethanol; slurry technique	[150]
	Fruits	Triton X-100; slurry technique	[121]

References

[1] B. Welz and M. Sperling, Atomic Absorption Spectrometry, 3rd Ed. Wiley-VCH, Weinheim, 1999.

[2] D. A. Skoog, F. J. Holler and T. A. Nieman, Principles of Instrumental Analysis, 5rd Ed. Harcourt Brace & Co. Orlando Fl, 1998.

[3] J. A. C. Broekaert, Analytical Atomic Spectrometry with Flames and Plasmas, Wiley-VCH, Weinheim, 2002.

[4] IUPAC, Spectrochim. Acta B 33 (1978) 247.

[5] Z. B. Alfassi, Determination of Trace Elements, VCH Verlagsgesellschaft mbH, Weinheim, 1994.

[6] B. Welz, S. Akman and G Schlemmer, Analyst 110 (1985) 459.

[7] D. C. Manning and W. Slavin, Appl. Spectrosc. 37 (1983) 1.

[8] W. Slavin, G. R. Carnrick and D. C. Manning, Anal. Chem. 56 (1984) 163.

[9] J. P. Byrne, M. M. Lamoureux, C. L. Chakrabarti and T. Ly, D. C. Grégoire, J. Anal. At. Spectrom. 8 (1993) 599.

[10] B. Welz, S. Akman and G. Schlemmer, J. Anal. At. Spectrom. 2 (1987) 793.

[11] B. Welz, Fresenius J. Anal. Chem. 325 (1986) 95.

[12] J. A. Holcombe, Spectrochim. Acta B 38 (1983) 609.

[13] S. Akman, B. Welz and N. Tokman, Spectrochim. Acta 60 (2005) 1349.

[14] N. Tokman and S. Akman, Spectrochim. Acta B 60 (2005) 291.

[15] G. Doner and S Akman, Spectrochim. Acta B 51 (1996), 181.

[16] M. Ozcan and S. Akman, Spectrochim. Acta B 55 (2000) 509.

[17] R.A. Newstead, W.J. Price and P. J. Whiteside, Prog. Analyt. Atom. Spectrosc. 1 (1978) 267.

[18] B. Welz, G. Schlemmer and J. R. Mukadavi, Anal. Chem. 60 (1988) 2567.

[19] R. Cruz and J. C. Van Loon, Anal. Chim. Acta 72 (1974) 231.

[20] S. Bektas and S Akman, Anal. Sci. 6 (1990) 547.

[21] G. Doner and S. Akman, J. Anal. At. Spectrom. 9 (1994) 333.

[22] W. Slavin, D. C. Manning and G. R. Carnrick, At. Spectrosc. 2 (1981) 137.

[23] S. A. Clyburn, T. Kántor and C. Veillon, Anal. Chem. 46 (1974)2214.

[24] G. Schlemmer and B. Welz, Spectrochim. Acta B 41 (1986) 1157.

[25] N. Coskun and S. Akman, Spectrochim. Acta B 60 (2005) 415.

[26] M.A.Belarra, C.Crepso, M.P.Martinez-Garbayo and J.R. Castillo, Spectrochim. Acta B 52 (1997) 1885.

[27] National Research Council, Recommended Dietary Allowances, 10th ed. National Academy Press, Washington, DC, 1989.

[28] WHO, Trace Elements in Human Nutrition and Health. World Health Oranization, Geneva, Switzerland, 1996

[29] WHO, Trace Elements in Human Nutrition. Technical Report Series No. 532, World Health Organization, Geneva, Switzerland, 1973.

[30] K. Eto, Neuropathology 20 (2000)14.

[31] F. H. Nielsen, Essential and Toxic Trace Elements in Human Health and Disease: An Update, Wiley-Liss, Inc. pages 355-376; 1993.

[32] D.B. Thompson, Iron. In *Trace Minerals in Foods*, pp 157-208 [K. T. Smith, editor]. New York: Marcel Dekker, 1988.

[33] R. Eisler, Patuxent Wildlife Research Center, Contaminant Hazard Reviews Report No. 12; January 1988.
[34] E.B. Hayes, M.D. McElvaine, H.G. Orbach, A.M. Fernandez, S. Lyne and T.D. Matte, Pediatrics 93 (1994) 195.
[35] R.D. Kimbrough, M. LeVois and D.R. Webb. Pediatrics. 93 (1994) 188.
[36] WHO. Environmental Health Criteria 165- Inorganic Lead. Geneva, 1995.
[37] R.A. Kehoe, J Cholak, and E.J.Largent, J. Am. Water Works.Assoc. 36 (1944) 637.
[38] A.Goldberg, Environ. Health Perspect. 7 (1974) 103.
[39] Z. Kerin, D.Kerin and D. Djuric, Int. Arch.Arbeitsmed. 29 (1972) 129.
[40] C.C. Patterson, Arch environ. Health 11 (1965) 344.
[41] National Academy of Sciences. Toxicologic effects of methylmercury. Washington, DC: National Research Council, 2000.
[42] P.W Davidson, G.J. Myers and B. Weiss, Pediatrics 13 (2004) 1023.
[43] American Academy of Pediatrics. Pediatrics. 108 (2001) 197.
[44] A.P.Alvares, S.Leigh, J. Conn and A.Kappas, J. Exp. Med.135 (1972) 1406.
[45] L.W. Chang, K.R. Reuhl and G.W. Lee, Environ. Res. 14 (1977) 414.
[46] Dr. I. Elmadfa, Expertengutachten zur Lebensmittelsicherheit Cadmium, Im Auftrag des Bundeskanzleramts; Institut für Ernährungswissenschaften der Universität Wien, 1999.
[47] K. Becker, P. Nöllke, E. Hermann-Kunz, H. Klein, C. Krause, C. Schulz, D. Schenker, Akt Ernähr-Med 23 (1998) 142.
[48] Codex Alimentarius Commission, Codex Committee on Food Add. Contam. – Draft Guideline Levels for Cadmium in Cereals, Pulses and Legumes. CX/FAC 97/20, 1997.
[49] Codex Alimentarius Commission. General Standard on Contaminants and Toxins in Food (GSCTF). Kloet DG (ed.), Food Safety Information Center, DLO, Wageningen, CX/FAC 98/13.
[50] European Commission (1996). Food science and techniques, Reports on tasks for scientific cooperation (SCOOP). Report of experts participating in Task 3.2.4 – Dietary Exposure to Cadmium. Brussels, 1998.
[51] FAO/WHO. Evaluation of Certain Food Add. Contam.. 33 rd report of the joint FAO/WHO Expert Committee of Food Additives (Technical Report Series 776) Geneva, 1989: 28-31
[52] L. Friberg, M. Piscator, GF Nordberg, T. Kjellstrom, (1974)*Cadmium in the environment.* 2nd ed. Ohio, Chemical Rubber Company.
[53] K. Tsuchiya. Keio J. Med. 18 (1969) 195.
[54] WHO, Selenium, Environmental Health Criteria 58, Geneva: World Health Organization, 1987.
[55] European Commission, Commission regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs, Official Journal of the European Communities, L77 (16 March 2001), 1–13 (2001).
[56] C. Reilly, Trends Food Sci. Technol. 9 (1998) 114.
[57] R.F. Martin, V.R. Young, J. Blumberg and M. Janghorbani, Am. J. Clin. Nut. 49 (1989) 862.
[58] J. Alexander and H.M. Meltzer, Selenium, In: A. Oskarsson Ed. Risk evaluation of essential trace elements - essential versus toxic levels of intake. Copenhagen: Nordic Council of Ministers, Nord 1995: 18, pp 15-65, 1995.
[59] S. Schwarz, Med. Clin. North Am. 60 (1976) 745.
[60] P.D. Whanger, Selenium and heavy metal toxicity. In: J.E. Spallholz, J.L. Martin, H.E. Ganther, eds. *Selenium in Biology and Medicine.* Westport, CT: AVI; 1981:230-255.
[61] M. Vinceti, E.T. Wei and C. Malagoli. Rev. Environ. Health 16 (2001) 233.

[62] Sample Preparation Techniques in Anal. Chem. Edited by Somenath Mitra ISBN 0-471-32845-6 Copyright *6* 2003 John Wiley & Sons, Inc.
[63] A. Taylor, S. Branch, D. Halls, M. Patriarca and M. White, J. Anal. At. Spectrom. 17 (2002) 414.
[64] M.L. Adams, A.M. Chaudri, I. Rousseau and S.P. Mcgrath, Intern. J. Environ. Anal. Chem. 83 (2003) 307.
[65] M. Cocchi, G. Franchini, D. Manzini, M. Manfredini, A. Marchetti, and A. Ulrici, J. Agric. Food Chem, 52 (2004) 4047.
[66] R. Pöykiö, H. Torvela, P. Perämäki, T. Kuokkanen and H. Rönkkömäki, Analusis, 28 (2000) 850.
[67] M.I. Skurikhin, J. AOAC Int. 76 (1993) 257.
[68] F. Fagioli, S. Landi, C. Locatelli and F. Reghini, *J. Anal. At. Spectrom.* 5 (1990) 519.
[69] A. N. Baklanov, Yu. V. Bokhan, and F. A. Chmilenko, J. Anal. Chem. 58 (2003) 489.
[70] M. Achilli, R. Barban, B. Zucchi and W. Martinotti, Water Air Soil Pollut. 57–58 (1991) 495.
[71] E.J. Gawalko, T.W. Nowicki, J. Babb and R. Tkachuk, J. AOAC Int. 80 (1997) 379.
[72] CEM Corp. HP-500 Plus and XP-1500 Plus Vessel Accessory Sets: Instructions for Use, p16. CEM Corp Matthews, NC 199X.314 M.L. ADAMS et al.
[73] H.T. McCarthy and P. Ellis, J. AOAC Int. 74 (1991) 566.
[74] J.E. Rechcigl and G.G. Payne, Commun. Soil Sci. Plant 21 (1990) 2209.
[75] I. Matejovic and A. Durackova, Commun. Soil Sci. Plant 25 (1994) 1277.
[76] I. Lavilla, A.V. Filgueiras and C. Bendicho, J. Agric. Food Chem. 47 (1999) 5072.
[77] D. Sun, J.K. Waters and T.P. Mawhinney, J. AOAC Int. 83 (2000) 1218.
[78] E. Vassileva and C.R. Quetel, Anal. Chim. Acta 519 (2004) 79.
[79] R. Milacic and B. Kralj, Eur. Food Res. Technol., 217 (2003) 211.
[80] M. Barwick and W. Maher, Mar. Environ. Res. 56 (2003) 471.
[81] T. Roychowdhury, H. Tokunaga and M. Ando, Sci. Total Environ. 308 (2003) 15.
[82] M. Kim, C. Kim and I. Song, Food Add. Contam. 20 (2003) 149.
[83] J.M. Anzano and M. Ruiz-Gil, Atom. Spectrom, 26 (2005) 28.
[84] L.A. dePaz, A. Alegria, R. Barbera, R. Farre and M.J. Lagarda, Food Chem. 58 (1997) 169.
[85] C. Cabrera, Y. Madrıd and C. Camara, J. Anal. Atom. Spectrom. 9 (1994) 1423.
[86] G. Doner and A. Ege, Anal. Chim. Acta 520 (2004) 217.
[87] M. Kim, Food Add. Contam. 21 (2004) 763.
[88] A. Ikem, A. Nwankwoala, S. Odueyungbo, K. Nyavar and N. Egiebor, Food Chem. 77 (2002) 439.
[89] D.H. Sun, J.K. Waters and T.P. Mawhinney, J. Agric. Food Chem. 45 (1997) 2115.
[90] D. Bellido-Milla, J.M. Moreno-Perez and M.P. Hernandez-Artiga, Spectrochim. Acta B 55 (2000) 855.
[91] C.F. Harrington, S. Elahi, P. Ponnampalavanar and T.M. D'Silva, Atom. Spectrom. 20 (1999) 174.
[92] E.J. Gawalko, T.W. Nowicki, J. Babb, R. Tkachuk and S.L. Wu, J. AOAC Int. 80 (1997) 379.
[93] P. Hocquellet, Analusis 23 (1995) 159.
[94] S.l. Jeng and C.P. Yang, Poultry Sci. 74 (1995) 187.
[95] P. Mantovi, G. Bonazzi, E. Maestri and N. Marmiroli, Plant and Soil, 250 (2003) 249.
[96] S.P. Dolan and S.G. Capar, J. Food Comp. Anal. 15 (2002) 593.
[97] C. Blake and B. Bourqui, Atom. Spectrom, 19 (1998) 207.

[98] M. Wilhelm, J. Wittsiepe, P. Schrey, A. Hilbig and M. Kersting, Sci. Total Environ. 343 (2005) 61.
[99] A. Wagner, J. Boman, Spectrochim. Acta B 59 (2004) 1125.
[100] Z.Y. Hseu, Biores. Technol. 95 (2004) 53.
[101] P.C. Aleixo, D. Santos, A.C. Tomazelli, I.A. Rufini, H. Berndt and F.J. Krug, Anal. Chim. Acta, 512 (2004) 329.
[102] P. Szefer, B.S. Kim, C.K. Kim, E.H. Kim and C.B. Lee, Environ. Pollut. 129 (2004) 209.
[103] D. Juresa and M. Blanusa, Food Add. Contam. 20 (2003) 241.
[104] J.M. Llobett, G. Falco, C. Casas, A. Teixido and J.L. Domingo, J. Agric. Food Chem. 51 (2003) 838.
[105] G.P.G. Freschi, C.S. Dakuzaku, M. de Moraes, J.A. Nobrega and J.A.G. Neto, Spectrochim. Acta B 56 (2001) 1987.
[106] U. Gottelt, K. Lusky, M. Stoyke and G. Henrion, Fleischwirtschaft, 76 (1996) 1175.
[107] C. Rubio, T. Gonzalez-Iglesias, C. Revert, J.I. Reguera, A.J. Gutierrez and A. Hardisson, J. Agric. Food Chem. 53 (2005) 6543.
[108] P.C. Aleixo, D. Santos, A.C. Tomazelli, I.A. Rufini, H. Berndt and F.J. Krug, Anal. Chim. Acta, 512 (2004) 329.
[109] L. Valiente, M. Piccinna, E.R. Ale, A. Grillo and P. Smichowski, Atom. Spectrom, 23 (2002) 129.
[110] N. Salvato and C. Pirola, Microchimica Acta, 123 (1996) 63.
[111] C. Cabrera, Y. Madrid and C. Camara, J. Anal. Atom. Spectrom. 9 (1994) 1423.
[112] J.C.R. Garcia, J.B. Garcia, C.H. Latorre, M.F. Rodriguez, S.G. Martin and R.M.P. Crecente, Talanta, 61 (2003), 509.
[113] V.R. Amorim, K.G. Fernandes, M. de Moraes and J.A.G. Neto, J. Braz. Chem. Soc. 15 (2004) 28.
[114] P. Vinas, M. Pardo-Martinez, I. Lopez-Garcia and M. Hernandez-Cordoba, J. Agric. Food Chem. 50 (2002) 949.
[115] P. Vinas, M. Pardo-Martinez, I. Lopez-Garcia and M. Hernandez-Cordoba, J. Anal. Atom. Spectrom. 16 (2001) 633.
[116] P. Vinas, M. Pardo-Martinez and M. Hernandez-Cordoba, Anal. Chim. Acta 412 (2000) 121.
[117] N. Campillo, P. Vinas, I. Lopez-Garcia and M. Hernandez-Cordoba, Analyst 125 (2000) 313.
[118] P. Vinas, I. Lopez-Garcia, M. Lanzon and M. Hernandez-Cordoba, J. Agric. Food Chem. 45 (1997) 3952.
[119] I. Lopez-Garcia, P. Vinas, N. Campillo and M. Hernandez-Cordoba, J. Agric. Food Chem. 44 (1996) 836.
[120] P. Vinas, N. Campillo, I. Lopez-Garcia and M.H. Cordoba, Analyst 119 (1994) 1119.
[121] P. Vinas, N. Campillo, I. Lopez-Garcia and M.H. Cordoba, Fresenius J. Anal. Chem. 349 (1994) 306.
[122] J.K. Oygard, A.K. Lundebye and K. Julshamn, J. AOAC Int. 82 (1999) 1217.
[123] E.C. Lima, F.J. Krug and M.A.Z. Arruda, Spectrochim. Acta Part B, 53 (1998) 601.
[124] C. Cabrera, M.L. Lorenzo and M.C. Lopez, J. AOAC Int. 78 (1995) 1061.
[125] N.N. Meeravali, M.A. Reddy and S.J. Kumar, Atom. Spectrom, 26 (2005) 68.
[126] Y. Kikuchi, T. Nomiyama, N. Kumagai, T. Uemura and K. Omae, J. Occ. Health 44 (2002) 240.
[127] D.Y. Velez, N. Banez and R. Montoro, J. Agric. Food Chem. 43 (1995) 1289.

[128] N. Ladra-Ramos, R. Dominguez-Gonzalez, A. Moreda-Pineiro, A. Bermejo-Barrera and P. Bermejo-Barrera, Atom. Spectrom, 26 (2005) 59.
[129] P. Vinas, N. Campillo, I. Lopez-Garcia and M.H. Cordoba, Food Chem. 50 (1994) 317.
[130] E.M. Garcia, M.L. Lorenzo, C. Cabrera and M.C. Lopez, J. Dairy Res. 66 (1999) 569.
[131] C.M. Mena, C. Cabrera, M.L. Lorenzo and M.C. Lopez, J. Agric. Food Chem. 45 (1997) 1812.
[132] D. Juresa and M. Blanusa, Food Add. Contam. 20 (2003) 241.
[133] A.L. Moretto and S. Cadore, Microchim. Acta, 146 (2004) 239.
[134] T. Klapec, M.L. Mandic, J. Gargic, L. Primorac, A. Perl and V. Krstanovic, Food Chem. 85 (2004) 445.
[135] P. Vinas, I. Lopez-Garcia, B. Merino-Merono, N. Campillo and M. Hernandez-Cordoba, Chromatographia 57 (2003) 611.
[136] F. Ronda, D. Sancho, C.A. Blanco, M. Gomez, P.A. Caballero, Zuckerindustrie 127 (2002) 763.
[137] L.C. Sander, K.E. Sharpless, M.B. Satterfield, T. Ihara, K.W. Phinney, J.H. Yen, S.A. Wise, M.L. Gay, J.W. Lam, M. McCooeye, G. Gardner, C. Fraser, R. Sturgeon and M. Roman, Anal. Chem. 77 (2005) 3101.
[138] V.A. Resta, K.G. Fernandez, M. de Moraes and J.A.G. Neto, Atom. Spectrom. 23 (2002) 7.
[139] P. Vinas, N. Aguinaga, I. Lopez-Garcia and M. Hernandez-Cordoba, J. AOAC Int. 85 (2002) 736.
[140] P. Vinas, M. Pardo-Martinez and M. Hernandez-Cordoba, J. Anal. Atom. Spectrom. 14 (1999) 1215.
[141] C. Locatelli and G. Torsi, Ann. Chimica, 91 (2001) 65.
[142] K.V. Nagaraja, S. Vibhakar, S.N.R. Rao and U. Rani, Fresenius J. Anal. Chem. 352 (1985) 33.
[143] Y.L. Liu, Spectros. Spec. Anal. 23 (2003) 987.
[144] C. Delgado-Andrade, M. Navarro, H. Lopez and M.C. Lopez, Food Add. Contam. 20 (2003) 923.
[145] J. Falandysz, M. Gucia, A. Brzostowski, M. Kawano, L. Bielawski, A. Frankowska, and S. Wyrzykowska, Food Add. Contam. 20 (2003) 247.
[146] J.C.R. Garcia, J.B. Garcia, C.H. Latorre, S.G. Martin and R.M.P. Crecente, Food Chem. 91 (2005) 435.
[147] M. Yaman and N. Cokol, Atom. Spectrom, 24 (2004) 185.
[148] A. Taylor, S. Branch, A. Fisher, D. Halls and M. White, J. Anal. Atom. Spectrom. 16 (2001) 421.
[149] J. Falandysz, K. Lipka, M. Kawano, A. Brzostowski, M. Dadej, A. Jedrusiak and T. Puzyn, J. Agric. Food Chem. 51 (2003) 2832.
[150] P. Vinas, M. Pardo-Martinez, I. Lopez-Garcia and M. Hernandez-Cordoba, J. Anal. Atom. Spectrom. 16 (2001) 1202.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 18

Electrochemical stripping analysis of trace and ultra-trace concentrations of toxic metals and metalloids in foods and beverages

Samuel B. Adeloju

Water and Sensor Research Group, School of Applied Sciences and Engineering, Monash University, Churchill, Gippsland, Victoria 3842, Australia

1. Introduction

The introduction of heavy metals and metalloids to the food chain via various anthropogenic sources poses a serious threat to human health. In particular, the husbandry and/or processing practices adopted during the growth and handling of foods represent a major source of contamination by inorganic and organic substances. Owing to the growing concern in this area, many national and international regulatory bodies on food quality have lowered the maximum permissible levels of toxic metals in human food. As an example, the maximum tolerable daily intake set by FAO/WHO Joint Expert Committee on Food Additives for cadmium and lead for all sources (including air, food and water) are 1.0-1.2 μg/kg and 3.5-4.0 μg/kg body mass, respectively [1]. Consequently, the control of the concentrations of trace metals in foods, and possible minimization of food pollution by improving husbandry and food processing practices has become a significant aspect in achieving high food quality. As food is one of the main sources of toxic metals, it constitutes a significant pathway through which toxic metals are ingested by human and animals. Therefore, the on-going monitoring of these substances in foods and beverages with a high degree of accuracy, reliability and sensitivity at trace and ultra-trace concentrations is necessary for the protection of human health.

Some of the more commonly used methods for the determination of toxic heavy metals and metalloids in foods include atomic absorption spectroscopy, inductively coupled plasma-optical emission spectroscopy (ICP-OES), inductively coupled plasma-mass spectroscopy (ICP-MS), x-ray fluorescence, and electroanalytical methods. Over the past 30 or more years, some of the most exciting developments in the determination of toxic heavy metals and metalloids in foods have focused on the use of electroanalytical methods. To date, the range of electroanalytical methods that have been employed for the determination of heavy metals and metalloids include amperometry, polarography, potentiometry and electrochemical (potentiometric and voltammetric) stripping analysis. Among these and compared with other analytical methods, electrochemical stripping analysis offers several unique advantages, such

as high sensitivity, selectivity, ability to identify a wide range and forms of metals and metalloids in various matrices, ease of identification and elimination of interferences and contamination, and substantially lower costs than most other trace analytical methods [2,3]. Over the years, several workers have successfully used stripping voltammetry and potentiometric stripping analysis for heavy metals and metalloids analyses in a wide range of foods and beverages. Variants of electrochemical stripping analysis, such as anodic stripping voltammetry, cathodic stripping voltammetry and adsorptive stripping voltammetry are now commonly employed in laboratory and in-field for the determination of a wide range of heavy metals and metalloids in foods. Even the more recent variant, based on the measurement of the potential-time response and commonly referred to as potentiometric stripping analysis or stripping potentiometry and its variants, such as constant current stripping analysis and adsorptive constant current stripping analysis has gained considerable interest for the determination of these substances in foods and beverages [4-18]. Generally, the use and further development of potentiometric stripping analysis has followed similar path to the development of stripping voltammetry and has equally gained on-going interest for the analysis of metals and metalloids in various sample materials, including foods and beverages, both in the batch and flow analysis modes.

Both variants of electrochemical stripping analysis have provided extremely sensitive and selective methods for the analysis of many substances at trace and ultra-trace concentrations, as well as for multi-component analyses in a single sample solution. Also, the non-destructive nature of these methods permits further analyses on the same sample solution by other analytical methods.

This chapter will discuss the underlying principles of electrochemical stripping analysis for the determination of toxic trace metals and metalloids. Then, it will focus on the application of these methods to the determination of these substances in foods and beverages. In particular, the need for adequate sample pre-treatment to enable the attainment of analytical reliability when applied to solid and mixed solid-liquid food samples will be highlighted. The diverse range of foods and beverages that these methods have been applied to is discussed. The need to be aware of potential inorganic and organic interferences with these methods is also emphasised. Furthermore, some of the recent developments that will encourage the continuing use of electrochemical stripping analysis for metal and metalloid analysis in foods, beverages and other samples in the future is also discussed.

2. Principles of Electrochemical Stripping Analysis

2.1. Stripping Voltammetry

The first analytical application of this method was reported by Zbinden in 1931 when he attempted to measure copper electrogravimetrically by measuring the current during the

stripping process [19]. He found that the magnitude of the stripping current is related to the amount of copper deposited on the electrode. However, major advances in stripping voltammetry did not begin till the mid 1950s when Kemula developed the first hanging mercury drop electrode (HMDE). By 1960s and the 70s stripping voltammetric techniques were readily applied to trace metal analysis in natural waters and other samples. The method employs an electrochemical preconcentration step which involves an initial deposition of the analyte into or onto an electrode for a set period, as a means of increasing the analytical sensitivity. This is usually performed by controlled potential electrolysis which requires the application of a constant reduction or oxidation potential to an electrode under a reproducible hydrodynamic (mass transport) conditions. Following this step, analytical determination is accomplished by the electrochemical removal of the deposited analyte from the electrode and, hence, the term 'stripping' is adopted. The magnitude of the resulting peak current or peak height obtained from the stripping process is used to quantify the amount of analyte present in a sample. The development and interest in the use of potentiometric stripping analysis followed soon after in late 1970s and this approach became more popular in 1980s.

As a trace analytical method, electrochemical stripping analysis involves three main steps [20]:

(a) Electrolytic concentration of the analyte into or onto an electrode with stirring, achieved either by rotation of the electrode or solution stirring with the aid of a suitable reproducible stirring device. This step is commonly referred to as the deposition or preconcentration step and the high sensitivity achieved by this method is attributable to this step. The rotation of the electrode or stirring of the solution helps to maximise the rate of deposition and can enhance the concentration of the analyte on the electrode by a factor of as much as 10,000 times relative to its concentration in solution.

(b) Equilibration or rest period occurs prior to the stripping step, achieved by turning off the stirring device used for the deposition to allow the solution to equilibrate for 15-30 seconds. This is necessary to avoid or minimise the undesirable effect of stirring during the stripping measurements.

(c) Stripping step enables the analytical determination of the analyte by the electrochemical removal of the deposited analyte from the electrode. During this step, the potential of the electrode is scanned either positively or negatively to enable removal of the analyte from the electrode.

There are three variants of this method that can be used for metals and metalloids analysis. Each of these is described below.

2.1.1. Anodic Stripping Voltammetry

Anodic stripping voltammetry (ASV) is the most widely used version of stripping voltammetry. It involves the deposition of metal ions into a mercury electrode as an amalgam

or into other solid electrode as a metal by the application of a sufficiently negative potential to initiate reduction:

$$M^{n+} + ne,^{-} \rightarrow M(Hg) \qquad (\textit{mercury electrode})$$

or

$$M^{n+} + ne,^{-} \rightarrow M^{o} \qquad (\textit{mercury-free solid electrode})$$

This process involves stirring of the solution or electrode rotation for a given period (usually 1-30 min), while the applied potential is held constant.

The surface area of the electrode, duration and rate of preconcentration are key factors that can influence the sensitivity of this method. At a constant electrode surface area and preconcentration time, the sensitivity is influenced by the stirring of the solution or electrode rotation rate and it is also dependent on the solubility of the analyte in mercury. The stirring of the solution maximises the rate of electrodeposition of analyte by constant supply to the electrode and speeding up the preconcentration process, thus enabling a preconcentration factor of up to 10,000 times. It also ensures uniform and reproducible concentration gradient at the electrode (reproducible hydrodynamic or mass-transport conditions).

The total amount of metal ions amalgamated, [M(Hg)tot], for a given potentiostatic deposition (preconcentration) period (tdep), in seconds, is given according to Ficks law of diffusion controlled mass transport as:

$$M(Hg)_{tot} \alpha \{ [M^{n+}] (D_M{}^{n+}) + t_{dep}\, d_p{}^{-1}\, A \}$$

where $[M^{n+}]$ is bulk concentration of M^{n+}; $(D_M{}^{n+})$ is diffusion co-efficient for M^{n+}; $d_p{}^{-1}$ is the diffusion layer thickness; and A is the active area of the working electrode.

In terms of solubility, generally the greater the solubility of the metal in mercury the higher the sensitivity. Table 1 gives the solubilities of some metals in mercury. Metals that have higher solubilities in mercury, such as Bi, Cd, In, Pb and Zn, usually give sensitive stripping voltammetric responses, while those of poor solubilities, such Co, Ni and Sb, do not give adequate response at the trace and ultra-trace concentrations.

It is important to note that the bulk concentration does not change much during the potentiostatic deposition due to the working electrode area being so small (usually around 0.005 – 0.020 cm2), but the [M(Hg)tot] is directly proportional to [Mn+] and, hence, the current or response associated with the removal of the deposited metal is related to its concentration in solution.

Following the deposition period, a brief equilibration period (15-30 sec) is allowed and then the deposited metals are oxidized from the electrode into the solution:

$$M(Hg) \rightarrow M^{n+} + ne,^{-} \qquad (\textit{mercury electrode})$$

or

$$M^{o} \rightarrow M^{n+} + ne,^{-} \quad (\textit{mercury-free solid electrode})$$

As the potential is scanned towards more positive potentials the anodic current increases rapidly until the concentration of the metal in the electrode approaches zero, causing the current to peak and then decay as the substance is depleted from the vicinity of the electrode surface, as illustrated in Figure 1 for Cd and Pb. The potential scan can be achieved by either a linear potential sweep or a potential sweep with superimposed potential modulations.

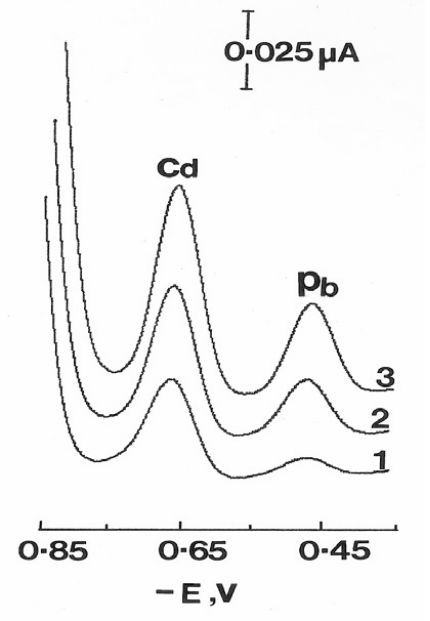


Figure 1. Simultaneous determination of cadmium and lead in oyster tissue sample by DPASV. Sample decomposition by wet digestion with a mixture of HNO3 and H2SO4. (1) sample only; (2) +2; and (3) +4 µg/l (Reproduced from [39] with permission from Royal Chemical Society© 1989).

The two important parameters obtained from the stripping voltammogram are peak potential (Ep), which is useful for qualitative analysis, and the peak current (ip), which is useful for quantitative analysis. Hence, a plot of ip versus analyte concentration gives a straight line within a defined linear concentration range. The range of metals that can be determined by ASV are listed in Table 2. Figure 1 shows the use of ASV for the simultaneous determination of cadmium and lead in an oyster tissue sample. Evidently, each response increased with increasing concentration of the analyte and quantification was achieved by standard additions method.

Table 1. Solubilities of some metals in mercury

Element	Solubility in Hg (atomic %)	Element	Solubility in Hg (atomic %)
Ag	0.066	Mg	2.5
Al	0.015	Mn	0.007
Au	0.133	Na	4.8
Ba	0.48	Ni	0.0021
Bi	1.1	Pb	1.3
Cd	10.0	Pu	0.013
Co	0.0003	Pt	0.10
Cu	0.006	Sb	0.000047
Ga	3.6	Sn	1.26
In	70.0	Ti	43.0
K	2.0	Zn	5.83

2.1.2. Cathodic Stripping Voltammetry

Cathodic stripping voltammetry (CSV) is useful for the determination of substances that are not soluble or insufficiently soluble in mercury. Instead of relying on the formation of an amalgam (as in ASV) with mercury, CSV measurement involves the formation of an insoluble deposit on the electrode surface. This usually requires the application of a positive potential, about 200-300mV more positive than E1/2 of the analyte, to the electrode. In this method, the electrode reactions are largely dependent on the chosen electrode material. For example, on a mercury or a silver electrode, the reaction involved is:

$$Hg \rightarrow Hg^{+} + e^{-} \qquad (\textit{oxidation of mercury})$$

$$Ag \rightarrow Ag^{+} + e^{-} \qquad (\textit{oxidation of silver})$$

Followed by the formation of an insoluble film:

$$2Hg^{+} + 2/n(X^{n-}) \rightarrow Hg_2^{2+}(X^{n-})_{2/n}$$

$$Ag^{+} + X^{-} \rightarrow AgX$$

The overall electrodeposition processes are:

$$2Hg + 2/n(X^{n-}) \rightarrow Hg_2^{2+}(X^{n-})_{2/n} + 2e^{-}$$

or

$$2\,Hg + 2/n\,(X_aO_b^{n-}) \rightarrow Hg_2^{2+}(X_aO_b^{n-})_{2/n} + 2e^-$$

$$Ag + X^- \rightarrow AgX + e^-$$

where $X^- = Cl^-, Br^-, I^-$; and $X_aO_b^{n-} = CrO_4^{2-}, MoO_4^{2-}, VO_3^{2-}, WO_4^{2-}, SeO_3^{2-}$. The factors that influence the amount of analyte deposited on the electrode include the kinetics of formation and solubility of the deposited substance, diffusion coefficient of the reacting species in the deposited film and the density of the film.

Then, following a brief equilibration period, the insoluble substance is reduced from the electrode surface back into the solution by scanning in the negative direction. An increase in the cathodic current is observed, as illustrated in Figure 2, when the potential is negative enough to reduce the film, as follows:

$$Hg_2^{2+}(X^{n-})_{2/n} + 2e^- \rightarrow 2Hg + 2/n(X^{n-})$$

$$Hg_2^{2+}(X_aO_b^{n-})_{2/n} + 2e^- \rightarrow 2Hg + 2/n\,(X_aO_b^{n-})$$

$$Ag\,X + e^- \rightarrow Ag + X^-$$

The sensitivity of the method is influenced by the dissociation rate of the insoluble compound from the electrode. Again, ip and the Ep obtained from the stripping voltammogram are the useful quantitative and qualitative parameters, respectively. Figure 2 shows the application of CSV to the determination of selenium in fish by standard additions method. Some of the other inorganic substances that can be determined by CSV are also listed in Table 2.

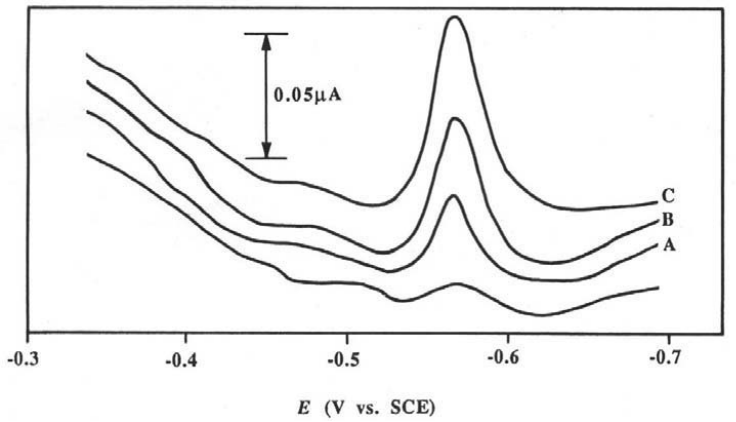


Figure 2. Cathodic stripping voltammetric determination of selenium in shark fish by DPCSV at a HMDE. Unlabelled response is for sample only; (a) +0.5; (b) +1.0; (c) +1.5 µg/l Se4+ (Reproduced from [46] with permission from Elsevier© 1983).

2.1.3. Adsorptive Cathodic Stripping Voltammetry

For substances that cannot be determined by ASV because of their poor solubility in mercury or by CSV because of their inability to form insoluble film, the third obvious choice of stripping voltammetric method is known as "adsorptive stripping voltammetry (AdSV)". The merits in the deliberate adsorption of species onto suitable electrode for analytical voltammetric measurement was first evident from Komarek's (a Czech Chemist) work in 1947 when he found that the adsorption of nickel as nickel dimethylglyoximate resulted in substantial enhancement of its polarographic response [21]. The concept was extended by a number of workers in 1980 to the AdSV determination of nickel and cobalt at picogram to nanogram range with increased sensitivity, improved selectivity, high specificity and better limits of detection, compared with conventional stripping voltammetric analysis.

Table 2. Some of the heavy metal and metalloids that can be determined by electrochemical stripping analysis

Analyte	Stripping Technique	Electrode(s)	Analyte	Stripping Technique	Electrode(s)
Aluminium	AdSV	HMDE, MFE	Germanium	ASV	HMDE, MFE
				AdSV	HMDE, MFE
Antimony	ASV, CCSA	HMDE, MFE	Gold	ASV, CCSA	Pt, GC
	AdSV	HMDE, MFE			
Arsenic	ASV, PSA, CCSA	Au, Pt, GC AuFE	Indium	ASV, PSA	HMDE, MFE
	CSV	HMDE, MFE			
Bismuth	ASV, PSA	HMDE, MFE	Iron	ASV, PSA, CCSA	Au, Pt, Ag, GCMFE
				CSV, AdSV	HMDE, MFE
Cadmium	ASV, PSA	HMDE, MFE	Lanthanum	AdSV	HMDE, MFE
	AdSV	HMDE, MFE			
Calcium	AdSV	HMDE, MFE	Lead	ASV, PSA	HMDE, MFE
				AdSV	HMDE, MFE
Cerium	AdSV, PSA	HMDE, MFE, Ag	Magnesium	AdSV	HMDE, MFE

Analyte	Stripping Technique	Electrode(s)	Analyte	Stripping Technique	Electrode(s)
Chromate	CSV	HMDE, MFE	Manganese	ASV, PSA	HMDE, MFE, Pt, Au
	AdSV	HMDE, MFE		CSV, AdSV	
					HMDE, MFE
Cobalt	ASV, CCSA	HMDE, MFE	Mercury	ASV, PSA CCSA	Au, Pt, GC
		HMDE, MFE			
	AdSV				
Copper	ASV, PSA	HMDE, MFE	Molybdenum	CSV, CCSA	HMDE, MFE
	AdSV	HMDE, MFE		AdSV	HMDE, MFE
Ferric hydroxide	CSV	HMDE, MFE	Nickel	ASV, CCSA	HMDE, MFE
				AdSV	HMDE, MFE
Gallium	ASV, PSA	HMDE, MFE	Palladium	AdSV	HMDE, MFE
Platinum	AdSV	HMDE, MFE	Thallium	ASV, PSA	HMDE, MFE
Praseodymium	AdSV	HMDE, MFE	Tin	ASV, PSA	HMDE, MFE
				AdSV, CCSA	HMDE, MFE
Rhodium	AdSV	HMDE, MFE	Titanium	AdSV	HMDE, MFE
Selenium	ASV	Au	Tungstenate	CSV	HMDE, MFE
	CSV, CCSA	HMDE, MFE			
		HMDE, MFE			
	AdSV				
Silicon	AdSV	HMDE, MFE	Uranium	AdSV, CCSA	HMDE, MFE
Silver	ASV, CCSA	Au, Pt, GC, MFE	Vanadate	CSV, AdSV	HMDE, MFE
Technetium	AdSV	HMDE, MFE	Zinc	ASV, PSA	HMDE, MFE
				AdSV	HMDE, MFE
Tellurium	ASV	Au			
	CSV	HMDE, MFE			

A major difference between AdSV and conventional stripping voltammetry is the utilization of a spontaneous adsorption process during the preconcentration step. This involves preconcentration by interfacial accumulation at an open circuit without any

electrolysis. The adsorbed species cannot be deposited by electrolysis and significantly more sensitive voltammetric responses are obtained for adsorbed species than for solution species alone. This results in a substantial improvement in the limit of detection by several orders of magnitude compared with corresponding solution-phase voltammetric measurements. This improvement in sensitivity for metals is due to the formation of metal complex(es) that possess surface active properties and that can be subsequently reduced during the potential scan. This is particularly beneficial for enhancement of the electrode processes of substances with extreme irreversible behaviour, such as nickel and cobalt. The sensitization factor of this approach is about 100 - 1,000 or more, resulting in improved limits of detection for the analytes. AdSV can be applied to the determination of both inorganic and organic substances in various samples.

The AdSV measurement of metals and metalloids involves three steps, similar to those described previously for the conventional stripping voltammetry, as follows [22,23]:

(a) Interfacial accumulation of the analyte as a metal complex or chelate after initial reaction with a complexing or chelating agent at an optimum pH:

$$M^{n+} + nL^{-} \rightarrow ML_n(aq)$$

The resulting metal complex or chelate is preconcentrated onto an electrode by adsorption at a carefully chosen and controlled potential, as follows:

$$ML_n(aq) \rightarrow ML_n(ads)$$

(b) Equilibration of solution by turning off the stirring device for 15-30s as for conventional stripping voltammetry.

(c) Reductive removal (stripping) of the preconcentrated chelate or complex by reduction, as follows:

$$ML_n(ads) + ne^{-} \rightarrow M^{n+} + nL^{-}$$

The potential sweep, in this case in the negative direction, is performed as for CSV. The resulting peak current is also directly related to the analyte concentration. It is widely accepted that the reduction peak current results from the reduction of either a reducible group on the complex or of the metal itself in the adsorbed complex [20,22,23,26]. In either case a necessary requirement is that the reducible component must be close to the electrode surface.

Figure 3 shows the responses obtained for nickel and cobalt in orchard leaves sample by DPAdSV with a mercury film electrode and quantification is achieved by standard additions method. Table 2 also provides a list of heavy metals and metalloids that can be determined by AdSV. A detection of limit within 0.01 to 1 nmol l-1 or lower is readily accomplished by AdSV due to the high efficiency of the reduction step and of the adsorption of the analyte. Lower detection limits have been accomplished by inclusion of oxidants, such as nitrite, nitrate, peroxide, chlorite and hydrogen ions [22]. The addition of these substances increases the peak current significantly by chemically oxidizing the reduction product generated during

the stripping step. This catalytic current generation has been successfully used for the AdSV determination of Co, Cr, Fe, Mo, Pt, Ti, W and other metals.

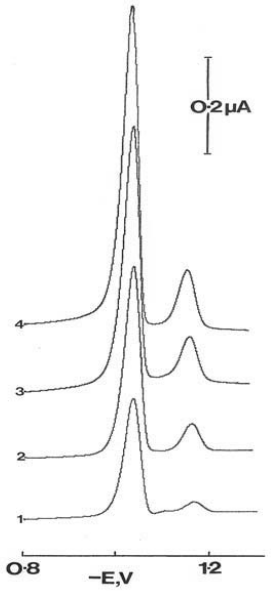


Figure 3. Simultaneous determination of Ni and Co in dry-ashed orchard leaves sample. (1) sample only; (2) +2.5 and 0.5; (3) +5.0 and 1.0; and (4) +7.5 and 1.5 μg/L of Ni and Co, respectively (Reproduced from [39] with with permission from Royal Chemical Society© 1989).

2.2. Stripping Potentiometric Analysis

Stripping potentiometry (SP) also employs an electrochemical preconcentration step to increase analyte concentration on electrode surface and, hence, to achieve considerable analytical sensitivity, as described previously for stripping voltammetry [24-26]. Generally, the steps involved in SP are identical to those described earlier for the stripping voltammetric methods, except that in this case the stripping of the analyte from the electrode is accomplished with the aid of a chemical oxidant (such as oxygen or mercuric ions) and the analytical response is dE/dt vs E. The different forms of SP that have been used for the analysis of metals and metalloids are briefly discussed below.

2.2.1. Potentiometric Stripping Analysis

The most commonly used form of stripping potentiometry is potentiometric stripping analysis (PSA), also sometimes refereed to as anodic stripping potentiometry (ASP). The

preconcentration step involves the deposition of metal ions into a mercury or solid electrode, as described previously for ASV. Also following the deposition period, a brief equilibration period (15-30 sec) is allowed, but in this case the deposited metals are chemically oxidised with the aid of a chemical oxidant according to their redox potentials as follows:

$$M(Hg) + n/m\ Ox \rightarrow \quad M^{n+} + n/m\ Red$$

where Ox is a chemical oxidant, such as dissolved oxygen or mercuric ions, used to strip the amalgamated metal.

As the diffusion layer thickness during preconcentration is equal to the diffusion layer thickness during stripping when the solution is stirred or the working electrode is rotated at the same rate during these processes, the time needed to obtain complete reoxidation of the metal-mercury amalgam, M(Hg), tstrip is given as:

$$t_{strip} \alpha [M^{n+}]\, t_{dep}\, d_S d_P^{-1} \{\Sigma n/m\ Dz_{j(ox)}[Ox]\}^{-1}$$

Furthermore, assuming that the stirring rate is constant and that the [Ox] and the temperature do not change, the stripping time (tstrip) is proportional to the concentration of metal ions multiplied by the deposition time as illustrated below [3]

$$t_{strip} \alpha [M^{n+}].\, t_{dep}$$

During the stripping step, the stripping time increases rapidly as the potential is scanned towards more positive potentials until the concentration of the metal in mercury or on a solid electrode approaches zero, causing the stripping time to peak and then decay as the substance is depleted from the vicinity of the electrode surface, as illustrated in Figure 4.

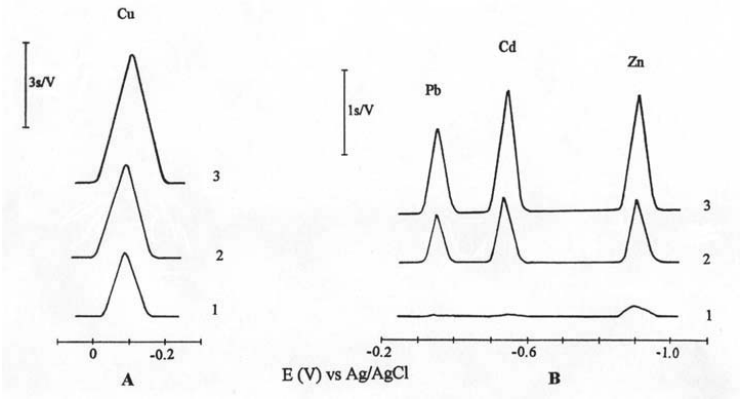


Figure 4. Determination of Cu, Pb, Cd and Zn in tap water by ASP. (1) sample only; (2A) +19.6; (3A) +38.5 µg/L Cu; (2B) +9.6; (3C) +18.7 µg/L Pb, Cd and Zn. Gallium was added to prevent intermetallic compound formation between Cu and Zn (Reproduced from [119]).

Again the two important analytical parameters that can be obtained from the stripping potentiogram are peak potential (Ep), which is useful for qualitative analysis, and the stripping time (tstrip), which is useful for quantitative analysis. Hence, a plot of tstrip versus analyte concentration will give a straight line within an operational linear concentration range. Some of the heavy metals and metalloids that can be determined by PSA are also listed in Table 2. Again the two important analytical parameters that can be obtained from the stripping potentiogram are peak potential (Ep), which is useful for qualitative analysis, and the stripping time (tstrip), which is useful for quantitative analysis. Hence, a plot of tstrip versus analyte concentration will give a straight line within an operational linear concentration range. Some of the heavy metals and metalloids that can be determined by PSA are also listed in Table 2.

2.2.2. Constant Current Stripping Analysis

Constant current stripping analysis (CCSA) is another form of SP which is similar in many respects to CSV and it is similarly useful for the determination of substances that are not soluble or insufficiently soluble in mercury. Like CSV, it relies on the formation of insoluble deposits on the electrode surface. The required processes and conditions for the preconcentration step in CCSA are identical to those of CSV, except that a constant current (anodic or cathodic) is applied to the electrode during the stripping step to remove the deposited material [34]. The resulting stripping time, t strip, is dependent on the electrode area, A, concentration of the analyte and the magnitude of the current applied, i.

$$t_{strip} \sim [M^{n+}].\ t_{dep}.\ A.\ i^{-1}.\ \text{constant}$$

The sensitivity of the technique is therefore determined by the current density rather than the concentration of the oxidant as required for PSA.

As for PSA, the important analytical parameters are peak potential (Ep) for qualitative analysis and the stripping time (tstrip) for quantitative analysis. Again, quantification by CCSA is achieved by plotting tstrip versus analyte concentration. Table 2 also list some of the metals and metalloids that can be determined by CCSA.

2.2.3. Adsorptive Constant Current Stripping Analysis

Adsorptive constant current stripping analysis (AdCCSA) is a form of SP which, like AdCSV, involves adsorptive preconcentration and subsequent stripping of the adsorbed analyte by application of a constant current, as for CCSA. As in conventional PSA the analytical response is given as a potential-time plot. However, in utilising surface adsorption in the preconcentration step a wide linear concentration range is often obtained at low suface coverage [27]. The use of shorter accumulation times, sample dilution or reduced stirring rates are beneficial in this case for minimising surface coverage.

Some successful application of AdCCSA include the determination of iron as iron(III)-SVRS complex [35], or as iron(III)-5-Br-PADAP complex [36], molybdenum as molybdenum(VI)-8-quinolinol complex [37], uranium as uranium (VI)-catechol complex [16], nickel and cobalt as nickel and cobalt (II)-DMG complexes [38]. However there is a considerable potential for further development in this area. Figure 5 shows the stripping potentiograms obtained for the determination of Ni by AdCCSA.

3. Analytical Applications of Stripping Analysis to Foods and Beverages

The important practical considerations for successful utilization of both SV and SP for the determination of heavy metals and metalloids in foods, beverages and several other samples include type of instrumentation, choice of electrodes, electrolysis potential, electrolysis time, supporting electrolyte, oxidant, oxidant concentration, and chelating/complexing agents. These considerations have been discussed in considerable details in several significant literature reviews and books devoted to SV and SP [22-24,26] and will therefore not be revisited in this chapter. This section of the chapter will instead be devoted to the several reported successful use of SV and SP for the determination of a wide range of heavy metals and metalloids in foods and beverages at trace and ultra-trace concentrations.

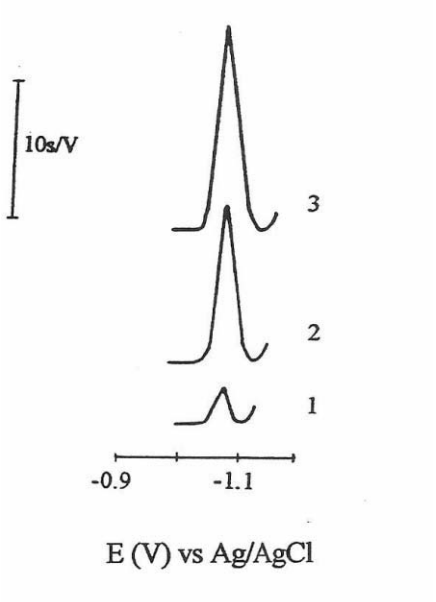


Figure 5. Determination of Ni in drinking water by AdCCSA. (1) sample only; (2) +9.9; and (c) +19.6 µg/L (Reproduced from [119]).

3.1. Animal Tissues, Fruits, Vegetables and Non-Dairy Products

Despite the extremely high sensitivity and selectivity of stripping voltammetry and stripping potentiometry, their direct application for reliable determination of heavy metals and metalloids in solid, complex liquid and mixed solid-liquid food samples, such as animal tissues (meat, fish and other seafoods), fruits and vegetables, is not possible. An important requirement for reliable and accurate use of these methods for such samples is the need for the removal or minimization of the enormous matrix effect that can affect reliable determination of the heavy metals and metalloids at trace and ultra-trace concentrations. In particular, the presence of organic matter can be very troublesome for these methods and often requires thorough and complete decomposition prior to the SV and SP measurements. Such decomposition needs to be performed carefully, not only to breakdown the undesirable organic matter, but to also ensure that the analytes in the food samples are retained quantitatively and is adequately presented for measurement in solution in a form(s) that are suitable for reliable quantification by SV and SP [5,12,27-34,39-43]. Owing to the reliance of SV and SP on the presence of the analytes in form(s) that are suitable for optimum detection, careful and complete decomposition of food samples is more critical for obtaining accurate and reliable results. If not adequately decomposed, organic residues in the sample solution will inhibit or interfere with associated electrode processes in SV and SP, consequently giving wrong results.

By far the most commonly used decomposition methods in conjuction with SV and SP for the determination of trace and ultra-trace concentrations of heavy metals and metalloids in various food samples and products are wet digestion and dry ashing [25,27,39,44,45]. Due to the reduced danger of losses associated with the use of wet digestion at lower temperatures, there has been considerable more interest in using this method of decomposition for SV and SP determination of heavy metals and metalloids in foods. Nevertheless, there is still considerable interest in using dry ashing, but the need to minimise or prevent losses often requires careful temperature manipulation and may even involve the use of ashing aids [12,15,25,27,39,41,46-48]. Whichever the choice of digestion method (wet or dry), extreme care needs to be taken to ensure at all times that the reagents used, such as acids, acid mixtures and ashing aids, do not contaminate the sample and render the results obtained by SV and SP for the food samples invalid. By careful choice and preparation of reagents used for decomposition of food samples by wet digestion or dry ashing, it is possible to minimize possible sources and extent of contamination.

In a further attempt to prevent or minimize contamination and losses of heavy metals and metalloids, other decomposition methods have been employed for food analysis by SV and SP. Notable among these is the use of closed systems, such as wet decomposition in pressure bombs, microwave digestion, combustion in oxygen flask. Others involve the use of special chemicals, such as tetraalkyammonium hydroxide and lumatom for direct dissolution of solid food materials [17,49,50].

The overall analytical considerations for successful and reliable determination of heavy metals and metalloids in solid food and /or mixed solid-liquid food samples by SV and SP is illustrated in Figure 6. As indicated, the sample may require additional pre-treatment (e.g. in the case of Se, As, Sb and some other analytes) to ensure that the analyte is present in a form that can be detected by SV and/or SP. For example, a sample decomposed by either wet digestion or dry ashing for selenium determination in food will have the analyte present predominantly as Se^{6+} in the final sample solution due to the oxidative nature of the decomposition method. The direct analysis of selenium in the resulting sample solution by CSV or CCSA is likely to indicate that the analyte is not present because Se^{6+} is electroinactive. However, if the sample digest is further treated with hydrochloric acid prior to the measurement, the selenium response, if the analyte is present in the sample, would be realised. This is a very crucial factor that must be considered when embarking on the determination of metals and metalloids in foods and other biological materials. Failure to ensure that a sample is adequately presented for analysis by SV and SP methods will render the results obtained meaningless and useless, wasting valuable time and effort. Even where there is no issue with the oxidation state of the analyte, incomplete decomposition of the sample will also give spurious analytical data, which may be much lower or higher than actual concentrations. In some cases the results obtained may be higher than the actual concentrations if the matrix components of the incompletely digested samples interferes positively with the stripping responses obtained by SV or SP.

A very useful way of confirming if the overall analytical methodology is adequate for heavy metals and metalloids analysis is to validate the method by either: (a) analysing a certified food or related reference material; (b) analysing samples spiked with known concentrations of the analytes to determine percentage recovery; or (c) compare results obtained by SV or SP with those of other analytical methods. The preferred validation approach is (a) or (b) as either of these focuses more properly on testing the adequacy of the sample solution to the electroanalytical methods. It is a well established fact that a sample digest that is suitable for one analytical method may not necessarily be suitable for a different analytical method.

For example, a sample digest that gives low concentrations for a particular analyte by SV or SP may well give higher concentrations if analysed by ICP-OES where the measurement may enable further decomposition of the sample. This is why precaution must be taken in using approach (c) for validation of SV or SP determination of metals and metalloids in foods. If this approach is to be used properly for validation, there is a particular requirement to ensure that the sample is presented to each different method in the best suitable form. Otherwise, the whole exercise will be nothing more than comparing apples with oranges. In general, as an advantage, a sample adequately digested for SV and SP determination of heavy metals and metalloids in foods and other biological materials will be suitable for verification by other methods, but the reverse is not necessarily true. However, this can be easily

identified from the extent of agreement or disagreement in the analytical data obtained by both methods. Where there is a huge difference, it does not mean that one or the other method is not adequate, it may just be that the digested sample has not been adequately presented for analysis by the chosen analytical methods.

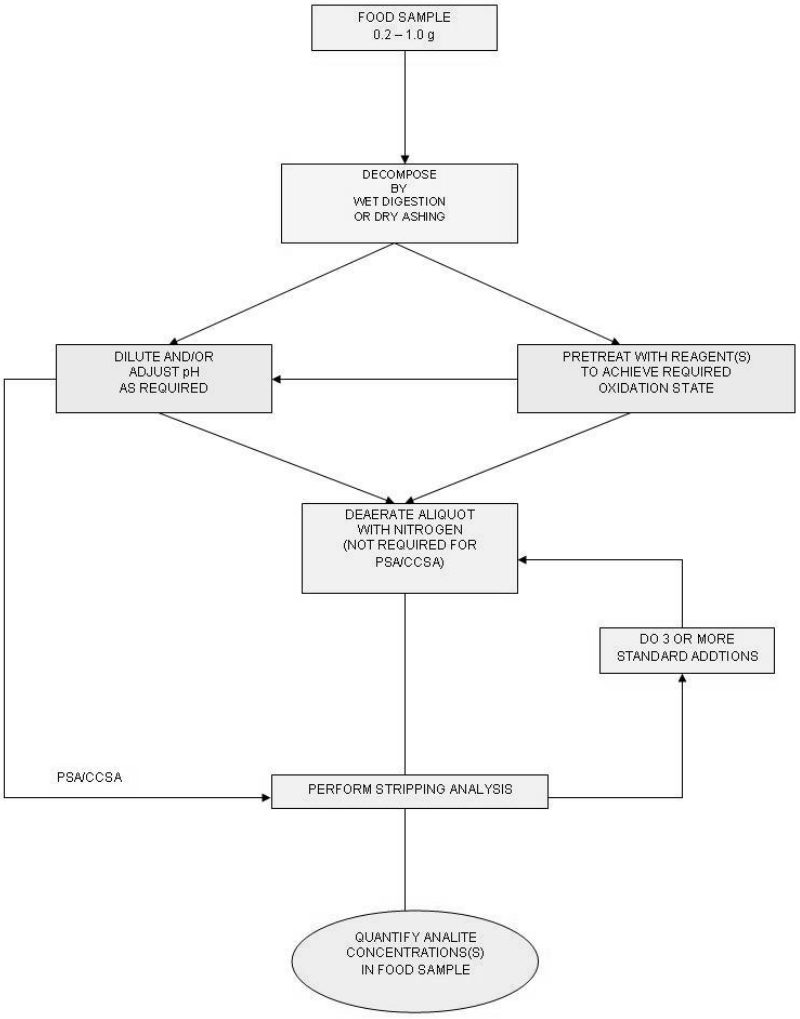


Figure 6. Overall analytical scheme for the determination of heavy metals and metalloids in foods by electrochemical stripping analysis.

Table 3 lists some of the foods that have been successfully analysed, after adequate sample decomposition or other pre-treatment, for a wide range of heavy metals and metalloids by SV and/or SP. In many cases, simultaneous determination of a number of metals and metalloids in a single sample solution is achieved.

Table 3. Some heavy metals and metalloids that have been determined in various food samples by electrochemical stripping analysis

Food	Metals/ Metalloids	Stripping Technique	Electrode	Ref.
Animal Muscle	Zn,Cu,Pb,Cd,Se	DPCSV	HMDE	[40]
	Se	DPCSV	HMDE	[39,43,65]
Apple Leaves	Sb	PSA(ASP)	GCMFE	[82]
	Mo,V	AdSV	GCMFE	[42,83]
Apple Pomace	Cu,Pb,Cd,Zn	DPASV	HMDE	[78]
Beet Pulp	Cu,Pb,Cd,Zn	DPASV	HMDE	[78]
Beet Sugar	As	DPCSV	HMDE	[56]
	Pb,Cd,Zn	DPASV	HMDE	[55]
Bovine Liver	Cu,Zn	DPCSV	HMDE	[40]
	Ni,Co	AdSV	AuFE	[84]
	As	ASV	GCMFE	[85]
	Cu,Pb,Cd,Zn	ASP/PSA	GCMFE	[42,83]
	Mo,V	AdSV	HMDE	[39,43]
	Se	DPCSV	HMDE/ RAuDE	[65]
		DPCSV/ DPASV		
Bowens Kale	Cd,Pb	ASP	GCMFE	[86]
	Se	DPCSV	HMDE	[65]
Canned Foods	Pb,Sn	ASV	HMDE	[87]
	Pb,Sn,Cu	PSA(ASP)	GCMFE	[88]
Cereals	Cu,Cd,Hg	DPASV	HMDE	[89]
Cheese	Cu,Cd,Pb	PSA	GCMFE	[63]
Eggs	Pb	ASV	HMDE	[90]
Dried Fruits	Cu,Pb,Cd,Zn	PSA(ASP)	GCMFE	[27]
Fish	Hg,As,Se	CSV/ASV	HMDE/MFE	[46,65]
	Cu,Pb,Cd,Zn	DPASV/ PSA	AuDE	[91]
	Pb,Sn	DPCV, AdSV	HMDE/MFE	[46]
	Se	CSP/PSA	GCMFE	[85]
	Hg	PSA (ASP)	GCMFE	[70]
			AuDE	[48]
				[9]
Frankfurters	As	CSV	HMDE	[92]
Garlic and Ginseng	Ge	AdSV	HMDE	[113]
Grape Pomace	Cu,Pb,Cd,Zn	DPASV	HMDE	[78]
Hay Powder	Pb,Sn	DPCV, AdSV	GCMFE	[70]
Human Diet	Se	CSP/PSA	GCMFE	[48]
Ketchup	As	ASV	AuFE	[84]
Kidney	Se	CSP/PSA	GCMFE	[45,48]
Maize Meal	Cu,Pb,Cd,Zn	SWASV	HMDE	[93]

Food	Metals/ Metalloids	Stripping Technique	Electrode	Ref.
	As,Se	SWCSV	HMDE	
	Mn,Fe	SWV	HMDE	
Meat	Cu,Pb,Cd,Zn	DPASV	HMDE	[40, 94]
	Se,As	DPCSV	HMDE	
	Ni, Co	AdSV	HMDE	
Milk	Cu,Pb,Cd	PSA/ASV	GCMFE/ HMDE	[16,62]
	Se	DPCSV/	HMDE/ RAuDE	[65]
	Ni	DPASV	HMDE	[114]
		AdSV		
Milk Powder	Cu,Pb.Cd	PSA(ASP)	GCMFE	[16]
	Se	PSA(CSP)	CFMFE (Carbon Fibre)	[35]
Milk Protein	Pb	PSA	GCMFE	[64]
Mussel Tissues	Pb,Sn	DPV, AdSV	GCMFE	[70]
	Se	PSA(CSP)	GCMFE	[45]
	Hg	PSA (ASP)	AuDE	[9]
	Co,Ni	AdSV	HMDE	[115]
Mussels (Tinned)	Cu,Pb,Cd,Zn	PSA(ASP)	GCMFE	[85]
Nuts	Cu,Pb,Cd,Zn	PSA(ASP)	GCMFE	[27]
Oranges	As	CSV	HMDE	[92]
Orchard Leaves	As	DPCSV	HMDE	[95]
	Se	ASV	AuFE	[84]
	Cu,Pb,Cd	DPCSV	HMDE	[43]
		DPASV/	HMDE	[40]
	Ni,Co	PSA(ASP)	HMDE	[17]
	Sb	AdSV	HMDE	[25,40]
	Hg	PSA(ASP)	GCMFE	[82]
		PSA(ASP)	AuDE	[9]
Oyster Tissues	Cd,Pb	DPASV	HMDE	[39]
	Ni,Co	AdSV	HMDE	[43]
	Se	DPCSV	HMDE	[17]
	As	DPCSV	HMDE	
Palm Oil	Cu	DPASV	HMDE	[79]
Peach Leaves	Sb	PSA(ASP)	GCMFE	[82]
	Mo,V	AdSV	GCMFE	[42,83]
	Cu,Pb,Sb,Bi	ASP (PSA)	Rotating GCAuFE	[52]
Pineapple	Pb	PSA	GCMFE	[66]
Rice Flour	Cu,Pb,Cd,Zn	SWASV	HMDE	[93]
	As,Se	SWCSV	HMDE	
	Mn,Fe	SWV	HMDE	[116]
	Al	AdSV	HMDE	
Shrimps	As	CSV	HMDE	[92]
Spices	Cu,Pb,Cd,Zn	PSA(ASP)	GCMFE	[27]
Sugar	Cu,Pb,Cd,Zn	ASV	HMDE	[96]
Tobacco Leaves	As	DPCSV	HMDE	[97]
Tomato Leaves	Pb,Cd	PSA(ASP)	HMDE	[17]
Tomato Pomace	Cu,Pb,Cd,Zn	DPASV	HMDE	[78]
Vegetables	Sn,Pb,Cd	DPASV	HMDE	[99]

Food	Metals/ Metalloids	Stripping Technique	Electrode	Ref.
Wheatmeal/ Wholemeal	Cu,Pb,Cd,Zn	SWASV	HMDE	[93]
	As,Se	SWCSV	HMDE	
	Mn,Fe	SWV	HMDE	
Yeast (Brewers)	Se	DPCSV	HMDE(SMDE)	[98]

DPASV: differential pulse anodic stripping voltammetry, DPCSV: differential pulse cathodic stripping voltammetry, ASV: anodic stripping voltammetry, CSV: cathodic stripping voltammetr, PSA: potentiometric stripping analysis, AdSV: adsorptive stripping voltammetry, CCS: constant current stripping analysis, AdCCSA: adsorptive constant current stripping analysis, SWAS: square wave anodic stripping voltammetry, SWCSV: square wave cathodic stripping voltammetry, SWV: square wave voltammetry, DPV: differential pulse voltammetry, HMDE: hanging mercury drop electrode, GCMFE: glassy carbon mercury film electrode, AuFE: gold film electrode, RAuFE: rotating gold film electrode, MFE: mercury film electrode, AuDE: gold disc electrode, RAuDE: rotating gold disc electrode, CFMFE: carbon fibre mercury film electrode, GCAuFE: glassy carbon gold film electrode, SMDE: static mercury drop electrode.

The approach used in a number of studies for the SP determination of heavy metals in spices, dry fruits and nuts [51], fruit leaves [52], grain [53,54], and pine needles [17] is worth a mention. The samples were washed, digested with lumatom [17] by heating for several hours, filtered, supporting electrolyte and methanol were then added prior to measurement by PSA.

As evident from the data in Table 3 both SV and SP methods can be used to determine these analytes in any food samples, provided the measurement is preceded by adequate sample decomposition and/or pretreatment.

3.2. Sugar

One of the food related areas where the use of SV and SP methods have been found useful for the analysis of heavy metals and metalloids is in sugar. Due to its nutritional roles in human diet and its presence as a major ingredient in various foodstuffs, the control of trace metal concentrations in sugar is very important. There is a correlation between sugar quality, price and metal content. Depending upon the sugar processing practices and on soil type (including extent of soil contamination) on which sugar beets are grown, trace metal concentrations in sugar can vary considerably. Therefore, a very important strategy for improving sugar quality has been to control trace metal concentrations and minimise sugar pollution through improved husbandry and processing practices.

As discussed in previous section for solid food or mixed solid-liquid food samples, adequate decomposition or pre-treatment is necessary prior to the SV or SP determination of heavy metals and metalloids in sugar and sugar beets. In one reported work on sugar, Sancho et al. [55] used DPASV at a HMDE to determine trace concentrations of zinc, cadmium and lead in refined beet sugar without prior treatment of samples. The method was successfully

applied to the determination of the metals in commercial beet sugar samples at concentrations below 10, 35, and 80 µg/kg for Cd, Pb and Zn, respectively. The direct determination of the metals in sugar beets reduced analysis time and risk of sample contamination considerably. In another study, Sancho et al. [56] also used DPASV and DPCSV at a HMDE to determine copper and arsenic, respectively, in refined beet sugar without pre-treatment. Again, they successfully determined Cu and As in commercial beet sugar samples and found concentrations below 15 and 50 µg/kg for arsenic and copper, respectively.

Other applications of ASV to the determination of heavy metals in sugar is identified in Table 3. Sattar et al. [57] have also reported on the use of PSA for the determination of heavy metals in oils, tea and sugar.

3.3. Milk, and Milk Products

Milk and other dairy products are consumed daily as part of a balanced diet because they contain a good balance of protein, fat and carbohydrate and essential nutrients such as vitamins and minerals. In particular, the presence of calcium in milk and milk products has been found to have an important role in bone health. However, the composition of milk can be affected by factors such as fat content, protein type and proportion, levels of sugar; various vitamins and minerals. Owing to the regular consumption of milk, there has been some interest in the determination of some heavy metals in milk and milk products by SV and SP.

Condensed, evaporative, sweetened, whole milk and non-fat dry milk have been analysed for heavy metals by various modes of SP [58]. Other milk and milk products have been analysed by PSA and CCSA for cadmium [18], copper [18,59,60], lead [18,59,60] and selenium [61]. The data in Table 3 also shows that the concentrations of Cd, Pb, and Cu in cheese, milk and milk protein have been analysed successfully by ASV and PSA [62-64]. Milk powder have also been analysed for Se by CCSA [35], DPCSV and DPASV [65]. The use of AdSV at a HMDE has also been reported for the determination of nickel in milk samples. In all cases the milk samples require decomposition [18,35, 58-65] by either wet digestion or dry ashing prior to the analysis by SV and PSA.

3.4. Fruit Juice and Soft Drink

There has been an extensive interest in the determination of a wide range of heavy metals and metalloids in fruit juices and soft drinks by SV and SP. As listed in Table 4, the range of juices that have been analysed for these substances include apple, grape, orange, peach, pear, and tomato. Soft drinks include canned cola, orangeade and lemonade. The two most commonly determined heavy metals in the fruit juices and soft drinks are lead and tin [66-68]. Except in some cases where the use of wet digestion and dry ashing is required [69], relatively simple pretreatment is often required prior to determination of these substances by SV and SP. However, care must be taken to ensure that the sample is not too acidic as lead and tin deposited onto the electrode during the preconcentration step may be stripped off at the same

potential under this condition and will lead to the superimposition of both lead and tin peaks. However, by judicious control of pH, it is possible to establish a condition where adequate peak separation is achieved. Another possible solution is to use a combination of AdSV and DPASV to determine tin and lead, respectively, in the fruit juices [70]. Where Pb, Cu and Cd interferes with the determination of tin in fruit juices by AdSV, the interferences were easily overcome with the addition of micromolar level of EDTA prior to the measurement [69].

3.5. Alcoholic Beverages

The interest in the determination of heavy metals and metalloids in wine, beer and spirits by SV and SP has continued to grow over the past three decades. As can be seen from the information in Table 4, some of the common heavy metals determined in beer, wine and vinegar by PSA [8,71-73] and DPASV [74] include cadmium, copper, lead and zinc. The required sample treatment for the analysis of these samples is relatively simple, often requiring only the addition of HCl for pH adjustment and of Triton X-100 to beer to prevent effervescence upon acidification [8]. Figure 7 shows the responses obtained by AdSV for the determination of iron in a wine sample. The only pre-treatment required was dilution with distilled water by a factor of 10 [80]. The adequate determination of copper in wine by PSA and SV requires more sample treatment as pH adjustment may not be sufficient [71].

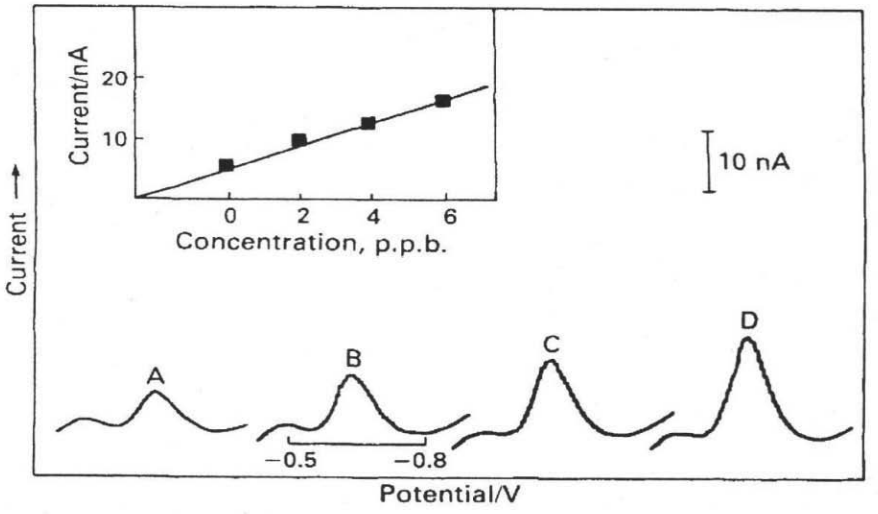


Figure 7. AdSV determination of iron in a wine sample. (a) sample only; (b) +2; (c) +4 and (d) +6 µg/L (Reproduced from [80] with permission from Royal Chemical Society© 1989).

Table 4 . Some heavy metals and metalloids that have been determined in various fruit juices and alcoholic beverages by electrochemical stripping analysis

Beverage/ Juice	Metals/ Metalloids	Stripping Technique	Electrode	Ref.
Apple Juice	Pb	PSA	GCMFE	[66]
Apricot Nectar	Sn	AdSV	GCMFE	[69]
Beer	Cu,Pb,Cd	PSA	GCMFE	[8]
Canned Cola	Pb,Sn	PSA	GCMFE	[101]
Canned Orangade/Soda	Pb,Sn	PSA	GCMFE	[67]
Coca Drinks	Cu,Pb,Cd	DPASV, PSA	HMDE, MFE	[112] [66]
Drinking Water	Pb	SWASV	BiFSPE	[100]
	Pb,Cu	ASV	BiFME	[74]
Fruit Juice	Cu,Pb,Bi,Sb,Sn	PSA	GCMFE	[52,66,67]
Grape Juice	Pb,Sn	PSA	GCMFE	[101]
	Sn	AdSV	GCMFE	[69]
Lemonade	Pb,Sn	PSA	GCMFE	[101]
Orange Juice	Sn	AdSV	HMDE	[102]
	Pb,Sn	PSA	GCMFE	[101]
Peach Nectar	Pb	PSA	GCMFE	[66}
	Sn	AdSV	GCMFE	[69]
Pear Juice	Pb,Sn	PSA	GCMFE	[101]
Pineapple Juice	Sn	AdSV	GCMFE	[69]
Spring and Mineral Water	As	SWCSV	HMDE	[103]
	Ni	AdSV	HMDE	[117]
Spirit (Brandy)	Tl (Thallium)	DPASV	Dental Amalgam	[104]
Spirits	Cu,Pb,Cd	PSA	GCMFE	[8]
Sugar Cane Spirits	Cu,Pb,Zn	ASV	HMDE	[77]
Tap Water	As	DPASV	AuDE	[105]
Tea	Cu,Ni,Pb,Co,Cd	AdSV	HMDE	[118]
Tomato Juice	Sn	AdSV	GCMFE	[69]
	Pb,Sn	PSA	GCMFE	[67]
Vinegars	Pb	PSA	GCMFE	[31]
Wine	Cu,Pb,Cd	PSA, DPASV	GCMFE	[8,71]
	Cu,Hg	DPASV	HMDE	[106]
	Pb,Cu	ASV	HMDE,	[107]
	Fe	AdSV	GCE	[108]
	Ni,Co	AdSV	MFE (micro-electrode) HMDE	[80] [109,110]
	Cu,Pb,Cd,Zn	ASV	HMDE	
	Pb,Zn	DPASV		[111]
	Cu, Pb	ASV	TFMGE	[104]
			Dental Amalgam BiFME	[74]

Pretreatment with HClO4, HNO3 and H2SO4 or low temperature ashing has also been used for the ASV determination of Cu, Pb, Cd and Zn in alcoholic beverages at a HMDE [75]. A mixture of HCl and methanol has also been used as supporting electrolyte for the determination of Sn in alcoholic beverages by ASV [76]. ASV has also been successfully used to determine the concentrations of Cu, Pb and Zn in sugar cane spirits, without the need for prior preteatment or the addition of supporting electrolyte [77]. Similar application to other spirits, including brandy, has been reported.

Table 5. Solubilities of some intermetallic compounds in mercury

Metal 1	Metal 2	Compound	Solubility in Hg (atomic %)
Cu	Ga	Cu-Ga	2×10^{-6}
Cu	Zn	Cu-Zn	5×10^{-18}
Cu	Sn	Cu-Sn	4.6×10^{-6}
Cu	Sn	Cu_3Sn	2.8×10^{-12}

Data from [81].

3.6. Other Food Related Materials

Table 4 indicate that SV and SP have been successfully applied to the determination of heavy metals and metalloids in other food related materials such as drinking (tap) water, spring and mineral water and tea. The range of these substances that have been determined in these samples include Pb, Cu, As and Ni. In general, these materials require little or no sample pretreatment. However, this is not always the case and care must still be taken to ensure that the sample is adequately presented for reliable determination of heavy metals and metalloids by SV and SP. For example, the successful simultaneous determination of copper, nickel, lead, cobalt and cadmium in tea samples by AdSV required prior decomposition of the samples with a mixture of nitric, perchloric and sulfuric acids [118]. The extent and nature of the reagents used for the decomposition highlights the complex nature of the tea samples.

Another unusual food related material to which SV and SP have been applied for the determination of heavy metals is in animal feed. Lippolis et al. [78] successfully used DPASV to determine the concentration of Cd, Pb, Cu and Zn in 12 different animal feeds, including crop residue such as straws and lignocellulose by-products from food industries. The samples which included straw, bagasse, sainfoin, beet pulp, apple pomace, tomato pomace and grape pomace were digested by dry ashing in a muffle furnace at 550oC overnight and dissolved with HClO4 prior to measurement by DPASV. The concentrations of metals found range from 0.02 - 0.30, 0.01 - 0.80, 0.6 – 100 and 3 – 50 mg/kg for Cd, Pb, Cu and Zn, respectively.

Even more unusual is the use of DPASV to determine copper concentrations in palm oil [79]. The sample was initially decomposed by dry ashing prior to the ASV determination of the metal. The method was sensitive and reproducible, and could detect copper in palm oil within 1-31 µg/L. The method was very selective and common major metal ions present in palm oil, such as Fe(II), Fe(III) and Ni(II) did not interfere. The use of PSA for the determination of heavy metals in oils has also been reported [57].

4. Interferences in Stripping Analysis

In using SV and SP for the determination of heavy metals and metalloids in foods and beverages, it is important to be well aware of the likely interferences that may affect the analytical results. In general, a potentially serious interference problem in electrochemical stripping analysis of metals and metalloids is the formation of intermetallic (ternary) compounds. Such compounds are usually formed between 2 or more metals that are deposited simultaneously at the applied electrode potential. Serious consequences often arise from the formation of these compounds, such as variation in stability of the metals, partial masking of the metal with the more negative reduction potential, and peak splitting [23,26,80]. This usually occurs when the resulting intermetallic compound has low solubility in mercury, as demonstrated by the data in Table 5. Also, some metals are more prone to the formation of such compounds than the others. For example, copper and zinc are known to form an intermetallic compound (Cu-Zn) in mercury when the determination of zinc is performed by ASV and PSA [33]. The applied potential for the deposition of zinc is negative enough to also co-deposit copper and other metals. The formation of the Cu-Zn intermetallic compound is more prevalent when MFEs are used due to the higher concentrations of the metals in the smaller volume of mercury in the film. It can be seen from the data in Table 5 that the solubility of the Cu-Zn intermetallic compound in mercury is considerably lower than their individual solubilities (Table 1). However, it is possible to prevent such intermetallic interference of copper in ASV and PSA determination of zinc by adding excess gallium ions to the solution. In this case, the formation of Cu-Ga intermetallic compound is favoured and the zinc ions are left free in solution for determination by ASV and PSA [33].

Another way of overcoming interference and, hence improving selectivity in sample solutions which contain more than one metals or metalloids is by selective preconcentration of the analyte based on a careful choice of the electrolysis (preconcentration or deposition) potential. This is particularly beneficial when the sample contains a number of metals that are electroactive and have peak potentials that are in close proximity. As shown in Figure 8, bismuth can be determined simultaneously with copper and lead by application of an electrolysis potential of -0.6 V. Under this condition, it can be observed that the copper peak is so close and impacted on the resolution of the bismuth peak. Therefore, in order to determine bismuth selectively in the presence of copper, the application of a less negative electrolysis potential (-0.4 V) is preferred, as illustrated in Figure 8b.

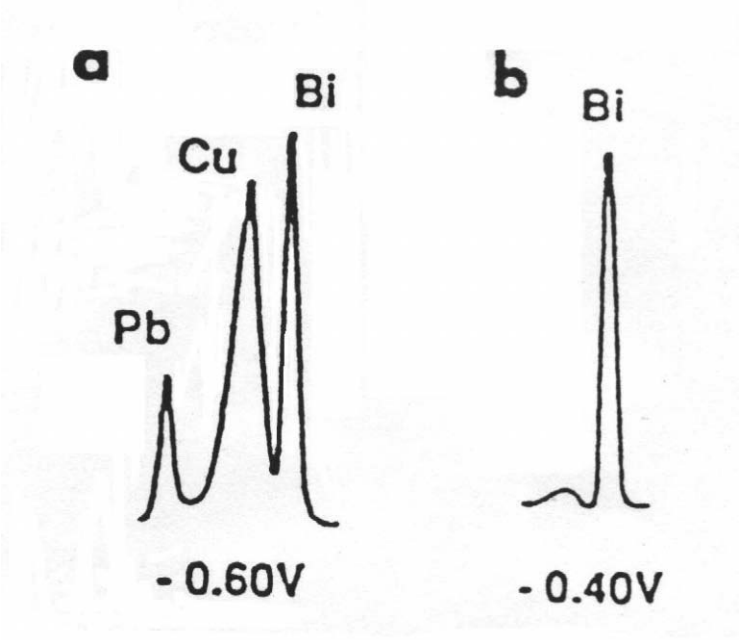


Figure 8. Selective determination of bismuth in the presence of other metals by ASP. Electrolysis potential: (a) -0.6 V vs SCE ; and (b) -0.4 V vs SCE. (Reproduced from [120]).

The interference experienced in AdSV and CCSA are usually caused by either competitive adsorption of surface-active substances, usually organic compounds, but can also be inorganic substances such as halide ions, or by competitive complexation of other metals by the chosen complexing agent [22,23,26]. The latter can result in a competition for sites on the electrode

surface and, consequently, result in a significant depression of the analytical response. The extent of interference depends on the relative affinities of the analyte and interferant(s) to the electrode surface and the concentration ratio of the analyte to the interferant(s).

Interference by surface-active compounds is also a common problem in ASV, PSA, CSV and CCSA, often resulting in a considerable reduction in sensitivity. In all cases, the interference of surface-active compounds can be removed, as discussed in section 3 of this chapter, by destruction of the organic substances prior to electrochemical stripping analysis by use of ultraviolet irradiation or by appropriate digestion in presence of reagents, such as perchloric acid or hydrogen peroxide. Other approaches that can be used to reduce or eliminate interferences by inorganic and organic substances in stripping analysis of foods and beverages by these methods include selective deposition, use of shorter deposition (preconcentration) times, masking of metals with EDTA, more selective or specific choice of complexing agent, medium exchange method and quantification by standard additions method to compensate for matrix effects [22-24,26,69].

5. Conclusions and Future Trends

Electrochemical stripping analysis provides a range of highly sensitive and selective voltammetric and potentiometric stripping methods for reliable determination of heavy metals and metalloids at trace and ultra-trace concentrations in a diverse range of solid, liquid and mixed solid-liquid foods and beverages. When applied to fruit juices and beverages, it provides one of the best choices for reliable and rapid determination of these substances without resorting to an extensive sample pretreatment that can often compromise the reliability of the measurement through sample contamination or loss of analyte. Electrochemical stripping analysis still remains among the few methods that can be used for direct speciation of metals in most samples. It is expected that with the increasing use and design of new electrodes, these methods are likely to gain more use for in-field measurements, clinical diagnostics and on-line measurements in the future. In particular, the use of newer electrodes, such as the bismuth film electrode and conducting polymer-based electrodes, are already making their impacts in electrochemical stripping analysis, but this will gain more use in the future. An emerging new area is in the fabrication of nanocomposite electrodes, based on the use of nanoparticles, carbon nanotubes and nanolayers. These new developments will continue to transform and enhance the use of electrochemical stripping analysis for the determination of heavy metals and metalloids in foods, beverages and other biological/environmental materials in the next decade.

References

[1] FAO/WHO, Joint Expert Committee on Food Additives, WHO Technical Report Series, (1972), No 505,32.
[2] D.H.S. Richardson, Environmental Pollution (Series B), 10 (1985) 261.
[3] D.R. Turner, S.G. Robinson, and M. Whitefield, Anal. Chem., 56 (1984) 2387.
[4] J. Adam, Talanta, 29 (1982) 939.
[5] S. Mannino and M. Bianco, Analyst, 113 (1988) 87.
[6] D. Jagner and K. Aren, Anal. Chim. Acta, 100 (1978) 375.
[7] D. Jagner, Anal. Chem., 51 (1979) 342.
[8] D. Jagner and S. Westerlund, Anal. Chim. Acta, 117 (1980) 159.
[9] D. Jagner and K. Aren, Anal. Chim. Acta, 141 (1982) 157.
[10] D. Jagner, M. Josefson and S. Westerlund, Anal. Chim. Acta, 128 (1981) 128.
[11] D. Jagner, M. Josefson and S. Westerlund, Anal. Chim. Acta, 129 (1981) 153.
[12] C.C. Nanos and M.I. Karayannis, Fresenius Z. Anal. Chem., 340 (1991) 253.
[13] H. Huiliang, C. Hua, D. Jagner and L. Renman, Anal. Chim. Acta, 208 (1988) 237.
[14] H. Huiliang, “Automated Stripping Potentiometry Using Flow Fibre Electrodes”, Ph.D Thesis, Lund, 1988.
[15] S.V. Psaroudakis and C.E. Efstathiou, Analyst, 114 (1989) 25.
[16] L. Almestrand, D. Jagner and L. Renman, Talanta, 33 (1986) 991.
[17] M. Rozali bin Otham, J.O. Hill and R.J. Magee, Fresenius Z. Anal. Chem., 326 (1987) 350.
[18] M. Fayyad, M. Tutunji, R.S. Ramakrishna and Z. Taha, Anal. Lett., 20 (1987) 529.
[19] C. Zbinden, Bull. Soc. Chim. Biol., 13 (1931) 25.
[20] S.B. Adeloju, Inorganic Substances, Encyclopedia, of Analytical Sciences, 2nd Edition, Elsevier Science, (2005).
[21] K. Komarek, Coll. Czech. Chem. Commun., 12 (1947) 399.
[22] C.M.G. van den Berg, Anal. Chim. Acta, (1991) 250, 265.
[23] J. Wang, "Stripping Analysis. Principles, Instrumentation and Applications", VCH Publishers, Deerfield Beach, Florida, 1985.
[24] D. Jagner, Analyst, 107 (1982) 654.
[25] S.B. Adeloju, T.M. Young, D.Jagner and G. Batley, Analyst, 123 (1998) 1871.
[26] K. Brainina and E. Neyman, “Electrochemical Stripping Methods”, American Chemical Society, Washington DC, 1993.
[27] A. Sattar, M. Wahid and S.K. Durrani, Plant Foods Hum. Nutr., 39 (1989) 279.
[28] K.N. Thomsen, H.J. Skov and L. Kryger, Anal. Chim. Acta, 219 (1989) 105.
[29] H.J. Haase, Chem. Labor Betr., 41 (1990) 331.
[30] D. Jagner and A. Graneli, Anal. Chim. Acta, 83 (1976) 19.
[31] S. Mannino, G. Fregapane and M. Bianco, Electroanalysis, 1 (1989) 177.
[32] T.V. Nghi and F. Vydra, Anal. Chim. Acta, 80 (1975) 680.
[33] S.B. Adeloju, E. Sahara and D. Jagner, Anal. Lett., 29 (1996) 283.
[34] J. Obiols, R. Devesa and A. Malet, Analyst, 113 (1988) 1529.
[35] C. Hua, D. Jagner and L. Renman, Anal. Chim. Acta, 197 (1987) 257.
[36] M. Fayyad, Anal. Chem., 59 (1987) 209.
[37] C. Hua, D. Jagner and L. Renman, Anal. Chim. Acta, 192 (1987) 103.

[38] W. Jin and J. Wang, Anal. Chim. Acta, 245 (1991) 77.
[39] S.B. Adeloju, Analyst, 114 (1989) 455.
[40] S.B. Adeloju, A.M. Bond and M.H. Briggs, Anal. Chem., 57 (1985) 1386.
[41] S.B. Adeloju, A.M. Bond and M.L. Noble, Anal. Chim. Acta, 161 (1984) 303.
[42] S.B. Adeloju and F. Pablo, Anal. Chim. Acta, 288 (1994) 157.
[43] S.B. Adeloju, A.M. Bond and M.H. Briggs, Anal. Chem., 56 (1984) 2397.
[44] S.B. Adeloju, A.M. Bond and M.H Briggs, Anal. Chim. Acta, 164 (1984) 181
[45] S.B. Adeloju, D. Jagner and L. Renman, Anal. Chim. Acta, 338 (1997) 199.
[46] S.B. Adeloju, A.M. Bond and H.C. Hughes, Anal. Chim. Acta, 148 (1983) 59.
[47] T.T. Gorsuch, “The Destruction of Organic Matter”, 1st Edition, Pergamon Press, Oxford, 1970.
[48] S.B. Adeloju and T.M. Young, Anal. Chim. Acta, 296 (1994) 69.
[49] C. Watson, Trends Anal. Chem., 3 (1984) 25.
[50] G. Knapp, Trends Anal. Chem., 3 (1984) 182.
[51] D. Jagner, Trends Anal. Chem., 2 (1983) 53.
[52] E. Wang, W. Sun and Y. Yang, Anal. Chem., 56 (1984) 1903.
[53] D. Jagner and K. Aren, Anal. Chem. Symp. Series, Vol. 2: “Electroanalysis in Hygiene, Environmental, Clinical and Pharmaceutical Chemistry”, W.F. Smith (Ed), Elsevier, 1980.
[54] D. Jagner, L. Renman and S.H. Stefansdottir, Anal. Chim. Acta, 281 (1993) 305.
[55] D. Sancho, M. Vega, L. Deban, R. Pardo and G. Gonzalez, Analyst , 122 (1997) 727.
[56] D. Sancho, M. Vega, L. Deban, R. Pardo and G. Gonzalez, Analyst , 123 (1998) 743.
[57] A. Sattar, N. Ammad and L.A. Khan, Nahrung/Food, 37 (1993) 220.
[58] C. Hua, D. Jagner and L. Renman, Talanta, 35 (1988) 525.
[59] G. Wang, X. Li and S.Z. Hui, Fenxi Shyanshi, 8 (1989) 58.
[60] S. Mannino, M. Bianco, and A. Guidi, Latte, 9 (1984) 277.
[61] H. Eskilsson and C. Haraldson, Anal. Chim. Acta, 175 (1985) 79.
[62] D.G. Cornell and M.J. Pallansch, J. Dairy Sci., 56 (1972) 1479.
[63] A. Gargano, R. Giangiacomo and S. Mannino, Riv. Soc. Ital. Sci. Alim., 6 (1986) 461.
[64] S. Mannino, M. Bianco, F. Bobomi and A. Bocca, Food Chemistry, 24 (1987) 159.
[65] S.B. Adeloju, A.M. Bond, M.H. Briggs and H.C. Hughes, Anal. Chem., 55 (1983) 2076.
[66] S. Mannino, Analyst, 107 (1982) 1466.
[67] S. Mannino, Analyst, 108 (1983) 1257.
[68] M. Fayyad, M. Tutunji and Z. Taha, Anal. Lett., 21 (1988) 1425.
[69] S.B. Adeloju and F. Pablo, Anal Chim Acta, 270 (1992) 143.
[70] S.B. Adeloju and F. Pablo, Electroanalysis, 7 (1995) 750
[71] A. McKinnon and G. Scollary, Analyst, 111 (1986) 589.
[72] C. Hua, D. Jagner and L. Renman, Anal. Chim. Acta, 201 (1987) 263.
[73] D. Jagner and S. Westerlund, Anal Chim Acta, 117 (1980) 159.
[74] M. Baldo and S. Daniele, Anal. Lett., 37 (2004) 995.
[75] Y. Yamazaki, Bunseki Kagaku, 31 (1982) 309.
[76] S. Wang and Z. Ni, Yingyong Huaxue, 3 (1986), 81
[77] P.J.S. Barbeira, L.H. Mazo and N.R. Stradiotto, Analyst, 120 (1995) 1647.
[78] M.T. Lippolis, V. Concialini, G.C. Galletti and R. Piccaglia, Analyst, 114 (1989) 1621.
[79] K.H. Wong, Y.S. Fung and K.W. Fung, Analyst, 105 (1989) 30.
[80] J. Wang and S. Mannino, Analyst, 114 (1989) 643.
[81] R. Neeb, "Inverse Polarographie und Voltammetrie", Verlag Chemie GmbH, Weinheim, 1969.
[82] S.B. Adeloju and T.M. Young, Anal. Chim. Acta, 302 (1995) 225.

[83] S.B. Adeloju and F. Pablo, Electroanalysis, 7 (1995) 476.
[84] Davis et al, Anal. Chem., 50 (1978) 137.
[85] L.G. Danielsson, D. Jagner, M. Josefson and S. Westerlund, Anal Chim Acta, 127 (1981) 147.
[86] L. Kryger, Anal. Chim Acta, 120 (1980) 19.
[87] P. Debacker, J.L. Vandenbalck, G.J. Patriarche and G.D. Christian, Microchemical Journal, 26 (1981) 192.
[88] W.G. Shun, M. Min-Hui and Y.J. Fang, Huaxue Xuebao (Acta Chimica Sinica), 42 (1984) 283.
[89] M. Lugowska, M. Striewska and S. Rubel, J. Electroanal. Chem., 226 (1987) 263.
[90] B. Grinitz and N. Tepedino, JAOAC, 64 (1981) 1014.
[91] S. Mannino and G. Granata, Italian J. Food Sci. Technol., 2 (1990).
[92] W. Holak, JAOAC, 59 (1976) 650.
[93] C. Locatelli, Electroanalysis, 16 (2004) 1478.
[94] H.D. Narres, P. Valenta and H.W. Nurnberg, Fresenius Z. Anal. Chem., 317 (1984) 484.
[95] Zima and C.M.G. van den Berg, 289 (1994) 291.
[96] T.M. Karadakhi, F.M. Najib and F.A. Mohammed, Talanta, 34 (1987) 995.
[97] Kowalska, Electroanalysis, 11 (1999) 1301.
[98] W. Holak and J.J. Specchio, Analyst, 119 (1994) 2179.
[99] P. Bourbon, J. Esclassan and J. Vandele, Analysis, 2 (1982) 104.
[100] J. Wang, U.A. Kirgoz and J.M. Lu, Electrochem. Commun., 3 (2001) 703.
[101] S. Mannino, Analyst, 109 (1984) 905.
[102] J. Wang and J. Zadeii, Talanta, 34 (1987) 909.
[103] Ferreira and Barros, Anal. Chim. Acta, 459 (2002) 151.
[104[Mikkelson and Schroder, Anal. Chim. Acta, 458 (2002) 249.
[105] Kopanica & Novotny, Anal. Chim. Acta, 386 (1998) 211.
[106] G. Golimowski, H.W. Nurnberg and P. Valenta, Lebensmittelchem. Gerichtt. Chem., 34 (1980) 116.
[107] L. Sipos, G. Golimowski, H.W. Nurnberg and P. Valenta, Fresenius Z. Anal. Chem., 298 (1979) 1.
[108] M.A. Baldo, C. Bragato and S. Daniele, Analyst, 122 (1997) 1.
[109] J.L. Guinon and J. Garcia Anon, Anal. Chim Acta, 177 (1980) 116.
[110] S. Sanllorente, M.C. Ortiz and M.J. Arcos, Analyst, 123 (1998) 513.
[111] Kh.Z. Brainina, N.Y. Stozhko, G.M. Belysheva, O.V. Inzhevatova, L.I. Kolyadina, C. Cremisini and M. Galletti, Anal. Chim. Acta, 514 (2004) 227.
[112] R. Andruzzi, A. Trazza and G. Marrosu, Anal Lett., 16 (1983) 853.
[113] C. Schleich and G. Henze, Fresenius Z. Anal. Chem., 338 (1990) 145.
[114] Z.Q. Zhang, Z.P. Cheng, S.Z. Cheng and G.F. Yang, Talanta, 38 (1991) 1487.
[115] M.G. Paneli and A. Voulgaropoulos, Fresenius Z. Anal. Chem., 341 (1991) 716.
[116] E. Stryjewska and S. Rubel, Electroanalysis, 3 (1991) 995.
[117] P.A.M. Farias, A.K. Ohara, I. Takase, S.L.C. Ferreira and J.S. Gold, Talanta, 40 (1993) 1167.
[118] Z.Q. Zhang, S.Z. Chen, H.M. Lin and H. Zhang, Anal. Chim. Acta, 272 (1993) 227.
[119] E. Sahara, Masters Thesis, University of Western Sydney, Australia, 1996.
[120] TraceLab Manual, Radiometer Analytical, Lyon, France, 1992.

Food Toxicants Analysis – Y. Picó (Editor)

Chapter 19

Inductively coupled plasma mass spectrometry

Francesco Cubadda

National Centre for Food Quality and Risk Assessment, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

1. Introduction

Inductively coupled plasma mass spectrometry (ICP-MS) developed from the work of Gray and Houk, which in 1980 led to the first ICP-MS publication [1]. In 1983, the first commercial ICP-MS instrument was launched by the Canadian company Sciex. Somewhat later, other companies launched their own ICP mass spectrometers and in a relatively short time the technique established itself as the favourite tool for element determination at trace and ultratrace levels in all types of matrices, including food.

A number of desirable features contributed to this success. These include high sensitivity, multi-element capability, wide linear dynamic range, high sample throughput, and ability to discriminate between isotopes. With modern instruments and in the absence of spectroscopic interferences, the detection limits (DLs) of most trace elements are in the low ng L^{-1} range when digestates of real samples are analysed. Another advantage is the suitability of ICP-MS as a selective on-line detector in hyphenated methods for the determination of element species, which gained further popularity to the technique as the importance of elemental speciation in biological sciences, including food toxicology, began to be recognized.

Today, the technique has become popular for the elemental analysis of food, notwithstanding the expensiveness of the instruments and the high running cost in terms of consumables and maintenance. Many governmental institutions have turned to ICP-MS for their institutional and control activities. On the other hand, the technique is being increasingly used for quality control and compliance with food regulations by major food industries or independent analytical laboratories. As a result, in the international legislation laying down the analytical methods for the official control of trace elements in food, atomic absorption spectrometry (AAS) is no longer being prescribed as the exclusive detection technique. Rather, either performance criteria are established and any validated method that fulfils these requirements is accepted, or ICP-MS is explicitly placed in the list of recommended techniques.

The aim of this chapter is to briefly outline the fundamentals of ICP-MS and review its applications to the detection of toxic elements and elemental species in food and agricultural matrices. It is well known that all elements are toxic given enough dose, but in the present context the term ‘toxic elements’ is used to indicate (non-essential) elements (and species) that pose a significant risk to human health through dietary exposure. Even though the focus

is on food safety and a limited number of analytes of importance in this context will be addressed, it should be realized that nearly all the elements in the periodic table can be accessed by the technique. Those who are interested to a more comprehensive overview of the applications of ICP-MS in food science can refer to a recent review [2] and the references therein.

2. Description of the technique

There are several textbooks which describe in detail the principles of ICP-MS and only a relatively brief overview of the technique is given in the following sections. For an in-depth study of the subject, the work of Montaser [3] is recommended. Updated handbooks [4] are published from time to time and the reader can refer to them for an outline of recent developments in technology and newly available instrument options.

2.1. Principles of ICP-MS

An argon ICP, essentially identical to that of ICP-AES instruments, is the standard ion source on all commercial ICP-MS instruments (Figure 1). A plasma is generated by radiofrequency (RF) magnetic fields induced by a coil wound around the end of a quartz torch through which argon flows. A high-voltage spark is used to seed the argon with electrons which collide with Ar atoms, ultimately causing ionization and plasma ignition (Figure 2). This energy transfer is called inductive coupling, since it involves no electrodes. The motion of electrons and ions, which is continuously induced by the oscillating magnetic field, causes collisions with Ar atoms i.e. ohmic heating of the gas, resulting in temperatures as high as 10,000 K (Figure 3). The plasma operates at powers of between 0.75 and 1.6 kW, at a frequency of 40.68 MHz, and is at atmospheric pressure. Some RF generators use a frequency of 27.12 MHz and reach higher power levels (up to about 2 kW).

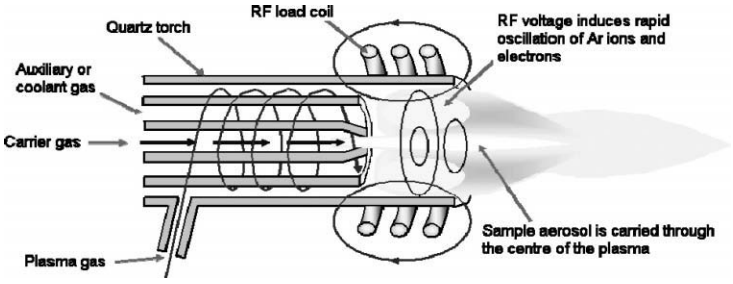


Figure 1. Schematic of the plasma showing the quartz torch and the induction coil. Courtesy of Agilent

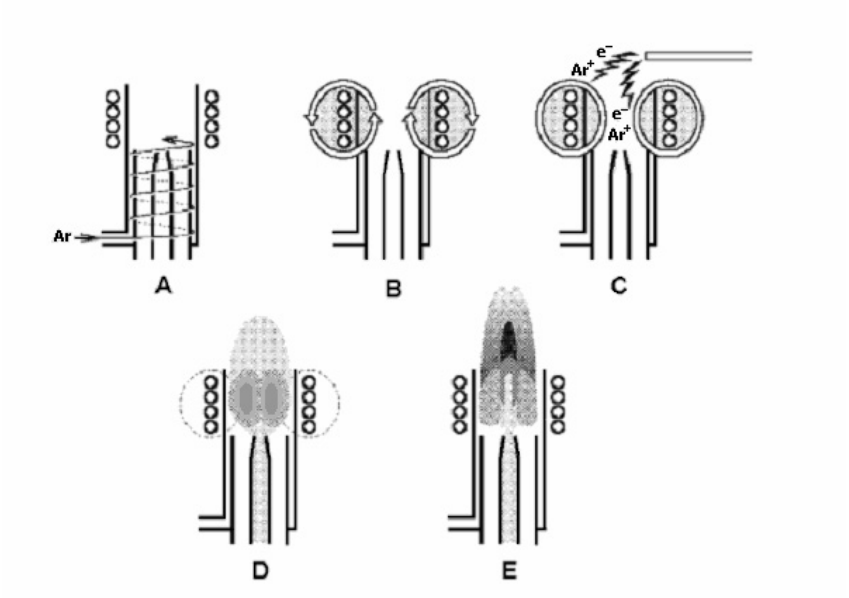


Figure 2 . Ignition sequence of an ICP: A) Argon gas is swirled through the torch, B) RF power is applied to the load coil, C) A spark produces some free electrons in the argon, D) The free electrons are accelerated by the RF fields causing further ionization and forming a plasma, E) The sample aerosol-carrying nebulizer flow punches a hole in the plasma. Courtesy of PerkinElmer Inc.

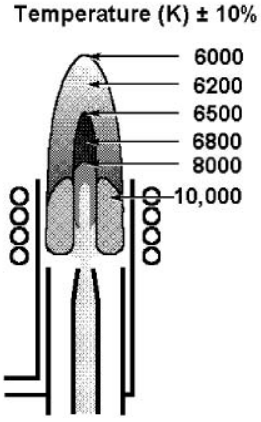


Figure 3. Temperature regions of a typical ICP discharge. Courtesy of PerkinElmer Inc.

Although numerous torch designs are available, the standard torches used on commercial instruments are usually of the Fassel-type and consist of three concentric quartz tubes (Figure 4). The outer tube is for the plasma gas, which is used at high flow rates to maintain and stabilize the plasma as well as to prevent it from melting the tube (Figure 5). In the intermediate tube the auxiliary gas flows, which positions the plasma within the torch and also prevents the overheating of the rim of the inner tube. Finally, the central tube (called injector tube) is used for the carrier gas, which can be the nebulizer gas when a nebulizer is used for the introduction of solutions, and has the function to carry the sample and introduce it into the centre of the plasma. Argon is commonly used as the operating gas for the three gas flows.

Figure 4. Fassel-type torch. Courtesy of Meinhard Glass Products.

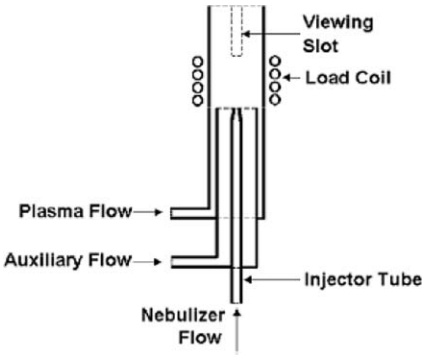


Figure 5. Cross section of the torch. Courtesy of PerkinElmer Inc.

Following ignition, the carrier gas flow essentially punches a hole in the centre of the elliptical plasma, giving it an annular or toroidal shape. Within this central channel created by the carrier gas the sample reaches the plasma and interacts with it. In standard liquid sample introduction, i.e. with the sample being presented to the ICP in solution, the sample is sprayed into the plasma as a wet aerosol by means of a nebulizer and a spray chamber. Once the aerosol created by the sample introduction system penetrates the plasma, a sequence of desolvation, vaporization, atomization, ionization takes place in a few milliseconds. All these processes require energy and in this region the temperature is lower than in the induction region of the plasma (Figure 3).

Analyte ionization in the plasma ($M \rightarrow M^+ + e^-$) takes place through different ionization mechanisms, including electron-impact, charge transfer, Penning ionization [3]. In localized region of the plasma, it can be assumed that a local thermal equilibrium (LTE) is established, which means that the rates of the processes occurring between electrons, ions, and neutrals are equal to that of the inverse processes and all particles are characterized by a common temperature. Under LTE conditions, the population distribution of the ionization products is described by the Saha equation:

$$\frac{n_{M^+} n_e}{n_M} = 2\frac{Z_{M^+}}{Z_M}\left(\frac{2\pi m_e kT}{h^2}\right)^{\frac{3}{2}} \exp\left(-\frac{E_{ion}}{kT}\right) \qquad \text{[eq. 1]}$$

where n_{M^+}, n_M, and n_e are the number densities (n per cm^3) of ions, atoms and free electrons, respectively, Z_{M^+} and Z_M are the partition functions for the ion and the atom, respectively, m_e is the electron mass, k the Boltzmann constant, T the absolute temperature, h the Planck's constant, E_{ion} the ionization energy of the element M.

The degree of ionization is:

$$\alpha = \frac{n_{M^+}}{n_M + n_{M^+}} \times 100 \qquad \text{[eq. 2]}$$

Houk calculated the degree of ionization for atomic ions at an ionization temperature of 7500 K, assuming an electron density of 1×10^{15} cm^{-3} (Figure 6) [5]. It is clear that singly charged positive ions are formed efficiently, α being ≥90 % for some 54 elements. All elements with a first E_{ion} ≤10 eV are expected to have $\alpha \geq 50$ %, and even a number of non-metals having a high first E_{ion}, such as P, S, As, and Se, give good yields of singly charged ions. Only three elements (He, F, and Ne), which possess a first E_{ion} greater than that of Ar, would not be ionized and could therefore not determined by ICP-MS with an Ar plasma. The percentages of M^{2+} formed are also indicated in (Figure 6). Only the elements with the lowest second ionization energy (especially Ba and rare earths) are expected to form some doubly charged ions. However the experimentally observed degree of double ionization under actual operating conditions is lower than that given in Figure 6. The ICP is therefore an efficient elemental source for MS since a simple mass spectrum almost entirely composed by singly charged ions is obtained even when several elements are present in the sample.

H 0.1																	He
Li 100	Be 75											B 58	C 5	N 0.1	O 0.1	F 9×10^{-4}	Ne 6×10^{-6}
Na 100	Mg 98											Al 98	Si 85	P 33	S 14	Cl 0.9	Ar 0.04
K 100	Ca 99(1)	Sc 100	Ti 99	V 99	Cr 98	Mn 95	Fe 96	Co 93	Ni 91	Cu 90	Zn 75	Ga 98	Ge 90	As 52	Se 33	Br 5	Kr 0.6
Rb 100	Sr 96(4)	Y 98	Zr 99	Nb 98	Mo 98	Tc	Ru 96	Rh 94	Pd 93	Ag 93	Cd 85	In 99	Sn 96	Sb 78	Te 66	I 29	Xe 8.5
Cs 100	Ba 91(9)	La 90(10)	Hf 98	Ta 95	W 94	Re 93	Os 78	Ir	Pt 62	Au 51	Hg 38	Tl 100	Pb 97(0.01)	Bi 92	Po	At	Rn
Fr	Ra	Ac															

Ce 98(2)	Pr 90(10)	Nd 99*	Pm	Sm 97(3)	Eu 100*	Gd 93(7)	Tb 99*	Dy 100*	Ho	Er 99*	Tm 91(9)	Yb 92(8)	Lu
Th 100*	Pa	U 100*	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lw

Figure 6. Calculated values of the degree of ionization for M^+ at T_{ion} = 7500 K, $n_e = 1\times10^{15}$ cm^{-3}. Figures in parentheses represent percentages of M^{2+} formed. Elements marked by asterisks yield significant M^{2+}, but partition functions were not available. Reproduced from [5] by permission of the American Chemical Society.

Since the ICP operates at atmospheric pressure while the mass spectrometer requires a vacuum better than 10^{-6} torr, the ions must be extracted from the central channel of the plasma into a vacuum system. This is achieved through a differentially pumped interface (Figure 7), which is maintained at about 2 torr using a mechanical roughing pump. The interface includes two water-cooled metal cones with ca. 1-mm orifice diameter, the sampler and the skimmer, which provide passage for the sample ions. The cones are usually made of Ni, Pt or Cu. The difference in temperature between the sampler cone and the plasma leads to the presence of a boundary layer, a cooler plasma region where analytes are supposed to recombine with oxygen to form oxides. In addition, condensation of solids can lead to plugging of the cone orifice when high-salt solutions are aspirated.

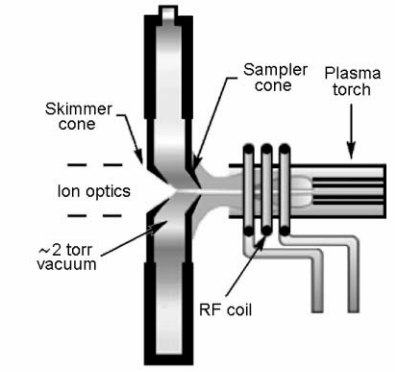


Figure 7. Schematic diagram of ICP-MS interface.

Because of the pressure differential over the sampler cone, gas flows through the orifice carrying some ions along. The reduction in pressure as the plasma gas passes through the sample cone gives rise to a rapid expansion of the gas. This is called supersonic expansion, or free jet, as within a space close to the cone the gas molecules reach supersonic speeds. This jet has a number of useful advantages. Molecules in a supersonic jet flow with non-turbulent, laminar motion. If the tip of the skimmer cone is placed in this region, the gas sampled will have a high component of motion in the axis of the instrument and will thus be easier to bring into focus. The central section of the supersonic jet flows through the central hole of the skimmer cone into a high vacuum. Here the mean free path is long enough for the ions to be focused into the mass spectrometer by electrostatic lenses. The first electrostatic lens (extraction electrode) is at a negative voltage in order to extract positive ions and transport them towards subsequent lenses. Negative ions are repelled and neutral particles diffuse to the vacuum turbomolecular pump.

Once entered the mass spectrometer, the analytes are separated from other ions and analysed. Afterwards, they reach the detector which converts the ion current into electrical pulses that are then counted by its integrated measurement circuitry. The magnitude of the electrical pulses corresponds to the number of analyte ions present in the sample. Trace element quantification in an unknown sample is carried out by comparing the ion signal with known calibration or reference standards. Depending on the type of instrument, different detection systems are used to count the number of ions emerging from the mass analyser.

2.2. Instrumentation

ICP-MS instruments vary in the type of mass spectrometer that is attached to the ICP ion source. Commercially available instrumentation at the time of writing includes quadrupole-based, sector-based and time-of-flight (TOF) based instruments. Quadrupole (Q) mass spectrometers only allow ions of a specific m/z value to reach a detector, ejecting all other ions. Sector instruments use magnetic and electrical fields to focus a beam composed of spatially dispersed ions. Focusing permits ions of a particular m/z to reach the detector through a slit. In TOF instruments, ions travel through a tube at different velocities, depending on their m/z. All ions are detected, with lighter ions reaching the end of the tube before heavier ions.

Quadrupoles are the cheapest of the three types. They are versatile and robust machines, and account for the majority of the ICP-MS instrument sales. Laboratories that routinely analyse samples use quadrupole instruments for their high-throughput capabilities.

Table 1 shows a list of commercially available ICP-MS instruments. Other machines, such as those based on ion traps or Fourier transform ion cyclotron resonance, are being developed for use in research laboratories.

2.2.1. Quadrupoles

These instruments are relatively easy to operate, have good stability, and are the most common instruments used by trace elemental analytical laboratories. A schematic representation of a generic quadrupole-based instrument is shown in figure 8. These mass spectrometers employ a quadrupole mass filter and have a resolution of something less than 1 amu (atomic mass unit). Thus, they are typically referred to as low-resolution instruments.

In their standard configuration, quadrupole instruments features a pneumatic nebulizer and a spray chamber for the introduction of liquid samples. A peristaltic pump completes the sample introduction system. Its function is to deliver the sample to the nebulizer preventing it from freely aspirating the sample. This is done in order to minimize physical interferences in sample aspiration, e.g. due to changes in viscosity between samples.

Table 1. Commercially available ICP-MS instruments

Quadrupoles

Instrument	Manufacturer	Collision/reaction cell
Elan 9000	Perkin Elmer - Sciex	None
Elan DRC-e, DRC II	Perkin Elmer - Sciex	Quadrupole bandpass reaction cell
7500a	Agilent	None
7500ce, 7500cs	Agilent	Octapole reaction cell
X series II	Thermo Electron	Hexapole reaction cell
Platform XS	GV Instruments	Hexapole reaction cell
810-MS, 820-MS	Varian	None[a]

Magnetic sector

Instrument	Manufacturer	Type
Element 2, XR	Thermo Electron	Double focusing
Neptune	Thermo Electron	Double focusing, multicollector
NU Plasma HR, 1700	Nu Instruments Ltd.	Double focusing, multicollector
IsoProbe	GV Instruments	Hexapole reaction cell, single focusing, multicollector

Time-of-flight

Instrument	Manufacturer	TOF orientation
OptiMass 8000	GBC Scientific Equipment	Orthogonal
Renaissance	LECO	Axial

[a] The 820-MS features a collision reaction interface (CRI) system

All the instruments use a similar interface with a sampler and a skimmer cone. Once in the high vacuum region of the mass spectrometer (maintained by a turbomolecular pump), the sampled ions are accelerated and focused into the mass filter by the ion lenses. These lenses consist of stainless steel plates or tubes supplied with precise, user controllable DC voltages,

which attract or repel the sampled ion beam. The positively charged ions are selected by the potentials applied, while neutral and negative particles are rejected. By entering the mass analyser, maintained at a vacuum of approximately 10^{-6} torr with a second turbomolecular pump, the ions reach the third vacuum stage of the mass spectrometer. A complete description of quadrupole mass analysers can be found elsewhere in this book and is not repeated here.

Quadrupoles of commercial ICP-MS instruments can be operated in different modes. The entire mass range or selected ranges may be scanned (scanning mode), or a selected number of masses can be measured (peak hopping mode). Rapid semiquantitative scans on the entire mass range are typically performed in a few minutes.

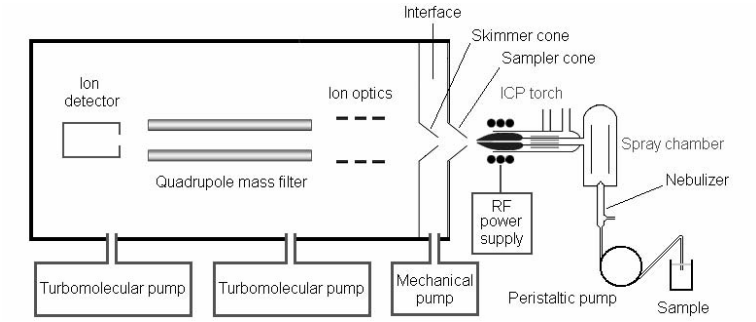


Figure 8. Schematic diagram of a Q-ICP-MS.

When the analytes reach the detector, the ion current is converted into an electrical signal. Today, ICP-MS systems generally use detectors that are based on discrete dynode electron multipliers. Such detectors use discrete dynodes to carry out the electron multiplication and feature high ion signal count rate with a very low background count rate, i.e. enhanced sensitivity compared to earlier detector designs. Detectors are generally positioned off-axis to minimize the background from stray radiation and neutral species coming from the ion source. Most instruments also include at least one photon shop, i.e. a metal disc mounted on the axis of the quadrupole with the aim to block as much plasma light as possible.

Present detectors operate in a dual-mode, i.e. are able to handle low and high ion-count rates by resorting to the pulse-counting mode or the analog mode, respectively. Digital counting provides the highest sensitivity, while operation in the analog mode allows extending the concentration range for which ion signals can be measured. The detectors of this type are capable of achieving approximately eight to nine orders of dynamic range in one simultaneous scan.

Due to limited resolution, spectroscopic interferences are a major problem in Q-ICP-MS (see below, § 2.4). Among them, polyatomic interferences, caused by two or more atoms forming an ion detected at the same m/z as the analyte of interest, are particularly deleterious.

To address this issue, collision and reaction cell technology has been developed and at present nearly all commercially available quadrupole systems can be purchased with an optional cell (Table 1). In general, a collision/reaction cell is a linear RF-driven multipole ion guide in an enclosed reaction volume that may be held under a constant pressure of a high-purity reaction and/or collision gas. The cells are used to promote reactive and non-reactive collisions, with resultant benefits in interference reduction, but also in thermalization/focusing of ions in ICP-MS. Several ion–molecule chemistry schemes can be employed, using a variety of reaction gas reagents selected on the basis of thermodynamic and kinetic principles and data.

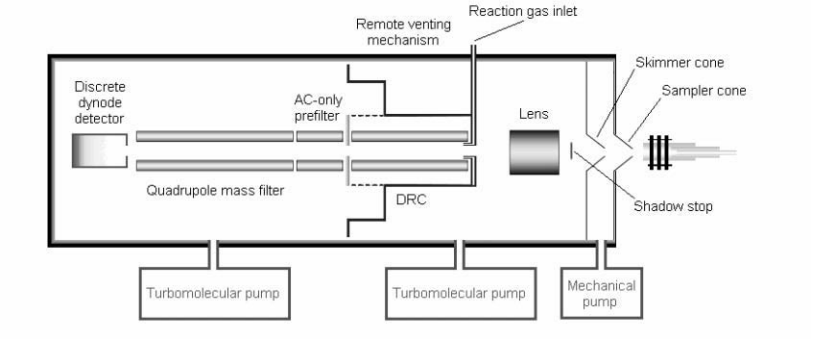


Figure 9. Schematic diagram of a Perkin Elmer SCIEX ELAN® DRC™. Modified from [6] by permission of Elsevier.

Basically two fundamental configurations exist, namely the quadrupole bandpass reaction cell (commercially known as dynamic reaction cell, DRC) and the collision/reaction cells based on higher multipoles. In the quadrupole-based design (Figure 9), reactive gases are used and unwanted precursor and/or product ions are selectively ejected by the quadrupole narrow bandwidth, which enables a high degree of mass-to-charge ratio specificity. Multipole cells use hexapoles or octopoles (Figure 10) as ion guides, which provide higher ion transmittance (i.e. a wide range of ion masses is simultaneously transmitted with higher efficiency than with quadrupoles), but require more selective ion chemistry with respect to the analyte/interfering ions of interest. In the DRC, only a narrow window of masses that are stable in the quadrupole field are selected by proper adjustment of operative parameters. In collision/reaction cells, post-cell kinetic energy discrimination (KED) is used to control secondary chemistry. A potential barrier between the cell and mass analyser is created, typically by operating the cell at a DC offset potential somewhat lower than that of the analysing quadrupole. Beam ions from the ICP retain a significant portion of their original kinetic energy and trajectory, whereas ions formed inside the cell are typically formed at lower kinetic energy and/or with off-axis trajectories. The lower energy ions formed in the

cell are unable to surmount the potential barrier and are thus prevented from passing into the analysing quadrupole and onto the ion detector.

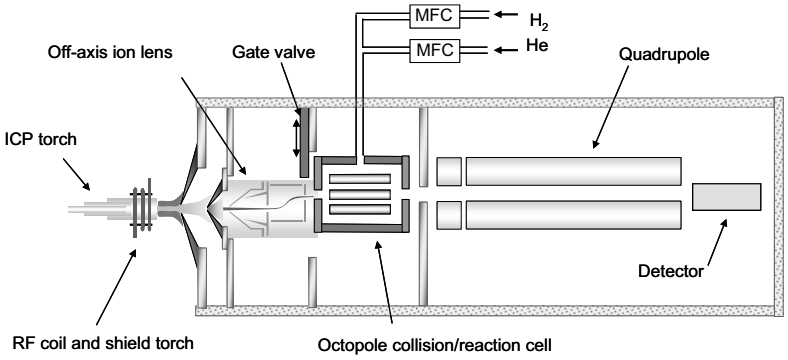


Figure 10. Schematic diagram of the Agilent 7500ce ICP-MS featuring a octapole reaction/collision cell. Courtesy of Agilent.

The gas-phase ion-molecule reactions employed for chemical resolution of spectroscopic interferences in ICP-MS are of the following types:

- charge exchange
- atom transfer
- adduct formation
- condensation reactions.

Charge-transfer reactions require a reagent gas with an ionization energy intermediate between the interfering ion and the analyte ion (in the following reaction, A denotes the analyte, I denotes an isobaric interference, and R denotes the reagent):

$A^+ + I^+ + R \rightarrow A^+ + I + R^+$ Eion A < Eion R < Eion I

Condensation, adduction, or atom-addition reaction reactions have no such restriction on ionization energy of the reagent gas, but may have thermodynamic and kinetic constraints. Hydrogen-based are important atom-transfer reactions, and include proton transfer, hydrogen atom transfer, hydride ion (H^-) transfer. Among atom-addition reactions, oxidation reactions are a particularly important class and have been successfully employed either for analyte or isobar shift of 16 amu (the mass of the oxygen atom). For the reasons explained above, clustering reactions with more reactive, less selective reagents like NH3 can only be employed with a quadrupole bandpass reaction cell. A list of reagent gases commonly used in collision/reaction cell ICP-MS is shown in Table 2. A more detailed description of the principles and functioning of cell devices can be found in recent reviews [6-7].

Table 2. Reagent gases employed in collision/reaction cell ICP-MS (routinely used reagent gases in bold)

Collision gases
He, Ar, Ne, Xe
Charge exchange gases
H_2, NH_3, Xe, CH_4, N_2
Oxidation reagent gases
O_2, N_2O, NO, CO_2
Reduction reagent gases
H_2, CO
Other reaction (adduction) gases
CH_4, C_2H_6, C_2H_4, CH_3F, SF_6, CH_3OH

Reproduced from [7] by permission of The Royal Society of Chemistry

2.2.2. Sector field analysers

Sector field (SF) spectrometers were the second type of ICP-MS instruments to be manufactured. They are more expensive than the other types of ICP-MS instruments and require more expertise on the part of the operator. However, these spectrometers have a very high detection power and provide the only general method for overcoming spectroscopic interferences from polyatomic species, i.e. the use of high mass resolution. This feature is achieved in most present instruments with a double focusing mass analyser, i.e. combining a magnetic sector with an electric sector field analyser.

A SF instrument is represented in Figure 11. The ICP source and the sample introduction system are similar to those used for quadrupole ('low-resolution') spectrometry. Then, depending on instruments, two different options exist, i.e. the regular geometry or the reverse one, which features the magnet before the electric sector. A five-stage vacuum system is normally required for the functioning of the mass spectrometer. A description of SF mass analysers can be found elsewhere in this book and is not repeated here.

Precise mass calibration is critical for SF-ICP-MS, and must be performed more frequently than with quadrupole instruments. A range of resolutions, from low (e.g. 300) to high (e.g. 10,000), can be selected. However resolution is typically increased by decreasing the slit width, which causes a reduction in ion transmission and thus a parallel loss in sensitivity. Besides single collector instruments, several instruments with multiple collectors (MCs) are also available for precise isotope ratio measurements (Table 1).

Figure 11. The ELEMENT 2 double focusing ICP-MS. Courtesy of Thermo Electron Corporation.

Owing to the greater complexity of the mass spectrometer, high-resolution instruments have larger dimensions than the quadrupole-based ones. Being more cumbersome and requiring highly specialized staff to be operated, SF instruments are used for specific applications that require superior accuracy and very low DLs, and for precise isotope ratio measurements (MC instruments).

2.2.3. TOF analysers

The latest addition to the family of ICP-MS instruments has been the TOF-type (Figure 12). In TOF instruments, ions are electrostatically accelerated to a uniform kinetic energy and travel at different velocities in the flight tube depending on their m/z. The lightest ions arrives first at the detector, the heavier ones arrives later. Using flight tubes of 1 m in length, even the heaviest ions typically take less than 50 μs to reach the detector. This translates into 20,000 to 30,000 mass spectra per second, approximately 2–3 orders of magnitude faster than the sequential scanning mode of a quadrupole system.

Basically two sampling approaches are used in commercial TOF mass analysers. In the orthogonal design the flight tube is positioned at right angles to the sampled ion beam, while in the axial design the flight tube is in the same axis as the ion beam. In both designs, all ions that contribute to the mass spectrum are sampled through the interface cones, but instead of being focused into the mass filter in the conventional way, packets (groups) of ions are electrostatically injected at exactly the same time into the field-free tube, where the flight time of all the ions is measured. Further details on TOF mass analysers and their fundamentals can be found elsewhere in this book.

TOF's simultaneous nature of sampling ions offers distinct advantages over traditional scanning (sequential) quadrupole technology for ICP-MS applications where large amounts of data need to be captured in a short amount of time, i.e., multielement determinations of rapid transient signals generated by sampling accessories such as laser ablation and electrothermal vaporization devices (see below, § 2.3), high-precision isotope ratio determinations,

multielement analysis of small-volume samples. On the other hand, as only <20% of ions are accelerated to the TOF tube, the detection limits are about one order of magnitude worse than those obtained by quadrupole-based instruments. As a result, TOF instruments are likely to find application in specific areas but are not expected to reach the popularity of quadrupole ICP-MS in the near future.

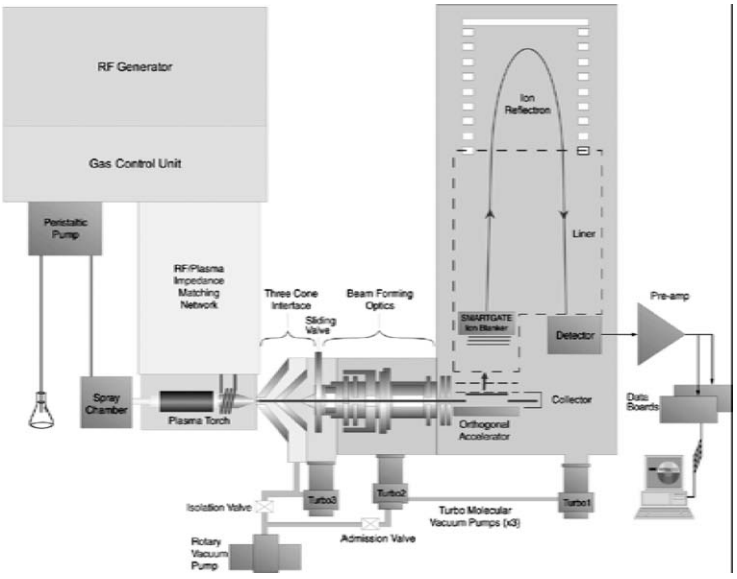


Figure 12. Schematic diagram of the OptiMass 9500 TOF-ICP-MS. Courtesy of GBC Scientific Equipment.

2.3. Sample introduction

Liquid sample introduction is the most common means for sample presentation to plasma. It ensures sample homogeneity, ease of handling and the possibility of simple calibration procedures. Generally, liquids are dispersed into fine aerosols before being introduced in the ICP. The analytical performance is critically affected by the quality of the aerosol generated. The latter is typically improved by removing large droplets from the aerosol stream via a spray chamber. Some spray chambers are externally cooled (either by water or through a Peltier system) to minimize the amount of solvent going into the plasma. This can have a number of beneficial effects, the main benefits being reduction of oxide species and the ability to aspirate volatile organic solvents. In some cases, a desolvation system is also used to

further reduce droplet size and solvent loading. Finally, an injector tube introduces the aerosol into the plasma base.

Aerosols can be originated by the kinetic energy of a high-velocity gas stream (pneumatic nebulizers, PNs) or the liquid itself (hydraulic nebulizers). Nebulization can also take place as the result of mechanical energy applied externally through a rotating (rotating nebulizers) or vibrating device (ultrasonic nebulizers, UNs).

The most commonly used devices for liquid sample introduction are PNs, which have two basic configurations (Figure 13). In the concentric type, the sample solution passes through a capillary surrounded by a high-velocity gas stream parallel to the capillary axis. In the crossflow type, the capillary is set at a right angle with respect to the tube carrying the gas stream. In both configurations, the pressure differential created across the sample capillary draws the sample solution through it, even though a peristaltic pump is generally used to force the sample solution through the nebulizer in order to overcome the viscosity effects resulting from self-aspiration.

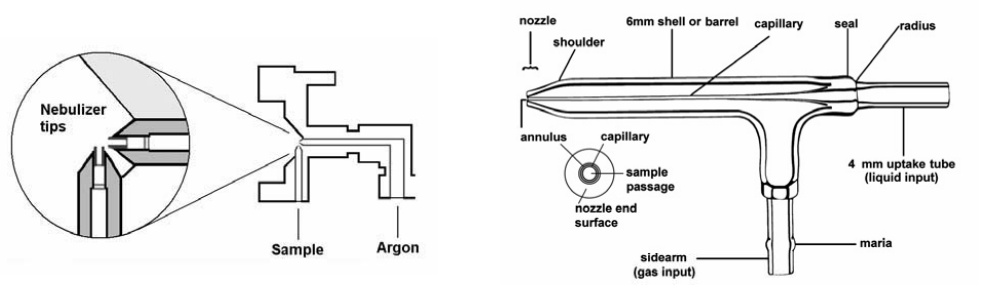


Figure 13. Schematic diagram of (a) a crossflow nebulizer, (b) a Meinhard concentric nebulizer ((b) courtesy of Meinhard Glass Products).

Common PNs are relatively inexpensive and simple devices, which provide good performances in most ordinary applications. Meinhard nebulizers are widely used glass or quartz concentric devices supplied in several configurations, with a range of operating specifications (nominal gas pressure, natural aspiration rate, etc.) and a choice of nozzle types (Figure 14). Cross-flow nebulizers are generally preferred when spraying samples having high solute concentrations, as they are generally less prone to salt build-up at the capillary tip than concentric nebulizers. The Babington-type are cross-flow nebulizers especially designed for the analysis of liquids with a significant solute content, including slurries. These devices, also called high-solids nebulizers, include the V-groove type, the Hildeberand dual grid-type, and the conespray type. Other nebulizers suitable for high-solids analysis are available, e.g. the parallel path-type which features parallel PTFE gas and sample capillaries.

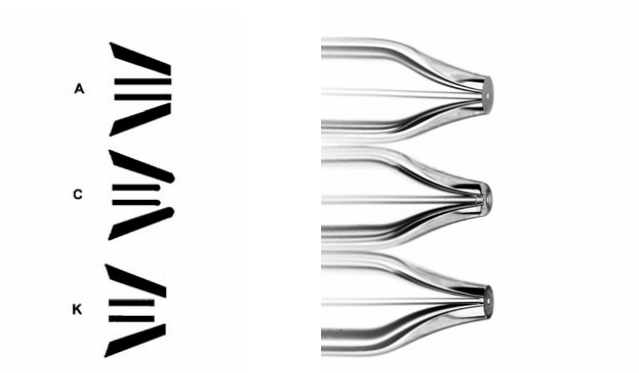


Figure 14. Geometries of nozzles in concentric nebulizers. Application: A - general purpose; C - high solids; K - Ar low-flow. Courtesy of Meinhard Glass Products.

The major drawback of common PNs is that they produce highly polydispersed aerosols in terms of droplet size. Rapid desolvation, volatilization, and atomization of the aerosol in the plasma require droplet diameters ≤ 10 μm. Thus larger droplets have to be removed by means of spray chambers, which usually are of the Scott type (also called 'double-pass', Figure 15) or the cyclonic type (Figure 16). This result in aerosol transport efficiency, defined as the percentage of the mass of the nebulized solution that actually reaches the plasma, as low as 2-3%. The resulting DLs can be inadequate for ultra-trace determinations in foods. Furthermore, the sample consumption rate of PNs is about 1-2 ml/min, which can be a problem when multielement analyses have to be performed on very small samples.

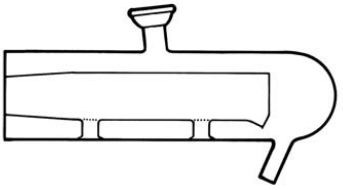


Figure 15. Cross section of double-pass spray chamber. Courtesy of Meinhard Glass Products.

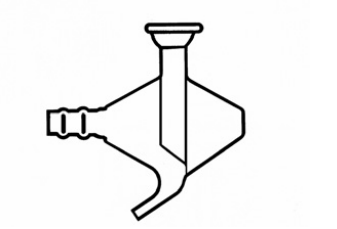


Figure 16. Cyclonic spray chamber. Courtesy of Meinhard Glass Products.

A great research effort has been made in recent years to develop nebulizers with higher aerosol transport efficiencies. Of these so-called high-efficiency nebulizers, the ultrasonic is probably the most used, followed by the thermospray, the hydraulic and others. In general terms, the analytical performance of these devices is superior to that of the conventional PNs but they are also more expensive and difficult to use, and often require a desolvation unit to avoid the negative effects of an excessive solvent load to the plasma.

In ultrasonic nebulizers (USNs), the solution is pumped to the surface of a piezoelectric transducer. As a consequence of the interaction between the ultrasonic waves and the liquid film, a very fine aerosol is obtained. This is carried by a stream of Ar through the desolvation system, consisting of a heated tube followed by a refrigerated tube that condenses out the solvent. With this nebulizer, improvements in detection limits up to 10-fold are achieved. A membrane desolvator can also be added after the condensor for further removal of sample solvent vapour. This second desolvation stage leads to improved DLs and enables removal of volatile organics, which can otherwise destabilize/extinguish the plasma and give rise to carbon-based interferences and carbon build-up on hardware components such as the ICP torch or the interface. Ultrasonic devices have been successfully used for reduction of polyatomic interferences [8-9] or accurate determinations at the ultratrace level [10-11]. However care is needed in their use as they are particularly prone to matrix effects and desolvation-type interferences, particularly for volatile elements.

The so-called thermospray, or thermal nebulizer (TN), consists of a capillary electrically heated to just above the boiling point of the solvent. A portion of the sample solution is vaporized and burst out in a jet, entraining nebulization of the rest of the solution. This process is very efficient and a fine aerosol is produced. In the hydraulic high-pressure nebulizer (HHPN), the aerosol is generated when a high-velocity liquid jet, which emerges from a narrow orifice, impacts on a solid surface placed in front of the nebulizer nozzle. For both of these nebulizers a desolvation system is needed, as with the USNs. Several chromatographic food-related applications of the TN [12] and HHPN [13-15] can be found in the literature.

Another increasingly important trend in recent years is the development of nebulizers with low sample consumption and low dead volumes. These so-called micronebulizers are particularly useful for ultratrace analysis of sample types that are of limited volume, toxic, or expensive. Their use minimizes sample consumption and waste production, features that are growing in demand in the analytical community. On the other hand, their low dead volumes allow increased sample throughput, reduced memory effects, ease of hyphenation with (micro)-chromatographic techniques. These nebulizers and their components are typically constructed from chemically resistant polymer materials such as polytetrafluoroethylene (PTFE), tetrafluoroethylene-perfluoroalkylvinylether copolymer (PFA, Figure 17), or polyvinylidene fluoride (PVDF), thus ensuring naturally low blank levels.

Various benefits of micronebulizers have been demonstrated for specific applications in food analysis. The use of a microconcentric nebulizer (MCN) in a micro-scale flow injection (FI) system has been reported to overcome matrix effects affecting As determination in wine [16]. A MicroMist MCN and a mini-cyclonic spray chamber have been found beneficial in reducing memory effects and wash-out time in Hg determination [17]. MCNs have been advantageously employed for multielement determinations in human milk and infant formulas [18] and alcoholic beverages [19-20], respectively. They have also been resorted to for the measurement of Pb isotope ratios in studies that compared quadrupole, sector-field and multicollector instruments for this purpose [21-22] as well as in hyphenated systems with ICP-MS detection for speciation purposes [23-24].

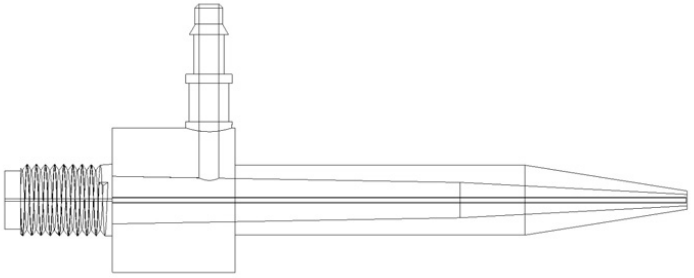


Figure 17. Cross section of a microflow PFA concentric nebulizer. Courtesy of Elemental Scientific, Inc.

Another MCN is the high efficiency nebulizer (HEN, Figure 18), a modified version of the Meinhard concentric nebulizer which can operate at low solution uptake rates (few μL/min) and is especially suited for microscale HPLC and FIA ICP-MS applications [25]. The behaviour of the HEN and other pneumatic micronebulizers has been characterized along with their performance in food analysis in a recent comparative study [26].

Figure 18. The high efficiency nebulizer (HEN). Courtesy of Meinhard Glass Products.

Total consumption nebulizers are also suitable low sample-consumption devices. They directly introduce micro-volumes of sample into the plasma and thus operate without the need of a spray chamber. Also called direct injection nebulizers (DINs), they have a nebulization efficiency of about 100% but must be operated at a low Ar flow rate in order to avoid plasma extinction. As a consequence, relative (concentration-based) DLs are often comparable to conventional PNs. However, the low dead volume and very fast washout times minimize both postcolumn band broadening in chromatographic applications and analyte memory effects. Therefore, the DIN is particularly useful in μ-HPLC- and CE-ICP-MS setups for speciation studies [27-29] as well as for the determination of volatile elements such as Hg and its compounds [30-31]. The latest version of this nebulizer, the direct injection high efficiency nebulizer (DIHEN), has a simple one-piece glass design and a lower cost than previous ones.

A number of other nebulizers have been described in the literature, e.g. the oscillating capillary type, the glass frit type, the jet impact, the recycling, the microultrasonic, and the microwave thermal nebulizer. Details on these devices can be found elsewhere [3, 32].

Improvements in the analytical performance of ICP-MS can also be obtained by adding a desolvation system to a specific nebulizer or even a conventional PN/spray chamber system. Integrated sample introduction systems featuring aerosol desolvation for high sensitivity determinations are commercially available. The Aridus is an inlet system consisting of a PFA MCN nebulizer, a heated spray chamber, and a heated fluoropolymer membrane serving as desolvation system. A low-flow of nitrogen gas can be added after the membrane to further enhance ICP-MS sensitivity and minimize oxide formation. The Apex has a similar design (Figure 19), but features a Peltier-cooled multipass condenser to which an optional membrane desolvation system can be added for efficient removal of residual solvent vapour from the sample aerosol stream.

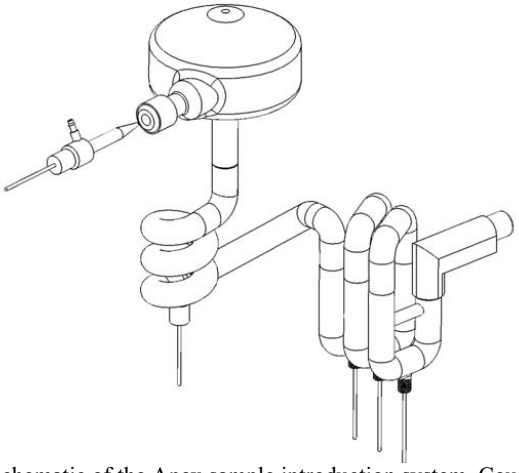


Figure 19. Schematic of the Apex sample introduction system. Courtesy of Elemental Scientific, Inc.

Some limitations and constraints of traditional continuous nebulization can be overcome by FI. Through an adequate selection of sample dispersion, this versatile automated sample handling tool can be used to (i) increase sample throughput with low sample consumption, (ii) perform microsampling, (iii) carry out on-line sample manipulations (including hydride generation, sample preconcentration, matrix separation, standard additions, dilutions), (iv) reduce memory effects. Moreover, FI enables solutions of high salt content, viscosity and acid strength to be analysed without blockage or distortion of the sample interface [33]. Multi-element determinations can be performed by time resolved software and the transient signals obtained can be processed in different ways by the analyst to optimize accuracy and precision [34]. A number of applications demonstrate the use of FI-ICP-MS in food analysis, e.g. on-line matrix elimination through a minicolumn of chelating iminodiacetate-based resin [35], analysis of wine and other liquid matrices after minimal dilution [36-37], analysis of samples with high levels of salts [38].

Vapour generation methods are an attractive alternative to aqueous sample introduction for analytes that can be turned into volatile compounds. A typical example is the generation of hydrides by reaction of hydride-forming elements, such as As, Bi, Pb, Sb, Se, and Sn, with appropriate reagents, e.g. $NaBH_4$ [39-40]. A gas-phase separator is usually employed to separate the chemical vapour from the sample solution after reaction of the latter with the reactant, but sometime a regular spray chamber is directly used. Vapour generation methods achieve essentially 100% transport efficiency, with a concurrent separation of the matrix resulting in reduced matrix effects in the plasma and lower detection limits with respect to conventional nebulization. In the case of elements prone to memory effects by continuous nebulization, such as Hg, the approach also significantly reduces memory and washout effects. Cold vapour generation with isotope dilution (ID) has been used for the sensitive and accurate determination of Hg in water and fish tissue and applied to Hg certification in standard reference materials (RMs) [41]. The conversion of the analyte into an active form for vapour generation and the effect of spectroscopic and chemical phase-phase interferences are critical issues in chemical vaporization methods.

An alternative to liquid and gaseous sample introduction is solid sampling. Solid sample introduction offers the advantage of reducing sample preparation time and, since no solvent is present, avoiding solvent-related spectroscopic interferences. Furthermore, certain solid sampling techniques provide spatially resolved information and enable depth profiling of the sample. Direct sample insertion, laser ablation (LA), electrothermal vaporization (ETV) are some of the available approaches for the introduction of solids into a plasma. LA and, above all, ETV have been used for food analysis by some authors. In LA-ICP-MS, the accuracy attainable strongly depends upon the calibration strategy. In food analysis, when using external calibration with a solid standard, roughly semiquantitative results are obtained, depending on the nature of the matrix and the resemblance between sample and standard [42]. In ETV-ICP-MS accurate calibration is more straightforward and the benefits mentioned

above for chemical vaporization (high transport efficiency, matrix separation) are warranted. Slurry analysis can be performed by means of an ultrasonic probe to mix the slurry prior to sampling by the autosampler. Moreover, besides being used for the analysis of solid samples [43] and slurries [44-47], ETV-ICP-MS represents an alternative for introduction of liquid samples [45, 48-50] and emulsions [51]. The potential for matrix clean up, either through selective volatilization of the analyte or destruction of the matrix, is of interest for many applications.

FI, LA and ETV are discrete sample introduction techniques which give origin to transient signals of short duration, such that the number of nuclides that can be monitored is limited. Multi-element capabilities can be fully exploited if a TOF mass spectrometer is used, since over 20,000 full mass spectra can be obtained per second, even though at the cost of a much lower sensitivity with respect to other ICP-MS systems.

2.4. Interferences

One major problem connected with the limited mass resolution affordable by ICP mass spectrometers, with the exception of sector-based instruments used in the high-resolution mode, is spectroscopic interferences. These interferences are caused by isobars, doubly charged ions, and polyatomic ions – such as oxides (MO^+), hydroxides (MOH^+), argides (ArM^+) – having the same nominal mass to charge ratio (m/z) as the nuclide of interest.

Isobaric overlaps are easy to predict and to overcome by selection of alternative isotopes or by elemental correction equations based on relative natural abundances. For instance, the instrument software corrects the overlap of ^{112}Sn with ^{112}Cd by means of the following equation

$$I(^{112}Cd) = I(^{112}M) - \{I(^{118}Sn) \times [a(^{112}Sn) / a(^{118}Sn)]\} \quad \text{[eq. 5]}$$

where I is the intensity of the specified mass and a is the natural abundance of the isotope.

Doubly charged species are formed only by elements with low second E_{ion} to an extent which generally does not pose serious analytical problems. However their signal varies in response with plasma parameters and can be minimized, e.g., increasing the aerosol carrier gas flow rate.

Polyatomic ions are formed already when pure water is nebulized (Figure 20). They mainly originate from Ar, O, H, and N atoms present in the plasma, entrained air, or the solvent. Most polyatomic ions consist of only two components (sometimes three with hydrogen) and, since ^{40}Ar is the heaviest nuclide present in high concentrations, they mainly occur at m/z $\leq$81 ($^{40}Ar^{40}Ar^{1}H$).

Usually samples are not dissolved in pure water but in an acid solution. The use of acids further complicates the background spectrum. When nitric acid is used, only the concentration of nitrogen in the plasma increases. Nitrogen is always present in the plasma and yields relatively small peaks in the blank spectrum due to its high Eion (14.53 eV). HNO_3 is therefore the ideal acid for ICP-MS, and the resulting interferences can be simply corrected by

blank subtraction. Some very intense peaks do represent an exception however, such as 40Ar, $^{40}Ar^{16}O$, $^{40}Ar^{40}Ar$, which preclude the measurement of the major isotopes of Ca, Fe, and Se, respectively.

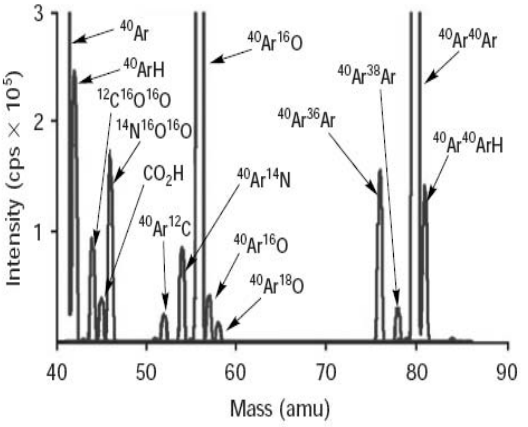


Figure 20. Mass spectrum of deionized water from mass 40 to mass 85.

When polyatomic species arise from the sample matrix, the solution of interference problems is more challenging, especially with quadrupole instruments. In multi-element analysis of food it takes considerable effort to develop methods which account for all the possible matrices and any associated interference [52-54]. The great variability of analyte and constituent levels among different food groups often cause a potential interference to be negligible in one matrix and not in another. Several potential matrix-induced spectroscopic interferences of importance in food analysis are summarized in Table 3.

For polyisotopic elements, in some cases spectroscopic interferences can be avoided by selecting an alternative interference-free isotope, provided it has a sufficient abundance for analyte detection. When the analyte is monoisotopic or all of the major isotopes are affected by spectral overlaps, the solution is not straightforward. A number of analytical approaches have been proposed to overcome spectroscopic interferences caused by matrix-induced polyatomic species in food analysis. Changes in instrument settings (e.g. RF power, aerosol carrier gas flow rate) may offer some solutions in order to reduce oxide levels or to realize 'cold' plasma conditions. The latter in conjunction with matrix separation have been used to reduce interferences in the determination of Cr and Fe in rice RMs [55]. Desolvation is a means to reduce oxides and hydroxides and can be realized by different methods, e.g. simply cooling the spray chamber, or more effectively, using membrane interfaces or cryogenic desolvation devices.

Table 3. Selected examples of potential matrix-induced spectroscopic interferences in food analysis.

Matrix element	Polyatomic ions	Analytical masses subject to interference
Ca	$^{40}Ca^{16}O$	^{56}Fe
	$^{40}Ca^{16}OH$	^{57}Fe
	$^{44}Ca^{16}O$, $^{40}Ca^{17}OH$, $^{42}Ca^{16}O$	^{60}Ni
Cl	$^{35}Cl^{16}O$, $^{37}Cl^{14}N$	^{51}V
	$^{37}Cl^{16}O$, $^{35}Cl^{17}OH$, $^{35}Cl^{18}O$	^{53}Cr
	$^{40}Ar^{35}Cl$, $^{40}Ca^{35}Cl$	^{75}As
	$^{40}Ar^{37}Cl$, $^{40}Ca^{37}Cl$	^{77}Se
P	$^{31}P^{16}O_2$	^{63}Cu
	$^{31}P^{16}O_2H$, $^{31}P^{16}O^{17}O$	^{64}Zn
S	$^{32}S^{16}O_2$, $^{34}S^{16}O_2$, $^{32}S_2$	^{64}Zn
	$^{32}S^{16}O_2H$, $^{32}S^{33}S$, $^{32}S^{16}O^{17}O$, $^{33}S^{16}O_2$	^{65}Cu
	$^{34}S^{16}O_2$, $^{33}S^{16}O_2H$, $^{32}S^{16}O^{18}O$, $^{32}S^{17}O_2$, $^{33}S^{16}O^{17}O$, $^{32}S^{34}S$, $^{33}S_2$	^{66}Zn
C, N	$^{12}C^{15}N$, $^{13}C^{14}N$, $^{12}C^{14}NH$, $^{14}N_2$ spread	^{27}Al
	$^{40}Ar^{12}C$	^{52}Cr
	$^{40}Ar^{13}C$	^{53}Cr

Suited sample preparation protocols have been developed by several authors to cope with interferences affecting one or few 'difficult' elements. Sample digestion procedures which minimize C and Cl content of digestates have been described [56]. These procedures allowed reducing the interferences of carbon-containing polyatomic ions on Cr isotopes and of chlorine-containing polyatomic ions on As, Cr, Se, V isotopes. Residual interference is usually corrected by mathematical equations (see below). Addition of chemical additives to digested samples has also been reported. For example, use of methanol [57] or water soluble tertiary amines [58] has been found to enhance signals due to improved ionization efficiency and decrease polyatomic chlorine interferences in the determination of As and Se.

Analyte/matrix separation is another solution demonstrated by some authors [55]. For example, the use of a microcolumn of an iminodiacetate-based resin has been reported for on-line removal of interferents (i.e. sulfate, chloride, phosphate, sodium) from digestates of biological materials [35]. Generally, separation of the analyte from polyatomic interferents is easily achieved in most analytical protocols used for speciation purposes. Separation can also be carried out with means other than chromatographic columns, e.g. through analyte volatilisation by hydride generation (HG). Applications such as elimination of ArCl

interference on As in drinking water analysis have been reported [59], however it should be recognized that with HG new interferences may arise that need to be taken care of.

Alternative introduction devices can be effective in reducing some spectroscopic interferences, as demonstrated in the case of chromium determination with ultrasonic nebulization [8-9]. Ultrasonic nebulization has proven useful to achieve a reduction in solvent-related spectroscopic interferences while increasing analyte signal intensities with an improvement of the DLs. Solvent-related interferences are reduced with UNs because of the solvent removal apparatus that is included in the device. Desolvation has been shown to be effective also in removing Cl (as gaseous HCl) from sample aerosol, thus leading to a reduction of the interferences from chlorine-containing polyatomic ions.

Use of mixed-gas plasma has also been employed to reduce interferences in the analysis of foods. The foreign gas can be introduced in the central (carrier) gas, in the outer gas or directly mixed into the main argon supply. An excellent discussion of the properties of plasmas resulting from the addition of foreign gases and of the features and limitations of the various possible mixed-gas plasma approaches can be found in Beauchemin [60]. In general, nitrogen appears to be the most successful in the reduction of oxides and other polyatomic species such as ArO, ClO, ArCl. When adding N2, a more energetic plasma and a more efficient energy transfer to analyte aerosol particles are obtained. As a result, a less stable environment for the polyatomic ions is established and competitive formation of nitrogen-containing ions is favoured. The addition of N_2 to both the outer and the carrier Ar gas flow has been reported to decrease the $^{40}Ar^{35}Cl$ and increase the ^{75}As intensities in the analysis of food samples and certified reference materials (CRMs) [61-63]. Reduction of chlorine based interferences on As, Cr, Se, V, Zn has been reported in the determination of five food-related RMs following N_2 addition to the aerosol carrier gas flow [64]. However this caused a sacrifice in sensitivity (DLs were on average 2-3 times higher with the mixed-gas plasma). Use of a 12% air-argon mixed nebulizer gas plasma has proven successful in minimizing the ArC interference affecting Cr determination in a dogfish liver tissue CRM [65].

Correction equations have also been frequently used to achieve signal deconvolution by either the two following methods: (i) direct measurement of an isotopic analogue of the interfering polyatomic (e.g. correction of $^{40}Ar^{35}Cl$ interference by measuring the signal of $^{40}Ar^{37}Cl$ at *m*/*z* 77 and applying a correction based on known isotopic abundances of ^{35}Cl and ^{37}Cl), (ii) indirect measurement of the signal of a related species (e.g. monitoring chlorine signal during analysis and applying a correction based on a previously determined value for the ratio of the signals of $^{40}Ar^{35}Cl$ and the monitored chlorine isotope). The equations corresponding to former and the latter approach are, respectively:

$$I(^{75}As) = I(^{75}M) - \{I(^{77}M) \times [a(^{35}Cl) / a(^{37}Cl)]\} \quad \text{[eq. 6]}$$

$$I(^{75}As) = I(^{75}As) - [I(^{37}Cl) \times CF)] \quad \text{[eq. 7]}$$

where the correction factor CF is equal to the ratio of the net signals at mass 75 ($^{40}Ar^{35}Cl$) and 37 (Cl) produced by a pure Cl solution. This correction factor is determined experimentally.

In eq. 6, if the sample contains selenium, an additional term is introduced to take into account the contribution of ^{77}Se at *m/z*=77. The selenium isotope at *m/z*=82 can be employed for the purpose (the ratio of the abundances of ^{77}Se and ^{82}Se will be used). As shown in eq. 7, a linear relation is generally assumed between the signal of the polyatomic (e.g. $^{40}Ar^{37}Cl$) and the monitored isotope (^{35}Cl or ^{37}Cl). However, successful use of a second degree polynomial function allowing for the experimentally observed non-linear dependence of the signal of CaO(H) species at m/z 60 ($^{44}Ca^{16}O$, $^{43}Ca^{16}OH$) on ^{44}Ca signal has also been demonstrated [66]. The major advantage of mathematical corrections is the speed and ease of use, possibly *a posteriori* without additional experimental work. A number of applications have been reported in food analysis [54, 67-70], with good results when the interference is about 10% of the analytical signal or less. A different correction approach based on the use of bayesian analysis has been demonstrated for the deconvolution of spectra in the mass range 46-68 daltons [71].

In many cases, polyatomic spectral overlaps can be successfully dealt with by use of sector field instruments [72], due to their higher resolving power [Figure 21]. Much work has been done with SF-ICP-MS in the field of food and beverage analysis, especially in cases when high accuracy and superior detection power were needed. Examples are the determination of Pb in calcium supplements [73], of trace and ultratrace elements in milk and infant formulas [17] as well as mineral water samples, including candidate RMs [74]. However, not all common interferences can be resolved with the resolution attainable. Moreover, it should be kept in mind that higher mass resolution implies lower ion transmission efficiency and in some cases the resulting loss in sensitivity may not serve one's purpose [68]. Often a medium resolution is chosen to eliminate several interferences, with mathematical corrections applied for the remaining unresolved interference.

The presence of spectroscopic interferences in unknown samples can be established for polyisotopic elements by resorting to measurement of isotope ratios. Any difference between the measured ratios and the expected ones (i.e. that of standard solutions) indicates the presence of isobaric interferences. In the absence of spectroscopic interferences, ID analysis (IDA) does represent a very reliable quantification method and is used for highly accurate and precise determination of elements in foods, as those required in the certification of RMs [41, 55, 75-76]. Equilibration of the enriched isotopes and the sample, correction for mass discrimination and for dead time are critical requirements for accurate ID results. Isotope ratios measurements can considerably benefit by the use of multicollector and time-of-flight mass spectrometers. For example, improved isotope ratios precision with respect to quadrupole instruments have been reported for Pb [21] and Sr [77] in wine. The best results

were obtained by ICP-SF-MC-MS, whilst application of ICP-TOF-MS was limited by poor sensitivity.

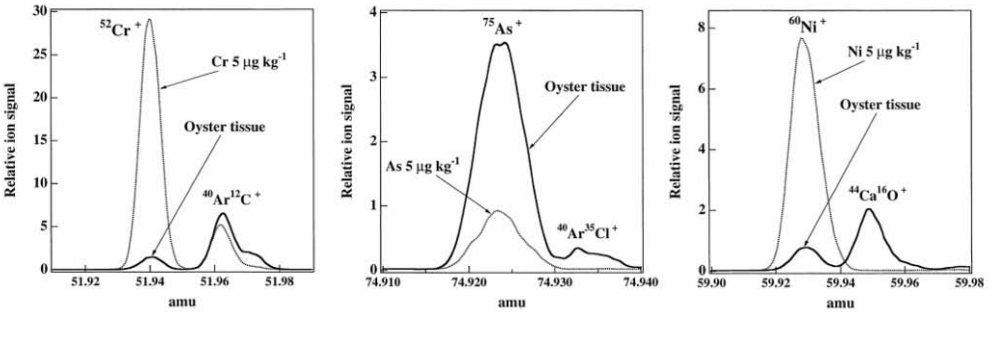


Figure 21. Resolution of interfering peaks in the analysis of an oyster tissue by SF-ICP-MS. Solid line, digested oyster tissue solution; dashed line, analyte spike. Reproduced from [72] by permission of Elsevierr

The recent availability of collision cell and dynamic reaction cell systems on most quadrupole instruments offers a different, and indeed very effective way to cope with the problem of spectroscopic interferences. As previously seen, in these systems interaction of the ion beam with a gas (or a mixture of gases) enables the chemical removal of polyatomic interferents through collisional dissociation and selective ion-molecule reactions. Table 4 summarizes some applications of ion-molecule chemistry techniques to food matrices.

The ICP-MS technique is also prone to non-spectroscopic interferences (‘matrix effects’), i.e. variations in the analytical signal due to matrix-induced changes in sample transport efficiency, ionization in the plasma, or extraction and transfer of ions in the mass analyser region. A variety of different mechanisms are involved in the generation of these interferences, and their ultimate effect can be either a signal enhancement or suppression. Their severity depends on the analyte, the sample physical properties (e.g. viscosity), the matrix composition, the instrumental setup and operating conditions. Regarding the instrument setup, both the sample introduction system and the interface/lens configuration and settings are important in determining the extent to which specific matrix effects are likely to occur.

Signal suppression in consequence of high concentrations of matrix elements in the sample is most frequently observed in analysis of foods. However, matrix effects are generally dependent on the absolute amount of matrix element rather than on the molar ratio of matrix element to analyte, hence they can be reduced by sample dilution. Internal standardization,

standard addition calibration, matrix-matching, ID, FI, matrix separation are some of the methods that have been used to eliminate (or substantially reduce) matrix interferences in food analysis. Internal standardization is routinely used in almost all ICP-MS applications because it is not only a remedy for matrix effects but it also compensates for instrumental drifts. However, judicious choice of the internal standard has to be made if effective correction for matrix effects is desired.

Table 4. Applications of collision and reaction cell technology to element determination in food.

Analytes	Samples	Cell gas	Cell	Ref.
Cd, Cr, Pb, Zn	Milk powder	NH_3	Quadrupole	[47]
Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Se, Sn, V, Zn	Seafood	NH_3	Quadrupole	[66]
P pesticides	Water	Collision gas: He	Octapole	[199]
Cr species	Water	NH_3	Quadrupole	[209]
As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Pb, Se, Sn, Sr, Th, U, V, Zn	Oyster tissue	Collision gas: He; reaction gas: H_2	Hexapole	[220]
Cr, Cu, Cd, Hg, Pb	Rice flour	NH_3	Quadrupole	[221]

It has been suggested that when non-spectroscopic interferences are due to space charge effects, the effectiveness of the internal standard would be dependent on the similarity in mass between the internal standard and analyte. If ionization suppression or enhancement is the dominant effect, the similarity in first Eion is crucial. The prevalence of one mechanism or another depends on the instrument used and a variety of other factors. Thus different internal standards should be tested to make sure that matrix induced variations of the analyte signal are correctly compensated. Internal standardization for compensation of the enhancement effect of ethanol on 208Pb in lead determination in wine after 10-fold dilution with 1% nitric acid is a suitable example. Goossens et al. compared several internal standards (Lu, Ta, W, Ir, Pt, Hg, Tl, Bi) and found that 205Tl was the most effective one in the experimental conditions of their study [78]. A slightly different approach is commonly pursued when multielement analyses have to be performed. In this case compromise conditions are chosen, and one or few

elements are used as ISs for all of the analytes. Selecting two or three ISs spanning the whole mass range is a common practice when many elements have to be determined in each single run.

In some cases, matrix effects can be advantageously employed to improve the analytical performance. Addition of carbon as 3% methanol in combination with increased plasma power has been found to enhance signal intensities of elements having a first E_{ion} in the range 9-11 eV (As, Se, I, Hg), and has been used to increase sensitivity in the determination and speciation of these elements in food matrices [57]. This enhancement is likely due to an increase of the ionization degree through charge transfer with the population of carbon-containing ions created in the central channel of the plasma by introducing a C-containing chemical. The carbon enhancing effect on elements with high E_{ion} must be kept in mind in case of, e.g., As determination in mineral waters with high bicarbonate levels [74].

2.5. Comparison with other atomic spectrometric techniques

Besides ICP-MS, the most widely used techniques for elemental analysis of food are AAS (which includes ETAAS, HG-AAS, CV-AAS, and FAAS) and inductively coupled plasma atomic emission spectrometry. The major advantages of ICP-MS over AAS detectors are its multi-element capability, which enables to analyse many elements in different samples with a high throughput, and the lower DLs. Other important features are the wide linear dynamic range, the ability to discriminate between isotopes, and the high versatility, i.e. easy coupling with different sample introduction systems and sample treatment devices, including chromatographic techniques for elemental speciation.

Compared to ICP-AES, which is also a fast multi-element detector, ICP-MS provides isotopic information and much lower DLs. The main disadvantage of the technique is its expensiveness, due to both the initial cost of the instruments and the running cost in terms of consumables and maintenance. Furthermore, a specialized staff is necessary to carry out good quality analytical work. When complex analyses are performed, large amounts of data can be generated which require careful processing and such procedures can be very time-consuming. Purpose-written software (e.g. spreadsheet macros) can be very effective to handle multi-element and multi-isotope data, although visual inspection of analytical results by an experienced specialist remains essential.

3. Analysis of toxic elements and elemental species in food

3.1. Element determination

3.1.1. Sample treatment

For solid foods, total element determination using conventional (i.e. liquid) sample introduction requires a dissolution step for analyte presentation to the instrument. The choice of the digestion method is made on the basis of several considerations, including efficiency in analyte solubilization, potential for loss of volatile elements and contamination of samples, reagent-related interferences and matrix effects, sample throughput, DL requirements, and safety aspects. Microwave (MW)-assisted digestion with HNO_3, generally in combination with H_2O_2, is a widely used method for the dissolution of food samples. In particular, microwave digestion with closed vessel systems has gained popularity, being a simple and fast dissolution technique that minimizes acid consumption, the risk of sample contamination and the loss of volatile elements. HNO_3 is the acid of choice for ICP-MS analysis as it causes minimal interferences. Small amounts of other acids can be added for specific analytical purposes, e.g., HF for the solubilization of Si-bound elements (Al, Co, Cr, Ni, Th, U, V) in vegetable matrices containing substantial amounts of silicon [79]. As previously stated, the use of HCl, $HClO_4$, H_2SO_4, is generally excluded to avoid resulting Cl, S interferences or cone blockage by salt formation (H_2SO_4). However, mixed acids can be successfully used for the heavier elements, where polyatomic interferences are less problematic. Alternative solutions to digestion with concentrated acids have also been reported for a rapid, multielement screening of biological and botanical materials, e.g., extraction with water-soluble tertiary amine solutions containing EDTA [80].

Solid foods in powder form can be analysed directly by means of LA- [42] or ETV-ICP-MS [43] to eliminate time-consuming sample dissolution procedures. However, this requires the preparation of homogeneous powdered samples and the subsequent analytical determination is not as straightforward as the one based on liquid sample introduction. Another way to perform direct analysis of solid foods is to grind and suspend them into slurries. The viability of slurry nebulization relies on the ability to prepare samples of fine particle size in a reproducible manner and on the adoption of suitable (e.g., high-solids) nebulizers. Otherwise, slurries can be analysed by ETV-ICP-MS resorting to the ultrasonic slurry sampling technique [44, 46-47].

As regards to liquid samples, these can be in many cases directly aspirated into the plasma following acidification or, alternatively, dilution and addition of suitable reagents. Sensitive multielement analysis of drinking water is generally straightforward and allows direct determination of analytes at ultra-trace levels, even though preconcentration/matrix separation, e.g., by means of ion exchange micro-columns [81], has been also reported.

Arsenic, Sb, Se and other hydride-forming elements can be determined by the hydride generation (HG) approach, which significantly enhances sensitivity [39]. SF instruments offer superior performance for the determination of ultra-trace and/or spectrally interfered elements without the need of any preliminary sample treatment [74]. Excellent DLs plus complete removal of ArCl overlap have been obtained when the technique was used in conjunction with HG for As determination [59]. The analysis of mineral water can be more challenging, especially in samples with a high mineral or bicarbonate content. In such cases, as well as with more complex liquid matrices, the addition of an appropriate internal standard is recommended.

Fruit juices, which have a more complex organic matrix with a significant carbohydrate content, can be analysed either following dilution, with subsequent centrifugation/filtration [82] or FI introduction [83], or after full sample digestion [84]. Dilution is usually applied to reduce the ethanol content of wine before analysis [78]. FI [85], standard addition and ID [86], matrix-matching [70], or ethanol removal by heating [87] have also been reported as viable solutions to deal with matrix effects in the analysis of alcoholic beverages. However, depending on the nebulizer used, not only ethanol but also other organic and inorganic components can produce significant matrix effects that need to be taken care of, especially when red wines are analysed [88]. The direct analysis of viscous or oily samples requires dilution and addition of surfactants such as Triton X-100, as reported in the analysis of honey [89]. An automated method based on on-line emulsion formation in a FI system was developed for multielement analysis of olive oil [90]. Comparison of MW digestion and emulsion preparation through use of Triton X-100 and tetralin has been addressed in another study, which focused on the analysis of synthetic vitamin E products [91].

Triton X-100 has been also used to measure Pb isotope ratios in milk powder samples prepared as slurries [92]. The pH of the slurries was adjusted at 7.5 with ammonia and analyses were performed with both ID and standard additions. Fresh milk dilution in alkaline media (in order to prevent protein precipitation) and multielement quantification with the standard addition method has been successfully applied by other authors [93], even though in most studies milk and infant formulae are analysed following full digestion [94-96].

For some elements, fit-for-purpose sample preparation is required in order to obtain reliable analytical results. Mercury is a well-known case, its determination being complicated by poor ionization and, above all, a marked memory effect. Addition of ammonia and Hg complexation with $(NH_4)_2H_2EDTA$ were found effective in shortening wash-out times in the analysis of nitric acid digestates of fish [97]. Small amounts of amines such as ethylenediamine and triethanolamine increased the sensitivity of Hg determination by improved nebulization–transport efficiency [98]. Gold chloride has also been used as stabilizing agent and additive in wash water to eliminate memory effects in mercury analysis of seafood [75] and drinking water [99]. Alternatively, sample introduction systems different

from conventional pneumatic nebulization have been successfully utilized, e.g. ETV [44] and cold vapour generation [41].

Prevention of sample contamination and quality assurance issues are of great importance in trace element analysis of food by ICP-MS. It is well known that, in such analytical determinations, method detection limits (Malls) are usually several times higher than instrument DLs. MDLs are critically affected by the samples being analysed, the dissolution procedure and the background levels of the analytes in reagents [52-53]. Employment of clean room facilities, ultrapure reagents, single-use disposable tubes and sample containers, and closed vessel digestion systems may enable to significantly lower MDLs and take full advantage of instrument sensitivity, especially when SF-ICP-MS is used. However the establishment of a strict quality control system is essential to warrant the maintenance of satisfactory levels of accuracy and precision in measurements over time.

Quality control in multi-element analysis of foods by ICP-MS has been discussed by Baxter et al. [100]. Defining and maintaining achievable quality levels for multi-element analyses of larger number of elements (>10) is considerably more difficult than when measuring smaller number of elements. Assessment of DLs, instrumental drift, recoveries from spiked blanks, agreement between replicates which were digested and measured in separate test batches (to eliminate within batch bias), and use of CRMs are all valuable means of quality control. Semiquantitative determination of potential interferents prior to analytical measurements and monitoring of the signal of major matrix interferents during analysis (in case with on-line correction with suitable elemental equations) can also be useful for routine determination of unknown samples, especially in the absence of suitable CRMs [54].

On the other hand, the sensitivity of multi-element determinations by ICP-MS has been employed to improve analytical quality assurance in the laboratory by identifying often overlooked sources of contamination during sample handling, e.g. laboratory gloves [101] or grinding/milling devices used for food homogenization before subsampling [102]. Furthermore, the technique is increasingly used in proficiency testing for the evaluation of laboratory performance and in collaborative trials for the certification of RMs [41, 55], not only for total element content but also for element species [75].

3.1.2. Applications

As already highlighted, ICP-MS has established itself as a major technique in the field of food safety and food control. The wide dynamic range and speed of multielement measurements makes it a particularly powerful tool for surveillance and legislative work, and proves valuable in dealing with emergencies such as contamination incidents. In a feed contamination incident in the UK (1989), rapid determinations of eleven elements in different matrices allowed the source of toxic effects noticed in beef, dairy cattle and other animals to be timely traced back to imported cattle feed, contaminated by Pb and other elements [103]. Ten years later, in the Jiangxi Province (China), more than 1000 people were seriously

poisoned by industrial lard contamination by organotin compounds. Screening of 11 analytes in lard, blood, urine and organ samples indicated the presence of high levels of several elements and a huge contamination by Sn, suggesting that organo-Sn compounds played a dominant role in poisoning. This hypothesis was confirmed by organotin determination by gas chromatography (GC)-FPD and GC-MS [104].

From the latter example the potential of the technique as a screening method for identification of toxic organometallic compounds, e.g. in pesticide residue analysis, can be appreciated. Anderson et al. [105] reported that an initial screening of total Sn by ICP-MS in a survey on cyhexatin (an organotin acaricide) allowed a limited number of suspect fruit samples to be identified for confirmatory analysis with a GC-MS method. This approach resulted in a ~30 % reduction of the survey cost in terms of staff hours.

The evaluation of food contact materials is another application of ICP-MS in the area of food safety. Q [106] and TOF [107] mass spectrometers have been utilized in the compositional analysis of paper and board material intended for food contact. ICP-MS detection has also been employed in studies on the migration of metals from food contact plastics using food simulants. Different simulants can be analysed without the need of any time-consuming preparation, i.e., aqueous acetic acid (3% w/v) directly, aqueous ethanol (15% v/v) after proper dilution, the olive oil simulant following emulsion preparation by means of tetralin and Triton X-100 [108].

ICP-MS potential has also been exploited in studies on the dietary intake of trace elements, which usually involve a very high sample throughput. Taking advantage of the speed of quadrupole instruments, it is possible to deal with a large number of samples within a reasonable time, thus reducing the delay between the execution of the survey and the availability of the results. This allows, if necessary on the basis of the resulting exposure data, timely adoption of appropriate measures to protect public health. Cadmium and Pb are the most widely investigated elements because of their toxicological significance and ease of determination by ICP-MS without major interferences [109-111]. Arsenic is frequently surveyed too, both alone [112-113] and together with Cd and Pb [114]. ICP-MS is also very suited for the determination of long-lived radionuclides, which are often difficult and time-consuming to measure with radioanalytical techniques. The determination of 129I, 232Th, 238U and other nuclides in various food groups has been reported by several authors, often with the aim of estimating their dietary intake [115-117]. In some total diet studies, similar food categories are combined into a limited number of major food groups and this made it possible to reduce the number of analysed samples and thus determine many elements (up to 30, [118]) in order to estimate the relevant intake.

ICP-MS is increasingly adopted in studies on trace element metabolism in man using stable isotope techniques, and this certainly represents a future growth area. Thermal ionization mass spectrometry is recognized as a reference technique for isotope analysis, but ICP-MS reduces sample preparation and analysis times, offering a precision that is generally adequate

for most tracer studies, and is indeed excellent in the case of MC instruments. A main advantage of stable isotope over radioisotopes is safety, i.e., studies can be performed using intrinsically labelled foods on all population groups including the most sensitive (i.e., infants, women, and the elderly), to whom administration of a radioisotope is undesirable. Several multiple-isotope labelling options exist and the duration of the experiment is not limited, which may be the case with short-lived radioisotopes. For this reason, even though tracers are costly, dose requirements are higher in comparison with radioisotopes, and external analysis of biological samples is required, stable isotopes are increasingly recognised as valuable tools for estimating the absorption and retention not only of nutrients, but also of harmful elements, providing ethical aspects connected with experiments on humans are properly addressed. For example, Crews et al. studied the absorption of Cd by feeding volunteers with porridge containing normal low dietary concentrations of intrinsically labelled cadmium (106Cd) [119]. The porridge was made from the flour of hydroponically grown wheat intrinsically labelled with the stable Cd isotope.

Many applications of ICP-MS to studies concerning the transfer of elements through the food chain have been reported. Element uptake from soil and transfer into the edible parts of plants has been addressed in several studies. Soil-to-plant transfer factors in fruit and vegetables grown in various agricultural conditions have been determined for, e.g., Pt (resorting to a HfO interference correction equation for accurate quantification by Q-ICP-MS) [68], Tl and other 11 elements [120], As, Cd Cr, Cu, Pb, Zn [121]. In a study on stable isotopes of fission product elements (Ce, Cs, Sr), an in vitro enzymolysis method has been applied to investigate the solubilization of the analytes from fodder in a simulated ruminant digestion [122]. The effect of inhibitors of fission product solubility was also considered and essential elements were determined simultaneously to evaluate potential nutrition problems for the animals from the use of such inhibitors. Selective leaching of individual classes of metal complexes with different ligands and sequential enzymolysis have been recently applied to estimate the potential bioavailability to humans of Cd and Pb in cocoa powder and related products [123].

The multielement capabilities of ICP-MS have been employed together with statistical analysis of concentration data with the aim of investigating the effects of soil types and agricultural practices on crop composition [124-125], possibly leading to discrimination of organic cultivations from conventional ones. Isotopic and elemental analyses have been used in authenticity studies aiming at identifying the production area of rice [126], and soft wheat [127]. Multivariate statistics, including discriminant partial least-square regression, principal component analysis, and canonical discriminant analysis, have been used in most of these studies. Similar approaches have been used to address quality and safety issues of aquaculture and fishery production. For instance, Bechmann et al. [128] determined the contents of 10 elements in blue mussels from seven sites in Denmark by SF-ICP-MS, resorting to a mass resolution setting of 4000 to resolve the analytical peaks of Cr, Ni, Cu from otherwise

interfering polyatomic ions. The mussels from the different sites could be separated using PCA and the comparison with the levels found in an earlier study allowed identification of temporal trends in trace element contamination. Cubadda et al. [66] used DRC-ICP-MS to determine sixteen elements in marine organisms and achieved discrimination of mussels from three farming sites in the Venice Lagoon characterized by different physico-chemical conditions and sources of element contamination by multivariate analysis of element fingerprints.

The final links in the food chain - processing and domestic preparation - may greatly affect element content and bioaccessibility in ready-to-eat foods. For instance, significant variations in the concentrations of several elements as a result of processing of durum wheat grain into semolina and pasta, and subsequent cooking, have been assessed in studies which employed multielement ICP-MS detection with ultrasonic nebulization of low levels of analytes in cereal-based matrices and water [129]. Another study on the effect of cooking used ICP-MS detection to investigate the possible migration of Pb from the bone of joints into beef meat [130].

Several application of ICP-MS to the determination of potentially toxic elements in water and beverages can be found in the literature. Owing to its excellent LDs and multielement capability, ICP-MS has established itself as a superior tool for water analysis and it has found widespread use for drinking water monitoring [40, 74, 99, 131-132]. In recent times, the technique has been resorted to in some surveys on the inorganic composition of bottled mineral waters, the consumption of which has markedly increased in several countries over the last few years. Two extensive studies carried out in Europe and in Canada reported on the determination of 39 and 21 elements, respectively, and found that a number of samples did not comply with existing regulations [133-134].

ICP-MS represents an ideal solution for the determination of long-lived radionuclides in water matrices. Several methods have been described for the measurement of natural and artificial radioisotopes in water. ^{226}Ra in mineral waters was determined by SF-ICP-MS following separation of matrix elements through a cation exchange resin, with a method DL of 0.01 pg L^{-1}, much lower than the United States Environmental Protection Agency required detection limit of 1 pg L^{-1} [135]. The effects of chemical form and instrumental memory on the determination of ^{99}Tc were studied and a method involving oxidation of complexes prior to analysis and the addition of Triton X-100 to the sample solution was developed [136]. Chelation column preconcentration and FI were used for ^{232}Th and ^{238}U determination at ultratrace levels [137].

The occurrence of Pb in wine is due to natural (soil related) and anthropogenic sources, including atmospheric pollution and contamination from the materials utilized for winemaking and storage. In recent years, the contribution from the latter sources has declined and the total Pb content of wine has been found to decrease. Several studies on Pb determination in wine employed ICP-MS detection [78, 86]. Pb isotope ratios have been

investigated as a tool to locate wine origin [86] and also to date the age of port wine after sample pre-treatment by UV-irradiation, filtration and 10-fold dilution [138]. Recently, MC and TOF instruments have been shown to substantially improve the precision of Pb isotope ratios with respect to Q-ICP-MS, even though laborious sample treatment and preconcentration are needed in the case of TOF [21-22]. On the other hand, MC-SF-ICP-MS appears ideal for the task, as more and more detection power is required for precise isotope ratio measurements due to the ever smaller Pb concentration in recent vintages. Other isotopic fingerprints ($^{87}Sr/^{86}Sr$), on the one hand, and multielemental analyses in conjunction powerful multivariate statistical methods, on the other hand, have been found useful for wine authentication as well [85, 139].

Apart from wine, several other works involving multielemental characterization of beverages by ICP-MS have been carried out. Ødegård and Lund determined 24 elements in tea leaves and infusions and calculated the extraction efficiency of the infusion process [140]. Wyrzykowska et al. used SF-ICP-MS with UN to determine 23 elements in an investigation of the interdependences among trace elements in beer through PCA [141].

The multielement capabilities and the detection power of ICP-MS have also been extensively employed for the characterization of human milk in studies aiming at assessing the expected ranges for several trace and ultra-trace elements, including potentially toxic ones [18, 94-96]. For those infants who are not breast-fed, the safety of substitutive formulae is of major concern. A number of studies on the composition of commercially available formulae and their suitability for infant nutrition from the standpoint of element content have been performed with ICP-MS detection [18, 94-95]. In these investigations both Q and SF instruments were employed to determine up to 28 elements, and among them Ag, Al, As, Au, Ba, Be, Cd, Cs, Hg, Li, Pb, Pt, Th, Ti, Tl, U.

3.2. Hyphenated techniques for element speciation

3.2.1. Hyphenated techniques for element speciation

The total amount of an element present in food tells us very little about its possible absorption and fate inside the body. To gain this information, the chemical form of the element must be assessed as this form, or 'species', critically influences its bioavailability and biological effects. In most cases, a large variation in toxicity among the various species of a given element is found. In order to carry out a correct assessment of possible health risks associated with dietary exposure to an element, the differences in toxicity among its chemical species must be taken into account. Thus, the assessment should be based on data concerning the chemical species of toxicological relevance rather than on the total concentration.

According to IUPAC guidelines, the chemical species of an element are specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure [142]. Speciation analysis consists in the analytical activities of

identifying and/or measuring the quantities of one or more individual chemical species in a sample. Measurement of isotopic ratios or isotopic composition has been already discussed in the preceding pages. In the following sections, the use of ICP-MS as a detector in hyphenated techniques for the qualitative and quantitative determination of oxidation states, ligand complexes or molecular compounds of potentially toxic elements in food is addressed.

As such, ICP-MS cannot be used for speciation analyses, because all the chemical species of each given element are broken down to atoms and ionized into the argon ICP. However, if these different species are separated from one another prior to their introduction into the plasma, ICP-MS can be used as a highly sensitive and selective on-line detector, also capable of multi-element determinations.

Liquid chromatography is the most commonly used technique for element speciation with ICP-MS detection. The mobile phase flow rates of HPLC columns match the typical liquid flow rate of common nebulizers making the coupling of the two components simple. The connection between the outlet of the chromatographic column and the nebulizer is a inert plastic tubing, whose inner diameter and length are small enough to minimize peak broadening. The application range is wide on account of the different chromatographic principles exploited, i.e. size-exclusion, reversed-phase, ion-exchange, also keeping in mind the combination of functional behaviours usually presented by a single column. The use of ion-pairing reagents both in reversed-phase chromatography (RPC), in order to resolve ionic analytes (reversed-phase ion-pairing chromatography, RPIPC), and in anion-exchange chromatography, with the aim to achieve simultaneous separation of anionic, cationic and neutral species, further extends the analytical capabilities of liquid chromatography.

A primary problem in the coupling of the two techniques is the low tolerance of ICP-MS for organic modifiers and salts (buffers, ion-pairing reagents) added to HPLC mobile phases to enhance separation. A compromise between chromatographic separation and detection must be adopted, and often additional measures to reduce the deleterious effects of organic solvents on the plasma and the interface - e.g. cooling of the spray chamber, desolvation, oxygen addition to the nebulizer gas and use of Pt cones - are required. Most HPLC-ICP-MS hyphenation is perfomed by using isocratic elution. Gradient elution may be used to improve retention times and partition functions but often difficulties arise when gradients are introduced into the ICP. Identifying optimal operating conditions for different mobile phase concentrations can be difficult. In addition, the changing load on the plasma due to variations in mobile phase composition can cause plasma instability.

Time resolved analysis is needed for acquisition of chromatographic signal. To this end, ICP-MS instruments are equipped with software capable of monitoring signal versus time at different m/z values.

The hyphenation with GC is more complicated than with HPLC, as a heated transfer line at about 250°C is needed in order to prevent condensation of the analytes. However GC-ICP-MS offers several analytical advantages, such as higher resolving power, 100% sample

introduction efficiency, obtainment of a more stable plasma, fewer spectroscopic interferences, reduced cone wear. Of course, the application is restricted to compounds which are volatile and thermally stable, or can be brought into such a form after derivatization. Capillary columns are preferred to achieve higher sensitivity and resolution, and DLs in the ppt range have been reported with solid-phase microextraction (SPME) as the sample introduction technique.

The coupling of capillary electrophoresis (CE) and ICP-MS has been the latest to be realized due to the complex interface needed. However, in recent years CE-ICP-MS has increasingly received attention as a technique for speciation of metal and metalloid species. CE offers highly efficient separation, rapid analyses and minute sample size requirements. Among the different modes of CE, capillary zone electrophoresis (CZE) is the most commonly used with ICP-MS detection to date. A major problem of CE-ICP-MS is that it still suffers from relatively high DLs. However recent developments delivered a number of improvements, making this hyphenated technique more promising.

3.2.2. Sample treatment

For HPLC-ICP-MS determination, the analytes are generally extracted from food matrices with aqueous solutions containing variable amounts of organic solvents. Extraction of organoarsenic compounds is often accomplished by use of methanol-water solutions. The treatment is typically repeated several times, and each time the supernatant solution is separated from the solid residue by centrifugation. An ultrasonic treatment is often applied to enhance the extraction efficiency. Extraction is followed by preconcentration (evaporation) of the extract. Defatting by non-polar solvent extraction before MeOH extraction may be necessary for lipid-rich animal tissues.

MW assisted extraction (MAE) [143-145] and accelerated solvent extraction [146-147] have been used to increase the speed of leaching and reduce the time required from hours to minutes. Enzymes (trypsin) and supercritical fluid extraction have also been applied for leaching of As [148] and As and organotin species in fish [149-151], respectively. Brisbin and Caruso investigated a variety of extraction procedures for leaching of As, Cd, Co, Mo, and Se species from lobster tissue [152]. Soxhlet, room temperature mixing, sonication, MAE, supercritical carbon dioxide and subcritical water extractions were evaluated for a variety of solvent systems. The solubility trends and solvents into which the analytes extracted gave an indication as to the polar/non-polar nature of the compounds present. In general, MAE yielded comparable or improved recoveries for all the analytes monitored and proved to be the mildest, fastest, and most reproducible technique evaluated. Several other works has been dedicated to the evaluation of extraction techniques for As species from selected matrixes, e.g. freeze-dried apples [153] and dogfish tissue [154]. Camara and coworkers used a focused ultrasonic probe and an enzymatic treatment with an aqueous mixture of protease XIV and α-amylase to reduce sample treatment time for As extraction in rice to only a few minutes [155].

SPME has recently attracted interest as a useful solvent-free extraction procedure [156-159]. SPME can collect volatile species (directly or following derivatization) from either the sample headspace or the liquid phase, and nonvolatile species from the liquid phase. Thus it can be used in conjunction with different separative techniques, i.e., besides HPLC, also GC and CE, which have been both coupled to ICP-MS for speciation work.

The widely different extraction efficiencies commonly observed in relation to the sample matrix and the solvent/extraction procedure utilized call for caution in the interpretation of the results of speciation studies. A compound may be found to largely predominate in an extract containing only 10% of the total element concentration and one should be aware that this compound could actually represent a minor species, 90% of the element content being in an unknown form. Moreover, the concentration of the investigated element in the other extracts (if any) and in the residue should be determined to check the accuracy of results. Establishing a mass balance of the analytes in all fractions of the analytical procedure is mandatory as it accounts for analyte extraction efficiency and helps evaluating the overall accuracy of speciation results.

For water and beverages, speciation analyses by hyphenated techniques are generally carried out without the need of any sample treatment, except, e.g., ethanol removal under reduced pressure in the case of wine [160]. More details on sample preparation and analytical approaches can be found in a recent review on elemental speciation in beverages [161].

A critical issue in speciation work is the preservation of species identity. In the case of liquid matrices, changes in speciation can be induced by erroneous sample storage procedures or during chromatographic separation and it is often difficult both detecting and avoiding them. In the case of solid food, care is needed to ensure that the chemical forms of an element are extracted and separated without modification of their original distribution in the analysed matrix. Sb and Se species transformation during extraction and storage has been traced by Lindeman et al. [162]. Artifactual formation of methylmercury during sample extraction has been detected by Hintelmann et al. using stable Hg^{2+} isotopes [163]. A species-specific ID technique has been proposed by these authors to correct Hg speciation results.

The application of IDA to quantitative elemental speciation offers a state-of-the-art approach for the obtainment of accurate results. IDA for elemental speciation can be performed under two different spiking modes, namely species-unspecific and species-specific. In species-unspecific ID, the spike solution containing the enriched isotopes is mixed with the effluent of the column containing the separated species to be determined (post-column spike). By this mode it is not possible to correct for any errors occurring before the spiking (e.g. during sample preparation), however this calibration strategy does represent the only alternative either when the structure and composition of the sought species is not exactly known or when the corresponding isotopically labelled compounds are not available. In species-specific ID an isotopically labelled analogue of the compound to be analysed is used for IDA. This means that the chemical identity of the compound is known and that it can be

obtained in an isotopically labelled form. In this mode, the spike solution is added to the sample prior to the separation of the species and, once complete isotope equilibration takes place, the traditional advantages of IDA can be exploited. When several elemental species need to be analysed, each compound can be enriched in a different isotope of the element (multiple spikes), which opens the way to new forms of quantification and to determination of degradation factors when serious species interconversion reactions occur. The theoretical basis of the above-named calibration strategies, together with the relevant mathematical equations and an excellent review of the literature can be found in a recent report [164].

An additional remark on speciation analysis by ICP-MS coupled techniques regards species identification. The latter is generally carried out by comparing retention times but, depending on the analyte, its concentration and the sample matrix, the risk for species misidentification can be high. Multi-dimensional approaches (i.e. combinations of two or more separation methods) or identity confirmation by molecular mass spectrometry (e.g. electrospray ionization-MS, GC-MS) have been resorted to with the aim of achieving reliable results in qualitative determinations. The unavailability of the standards of many naturally occurring element species makes often the latter as the only viable solution for species identification. However, the sensitivity of molecular mass spectrometry can be too low for many practical applications. The use of CRMs for trace element species would be the preferable approach for accuracy assessment in these cases, but only a few of them are currently present on the market. A wider availability of such tools could greatly simplify the overall accuracy assessment of speciation analyses.

3.2.3. Applications

Arsenic speciation has been the subject of an intensive research activity on account of the large number of naturally occurring inorganic and organic forms of this element (more than 30 water-soluble species have been currently described) and their widely different toxicity. Seafood contains fairly high As concentrations due to the presence of organoarsenic compounds with a low or negligible toxicity, such as arsenobetaine (AB), the dominating form in fish and crustaceans, and dimethylarsinoylribosides, which together with trimethylarsonioribosides are collectively known as arsenosugars. Some dimethylarsinoylribosides are the major As species in seaweeds and, together with AB, in bivalve molluscs. A range of minor compounds are also found in marine organisms (e.g. arsenocholine, AC, trimethylarsoniopropionate, TMAP, trimethylarsine oxide, TMAO, tetramethylarsonium ion, TETRA) and this stimulated research work to understand the biogenesis of these compounds and their position in the biotransformation pathways of As in the sea. On the contrary, As concentrations in foods of terrestrial origin are generally low and the highly toxic inorganic species, arsenious acid (arsenite or AsIII) and arsenic acid (arsenate or AsV) are found, together with dimethylarsinic acid (DMA) and monomethylarsonic acid (MA), compounds with a lower toxicity but suspected as cancer promoters. Mushrooms

represent an exception on account of both the high As levels often detected and the presence, in some species, of unexpected compounds such as AB, a species typical of marine animals.

HPLC-ICP-MS is the analytical technique that is most frequently used for arsenic speciation. ICP-MS is an ideal detector for the chromatographically resolved arsenicals because, besides the excellent detection power, it usually gives a uniform response for all As species which facilitates their quantification (e.g. a species can be quantified even though the relevant standard is lacking). Chloride-rich samples may disturb As detection due to 40Ar35Cl formation, however this polyatomic is in most cases easily separated from the analytes when chromatography is employed. Chromatographic separation of the different water-soluble As species can be performed in several ways, due to the fact that some compounds are permanently cationic (quaternary arsonium compounds such as AB, AC, TETRA), while the others are readily and differently ionized depending on the pH. This allows separation by either anion-/cation-exchange chromatography or RPIPC, when a suitable ion-pairing reagent is used. Larsen and colleagues detected several major and minor As species (including arsenosugars) in different food matrices by using anion- and cation-exchange chromatography, the former being used for the separation of AsIII, AsV, MA, DMA, i.e. the toxicologically relevant species [144, 165-166]. A thorough discussion of the use of ion-exchange chromatography (IEC) for As speciation as well as quality assurance issues in speciation work was also provided [167]. Recently this group developed a microwave assisted alkaline solubilization method that employed a mixture of sodium hydroxide in ethanol to solubilize AsIII from matrix components and convert it to AsV, thus allowing for subsequent determination of total inorganic arsenic (i.e. the most toxic fraction) as AsV by anion-exchange HPLC-ICP-MS [168]. It was stated that this method was able to liberate AsIII from the binding to –SH groups of protein and macromolecular constituents, which was thought to be the likely cause of the limited recoveries for AsIII found by various authors when methanol/water mixtures were employed for extraction. Analysis of 60 fish samples showed that in most cases inorganic As was lower than the DL of 3 ng g−1 , i.e. less than one per thousand of the total arsenic content in the fish samples. However the method was not suited for fat-rich fish such as mackerel, which showed incomplete recovery of AsIII.

Another separation mechanisms applied was SEC, which in combination with RPIPC was used by Shibata and Morita for the determination of the full range of As compounds in seafood [169]. SEC (or ‘gel permeation chromatography’) is typically employed for the separation of large proteins and polysaccharides. However, secondary adsorption and ion exchange effects can be advantageously used to resolve ions with a high charge-to-mass ratio, such as organoarsenic compounds. SEC has been applied by McSheehy and colleagues for the fractionation of As species and matrix removal in a series of elegant studies based on multi-dimensional approaches for As speciation. In these studies SEC was combined with anion-exchange chromatography [170], or with anion-/cation-exchange chromatography and RPC [171-172] for As speciation in a oyster RM and in edible seaweeds such as Hizikia fusiforme,

an algae that contain arsenate as the major As species, with identity confirmation/recognition by ESI-MS-MS.

Until today seafood attracted a lot of interest as regards to As speciation, however in recent years a wider range of food items containing lower levels of As, but with high proportions of inorganic and simple methylated species, have been increasingly investigated. Thus speciation of As in staples (e.g. rice, Figure 22), foodstuffs destined to sensitive population groups (e.g. infants), vegetables grown in As-rich soils has been addressed using HPLC-ICP-MS. Some examples are shown in Table 5, which summarizes the investigated matrixes and the chromatographic approach used. As far as non naturally-occurring compounds are concerned, the use of HPLC-ICP-MS for the quantification of As-containing residues that may enter the food chain should be mentioned. The determination of the growth promoter 4-hydroxy-3-nitrophenyl-arsonic acid (roxarsone) in chicken tissues by RPC after trypsin enzymolisis digestion and bulk matrix separation (by anion-exchange chromatography) [173] provides an example that highlights the potential of HPLC-ICP-MS for the determination of food residues containing metal (metalloid) heteroatoms.

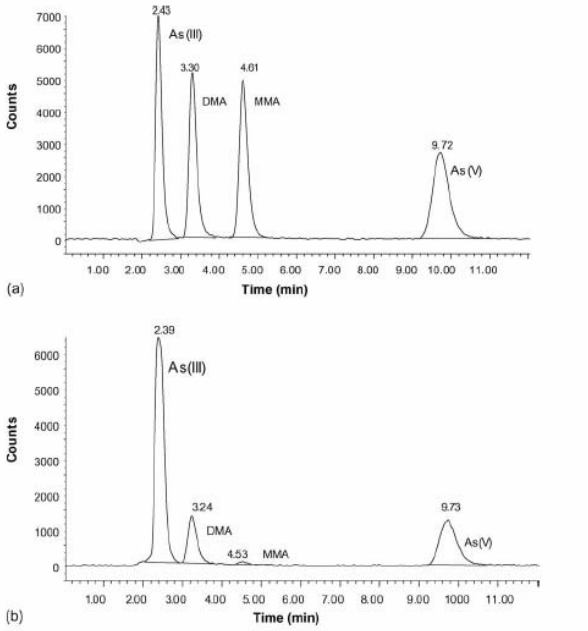


Figure 22. Arsenic speciation in rice by HPLC-ICP-MS: a) stock solution of As species at 10 μg L^{-1} (expressed as As) and (b) rice sample. See text for acronyms. Reproduced from [155] by permission of Elsevier.

A very recent trend of As speciation by HPLC-ICP-MS is the study of lipid-soluble As species (arsenolipids), a class of compounds largely unexplored from the chemical and toxicological point of view. The determination of these compounds is considerably more difficult than that of polar arsenicals and the lack of suitable analytical techniques has so far hindered investigation in this area. However, a recent work of Schmeisser et al. [174] provided a first attempt to separate and quantify arsenolipids by normal phase HPLC-ICPMS. Various mixtures of organic solvents were used as mobile phases and inherent problems of instability associated with the introduction of organics to the plasma were overcome by the use of reduced column flow, a chilled spray chamber, and the addition of oxygen directly to the plasma. The chemical structure of the arsenolipids could not be identified by this method, but analysis of ten fish oils with different origin revealed the existence of 4-6 major and several minor lipid-soluble compounds. Quantification was achieved by external calibration against triphenylarsine oxide or triphenylarsine sulfide, and the sum of species following HPLC of the oils matched well the total arsenic results (92–107%).

Table 5 . Arsenic speciation in various food by HPLC-ICP-MS

Species analysed [a]	Sample	Separation [b]	Chromatographic conditions	Ref.
Fruit, vegetables, and mushrooms				
AB, As^{III}, As^{V}, DMA, MA	Apple	AX	Hamilton PRP-X100 (250×4.1 mm), 10 mM ammonium phosphate and 10 mM ammonium nitrate at pH 6.2	[153]
AB, AC, As^{III}, As^{V}, DMA, MA, TMA, TMAO	Carrot	AX	Waters IC-Pak Anion HR (75×4.6 mm), 10 mM $(NH_4)_2CO_3$ adjusted to pH 10.0 with NH_3	[147]
AB, As^{III}, As^{V}, DMA, MA, TMAO	Mushrooms	AX, CX	AX: ION 120 (125×4.6 mm), 45 mM NH_4HCO_3 3% MeOH adjusted to pH 10.3 – CX: Chrompack Ionosphere-C (100×3 mm) 5 mM pyridinium formate, pH 2.65	[144]
Cereals				

Species analysed [a]	Sample	Separation [b]	Chromatographic conditions	Ref.
AB, AC, As^{III}, As^{V}, DMA, MA, TMA, TMAO	Rice	AX	Waters IC-Pak Anion HR (75×4.6 mm), 10 mM $(NH_4)_2CO_3$ adjusted to pH 10 with NH_4OH; Dionex AS7 & AG7 (150×4.6 mm & 50×4.6 mm), 12.5 mM HNO_3, pH 1.8; Hamilton PRP-X100 (150×4.6 mm), 10mM $NH_4H_2PO_4$, 10 mM NH_4NO_3 adjusted to pH 6.3 with NH_4OH	[222]
As^{III}, As^{V}, DMA, MA	Rice	AX	Hamilton PRP-X100 (250×4.1 mm), 10 mM $HPO_4^{2-}/H_2PO_4^-$, pH 6.0	[155]
Miscellaneous				
As^{III}, As^{V}, DMA, MA	Baby food	AX	Hamilton PRP-X100 (150×4.6 mm), 10 mM each ammonium phosphate and ammonium nitrate, pH=6.3 adjusted with ammonium hydroxide	[223]
AB, As^{III}, As^{V}, DMA, MA	Chicken	AX, AX IP	Supelcosil SAX1 (250×4.6 mm), gradient elution: eluent 1-0.1 mM phosphate buffer, pH 6.0, eluent 2-2.5 mM phosphate buffer, pH = 6.0; Ion Pac AS7 (250×4.0 mm), gradient elution: eluent 1-0.5 mM HNO_3, 0.5 % MeOH, 0.05 mM BDSA, eluent 2-50 mM HNO_3, 0.5 % MeOH, 0.05 mM BDSA	[224]
As^{III}, As^{V}, DMA, MA	Wine, kelp	RP IP	Discovery C_{18} (150×2.1 mm), 5 mM TBAH, malonic acid, pH 6.2	[225]
As^{III}, As^{V}, DMA, MA	Wine, club soda	AX	Wescan Anion/R IC (250×4.1 mm), gradient elution: eluent 1-2% propan-1-ol, eluent 2-50 mM carbonate buffer, pH 7.5	[226]

[a] AB = Arsenobetaine; AC = Arsenocholine; DMA = Dimethylarsinic acid; MA = Monomethylarsonic acid; TMA = Tetramethylarsonium ion; TMAO = Trimethylarsine oxide.
[b] AX = Anion Exchange Chromatography; CX = Cation Exchange Chromatography; RP = Reversed-Phase Chromatography; IP = Ion-Pair

Arsenic speciation in drinking and mineral water is another application that attracted interest. Generally inorganic As largely predominates in water, and thus the focus is in this case on the assessment of the concentrations and the relative proportions of AsIII and AsV, arsenite having a higher chronic toxicity. IEC [12, 175-178], RPIPC [179-181] and CE [23] have been coupled to ICP-MS for As speciation in water. To enhance sensitivity when CE was employed as the separation technique, HG was resorted to [182], also for simultaneous

speciation of As and Se [183]. Simultaneous separation of As and inorganic Se species has also been achieved by RPIPC [180].

Hg speciation mainly focuses on the distinction between the inorganic form and the main organic compound found in fish, methylmercury, which has a distinctly higher toxicity. The first HPLC method proposed for Hg speciation with ICP-MS detection was RPC, which has been applied to seafood RMs analysis both by direct coupling to the plasma sample introduction system [163, 184-185], or by post column vapour generation to improve DLs [186]. RPIPC with post column vapour generation has been reported for Hg speciation in water [187] and fish (Figure 23) [188]. However, the most widely used speciation technique is GC-ICP-MS following ethylation with sodium tetraethylborate (NaBEt4) [189], which has also been applied in conjunction with ID [163, 190], in alternative hyphenation systems including a low pressure He ICP mass spectrometer [191], or resorting to the purge and trap technique and multicapillary columns to further minimize DLs and analysis time [192]. Hg speciation following SPME for sample introduction has also been reported [156] and a precise and accurate method employing ID SPME-GC-ICP-MS has been applied to the analysis of a variety of fish CRMs, including the dogfish liver tissue DOLT-3 [157]. Some authors have been able to determine mercury species with other techniques, e.g. by CE with volatile species generation [193], or, in a very simple way, by a suitable extraction of organomercury and subsequent analysis using FI and ID, i.e. without any need of hyphenation [38].

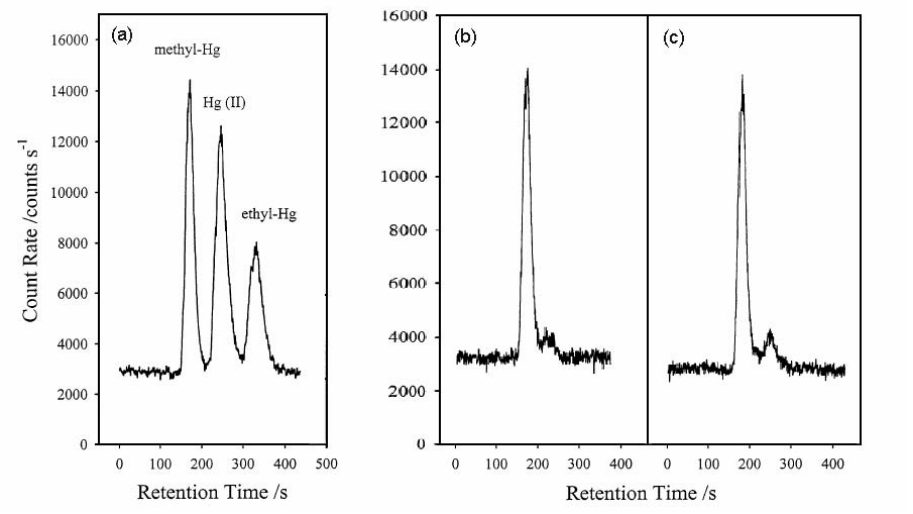


Figure 23. Mercury speciation in fish by HPLC-CV-ICP-MS: (a) Standard mixture, (b) Dogfish muscle CRM (DORM-2), (c) Swordfish sample. Reproduced from [188] by permission of Elsevier.

Hyphenated techniques with ICP-MS detection have also been used for the speciation of organotins. As regards HPLC-ICP-MS, RPIPC has been applied by the Caruso group to identify tributyltin and triphenyltin in fish following different extraction procedures [150-151, 194]. However, the technique of choice for organotin speciation is GC-ICP-MS, which has been applied to seafood analysis either by using multicapillary microcolumns (Figure 24) [195], or following SPME [158], or in conjunction with species specific ID [196]. Vercauteren et al. used SPME with GC-ICP-MS for the determination of the organotin pesticide fentin (triphenyltin) in mussels and potatoes [159]. The same group subsequently reported the successful application of a new extraction technique, stir bar sorptive extraction, to organotin speciation in mussels by GC-ICP-MS [197]. The combination of GC with a low pressure He ICP mass spectrometer has also been reported for organotin speciation in a fish RM [198]. Furthermore, the capability of the system to take fragment spectra providing structural information about gas chromatographic eluents was used for the separation of a mixture of organobromine standards, which prefigured future applications in halogenated pesticides analysis [198]. GC was also coupled with an octapole reaction cell ICP-MS for sensitive detection of organophosphorus pesticides [199].

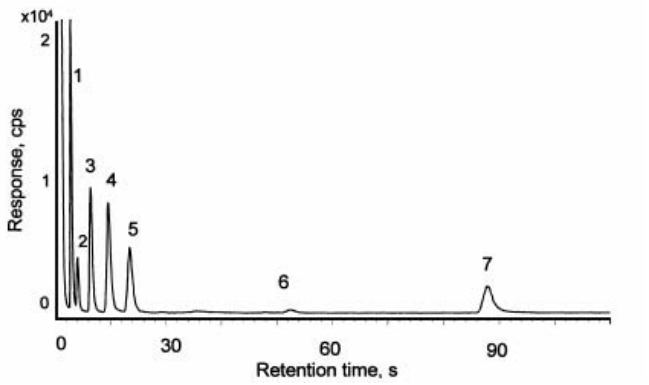


Figure 24. Organotin compounds in the BCR 477 Mussel tissue determined by microcolumn multicapillary GC-ICP-MS: (1) $BuSnEt_3$; (2) internal standard Pr_3SnEt; (3) $BuSnEt_2$; (4) $PhSnEt_3$; (5) Bu_3SnEt; (6) Ph_2SnEt_2; (7) Ph_3SnEt. Reproduced from [195] by permission of Elsevier.

Speciation of halogens has been investigated mainly for water analysis. The first to determine inorganic halogen species in drinking water and 'Miso' soup were Salov et al. [200]. They quantified six anions, i.e. $\tilde{I}$, $I\tilde{O}3$, $\tilde{Br}$, $Br\tilde{O}_3$, $\tilde{Cl}$, $Cl\tilde{O}_3$, by HPLC-ICP-MS. In the last years, interest in bromate and iodate determination in drinking water has risen, as they are formed by oxidation of bromide and iodide during the ozonation treatment for water disinfection. Ozonation has emerged as one of the most promising alternatives to chlorination, challenged for the possible production of carcinogenic disinfection by-products (e.g.

trihalomethanes). However, bromate formation represents a potential health risk because this species is classified as a probable human carcinogen by IARC. High-sensitivity bromate determination by IEC and ICP-MS has been demonstrated by various authors [24, 201-202], also with the use of UN [203] and ID [204]. Dudoit & Pergantis developed a IEC method for the simultaneous determination of As, Br, and I inorganic species in drinking water [176]. HPLC-ICP-MS has also been applied to the determination of bromate residues in bread and bakery products, following use of the additive potassiun bromate as flour improver [205-206].

Another species of interest in drinking water analysis is CrVI, which is considerably more toxic than CrIII and is classified as carcinogenic to humans. Cr speciation in water has been carried out by HPLC-ICP-MS using IEC in different setups [13, 207-208], e.g. either with the employment of a mixed-mode column and a HHPN [13] or with a microbore anion exchange column and a MCN [208]. RPIPC was used in another study, which reported improved DLs due to use of a DRC for the reduction of C- and Cl-related interferences affecting Cr isotopes [209].

Cadmium speciation has mainly been investigated in relation to the binding pattern of this element to proteins, including metallothioneins (MTs) and MTs-like proteins, and studies on meat (liver and kidney) and seafood have been reported. SEC [210] was selected in early studies as the coupled technique, while either CE [211] or anion-exchange fast protein liquid chromatography (AE-FPLC) with post-column IDA [212-213] have been employed in various recent studies carried out by Sanz-Medel group, all using preparative SEC a first fractionation step. In order to shed light on the potential bioavailability of protein-bound Cd, the changes of the element solubility following heat-induced protein denaturation during cooking should be addressed. Crews et al. investigate Cd speciation in cooked pig kidney following a simulated gastric and gastrointestinal digestion by SEC-ICP-MS and found that soluble cadmium was associated to a metallothionein-like protein containing 20% of the total Cd that was able to survive cooking and in vitro gastrointestinal digestion [210].

SEC-ICP-MS was also used to investigate metal–carbohydrate complexes in fruit and vegetable samples [214]. The same technique was employed to study the binding of Cd and Pb in cocoa [215], and of a number of elements in soybean flour & white bean [216].

A different application which has implications in the context of food safety is the quality control of vitamin B12 supplements. Baker and Miller-Ihli performed Co speciation in these supplements by CE (in both CZE and micellar electrokinetic chromatography modes) and succeeded in distinguishing cyanocobalamin (the form most commonly used in vitamin preparations) from free Co, other cobalamins, and a potentially harmful corrinoid analogue, i.e. cobinamide dicyanide [217]. Subsequently, Yanes and Miller-Ihli developed improved methods which employed a low flow, parallel path Mira Mist nebulizer for interfacing various capillary microseparation techniques, such as CE and μHPLC, to ICP-MS [218].

Besides those previously reported, additional applications concerning elemental speciation in beverages deal with the fractionation of metals in tea infusions and the determination of the

specific form of Pb in wine. Owen et al. [219] applied SEC-ICP-MS to study Al fractions in tea infusions and simulated gastrointestinal digests. Tea (*Camellia sinensis*) belongs to the small group of plants that take up Al in very high quantities in their aboveground tissues and thus Al speciation and bioavailability in tea infusions is of particular interest. After simulated gastrointestinal digestion, it was found that the soluble labile fraction, i.e., potentially available for absorption, corresponded to 15 % of the Al from the tea infusion [219]. Szpunar et al. [160] used SEC-ICP-MS to investigate Pb speciation in wine and found that the metal was mostly bound to a pectic polysaccharide, namely 6-deoxy-L-mannogalacturonan (rhamnogalacturonan II), with a molecular weight of 12 kDa (Figure 25). This compound, which form dimers (dRG-II) cross-linked by a negatively charged borate ester moiety, is present in the primary plant cell wall and released into wine during the vinification process. The dRG-II borate can bind at least ten times more Pb2+ than is typically present and wine could therefore have detoxifying properties due to the strong binding capacity of free dRG-II for inorganic Pb.

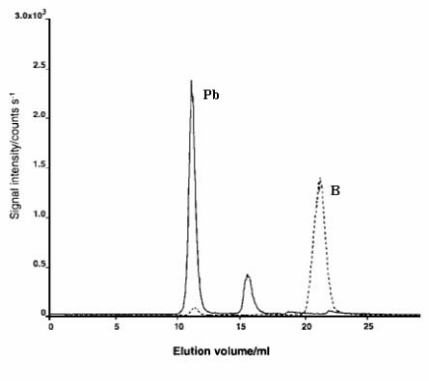


Figure 25 . Lead speciation in a wine sample by HPLC-ICP-MS using size-exclusion chromatography. Coelution of boron indicate that Pb is bound to dRG-II (see text). Reproduced from [160] by permission of The Royal Society of Chemistry.

4. Conclusions and future trends

Today, research in the area of toxic elements and elemental species in food greatly relies upon ICP-MS for precise and accurate analytical determinations. ICP-MS is an isotopic technique, and this is an important feature in a field where the application of IRs and ID methods is increasingly popular. Another important feature is the suitability of ICP-MS as a selective on-line detector in hyphenated methods for the determination of element species, which has gained further popularity to the technique due to the widely recognized importance of elemental speciation in food toxicology.

ICP-MS has many advantages also for high-throughput laboratories involved in food control. A first significant point is the low DLs achievable, since a high detection power is needed to meet current food standards. Other points include high throughput, multi-element capability, and wide linear dynamic range. These are the reasons why the technique is establishing itself as the favourite analytical tool in governmental and control laboratories as well, notwithstanding the expensiveness of the instruments and the high running costs.

Another issue to be emphasized is that in recent years progresses in instrumentation has overcome most of the past limitations of the technique, in particular the presence of heavy spectroscopic interferences at some m/z. The latter can now be adequately addressed by means of the collision and reaction cell technology. This and other developments are likely to favour applications in a variety of fields, e.g. the use of stable isotope tracers for studying the metabolic fate of elements in vivo.

ICP-MS has firmly established as a technique of critical importance in hyphenated methods for speciation analysis. This will clearly continue to be a very dynamic field of investigation and the integration of speciation and metabolism studies with the use of stable isotopes as species-(un)specific tracers is likely to become a research area of vital importance. On the whole, these technical developments and research trends can greatly contribute to a deeper knowledge of element bioavailability, and to understanding how it is influenced by the element chemical form.

Another issue concerning element speciation by ICP-MS-coupled techniques is that several approaches have been developed so far for species identification and quantification with ICP-MS detection in foods. For selected analytes/matrices, sufficiently practicable and reliable analytical protocols have already been established. In some cases, therefore, there is the potential for validating routine control methods and this should facilitate the laying down of species-specific regulations in the international legislation on trace elements in food.

References

[1] R.S. Houk, V.A. Fassel, G.D. Flesch, H.J. Svec, A.L. Gray. C.E. Taylor, Anal. Chem., 52 (1980) 2283
[2] F. Cubadda, J. AOAC Int., 87 (2004) 173
[3] Montaser (ed.) Inductively coupled plasma-mass spectrometry, Wiley-VCH, New York, 1998
[4] S. Nelms (ed.) Inductively Coupled Plasma Mass Spectrometry Handbook, Blackwell Publishing Ltd., Oxford, UK, 2005
[5] R.S. Houk, Anal. Chem., 58 (1986) 97A
[6] S.D. Tanner, V.I. Baranov, D.R. Bandura, Spectrochim. Acta B, 57 (2002) 1361
[7] D.W. Koppenaal, G.C. Eiden, C.J. Barinaga, J. Anal. Atom. Spectrom., 19 (2004) 561
[8] F. Cubadda, S. Giovannangeli, F. Iosi, A. Raggi, P. Stacchini, Food Chem., 81 (2003) 463
[9] F. Cubadda, A. Raggi, Microchem. J., 79 (2005) 91
[10] J.J. Thompson, J. AOAC Int., 76 (1993) 1378
[11] P. Evans, S. Elahi, K. Lee, B. Fairman, J. Environ. Monit., 5 (2003) 175

[12] S. Saverwyns, X. Zhang, F. Vanhaecke, R. Cornelis, L. Moens, R. Dams, J. Anal. Atom. Spectrom., 12 (1997) 1047
[13] Barnowski, N. Jakubowski, D. Stuewer, J.A.C. Broekaert, J. Anal. Atom. Spectrom., 12 (1997) 1155
[14] Chatterjee, Talanta, 51 (2000) 303 - K. Falk, H. Emons, J. Anal. Atom. Spectrom., 15 (2000) 643
[15] K.A. Francesconi, J. Gailer, J.S. Edmonds, W. Goessler, K.J. Irgolic, Comp. Biochem. Phys. C, 122 (1999) 131
[16] S. Wangkarn, S.A. Pergantis, J. Anal. Atom. Spectrom., 14 (1999) 657
[17] L. Perring, D. Andrey, Atom. Spectrosc., 22 (2001) 371
[18] T. Prohaska, G. Köllensperger, M. Krachler, K. De Winne, G. Stingeder, L. Moens, J. Anal. Atom. Spectrom., 15 (2000) 335
[19] Rodushkin, I., Ödman, F., & Appelblad, P.K. (1999) J. Food Compos. Anal. 12, 243
[20] Castiñeira, M.M., Brandt, R., von Bohlen, A., & Jakubowski, N. (2000) Fresen. J. Anal. Chem. 370, 553
[21] M. Barbaste, L. Halicz, A. Galy, B. Medina, H. Emteborg, F.C. Adams, R. Lobinski, Talanta, 54 (2001) 307
[22] J. Ruiz-Encinar, J.I. Garcia-Alonso, A. Sanz-Medel, S. Main, P.J. Turner, J. Anal. Atom. Spectrom., 16 (2001) 315
[23] M. Van Holderbeke, Y. Zhao, F. Vanhaecke, L. Moens, R. Dams, P. Sandra, J. Anal. Atom. Spectrom., 14 (1999) 229
[24] A.R. Elwaer, C.W. McLeod, K.C. Thompson, Anal. Chem., 72 (2000) 5725
[25] S.A. Pergantis, E.M. Heithmar, T.A. Hinners, Anal. Chem., 67 (1995) 4530
[26] S.E. Maestre, J.L. Todoli, J.M. Mermet, Anal. Bioanal. Chem., 379 (2004) 888
[27] G. Zoorob, M. Tomlinson, J. Wang, J. Caruso. J. Anal. Atom. Spectrom., 10 (1995) 853
[28] R. Garcia-Sanchez, J. Bettmer, L. Ebdon, Microchem. J., 76 (2004) 161
[29] Michalke, Electrophoresis, 26 (2005) 1584
[30] M.J. Powell, E.S.K. Quan, D.W. Boomer, D.R. Wiederin, Anal. Chem., 64 (1992) 2253
[31] S.C.K. Shum, H.M. Pang, R.S. Houk, Anal. Chem., 64 (1992) 2444
[32] J. Mora, S. Maestre, V. Hernandis, J.L. Todolí, TRAC-Trend. Anal. Chem., 22 (2003) 123
[33] M.J. Baxter, J.A. Burrell, H.M. Crews, R.C. Massey, D.J. McWeeny, Food Addit. Contam., 6 (1989) 341
[34] C.F. Harrington, S. Elahi, P. Ponnampalavanar, T.M. D'Silva, Atom. Spectrosc., 20 (1999) 174
[35] L. Ebdon, A.S. Fisher, P.J. Worsfold, H. Crews, M. Baxter, J. Anal. Atom. Spectrom., 8 (1993) 691
[36] J. Goossens, L. Moens, R. Dams, Anal. Chim. Acta, 293 (1994) 171
[37] S. Wangkarn, S.A. Pergantis, J. Anal. Atom. Spectrom., 14 (1999) 657
[38] Beauchemin, K.W.M., Siu, S.S. Berman, Anal. Chem., 60 (1988) 2587
[39] L.S. Zhang, S.M. Combs, J. Anal. Atom. Spectrom., 11 (1996) 1043
[40] Klaue, D.B. Blum, Anal. Chem., 71 (1999) 1408
[41] S.J. Christopher, S.E. Long, M.S. Rearick, J.D. Fassett, Anal. Chem., 73 (2001) 2190
[42] S.F. Durrant, N.I. Ward, Food Chem., 49 (1994) 317
[43] Y. Okamoto, Fresen. J. Anal. Chem., 367 (2000) 300
[44] M.J. Liaw, S.-J. Jiang, Y.C. Li, Spectrochim. Acta B, 52 (1997) 779
[45] Pozebon, V.L Dressler, A.J. Curtius, J. Anal. Atom. Spectrom., 13 (1998) 1101
[46] H.C. Liao, S.-J. Jiang, J. Anal. Atom. Spectrom., 14 (1999) 1583

[47] C.-Y. Ho, S.-J. Jiang, J. Anal. Atom. Spectrom., 17 (2002) 688
[48] C.C. Chang, S.-J. Jiang, J. Anal. Atom. Spectrom., 12 (1997) 75
[49] S.N. Willie, D.C. Gregoire, R.E. Sturgeon, Analyst, 122 (1997) 751
[50] Pozebon, V.L Dressler, A.J. Curtius, Talanta, 51 (2000) 903
[51] S.-J. Huang, S.-J. Jiang, J. Anal. Atom. Spectrom., 16 (2001) 664
[52] H.M. Crews, in J. Gilbert (ed.), Progress in food contaminant analysis, Blackie Academic & Professional, London, 1996, pp. 147-186
[53] P. Robb, H.M. Crews, M.J. Baxter, in S.J. Hill (ed.), Inductively coupled plasma spectrometry and its application, Sheffield Academic Press, Sheffield, UK, 1999, pp. 336-366
[54] Cubadda, A. Raggi, A. Testoni, F. Zanasi, J. AOAC Int., 85 (2002) 113
[55] C.J. Park, J.K. Suh, J. Anal. Atom. Spectrom., 12 (1997) 573
[56] Krushevska, S. Waheed, J. Nóbrega, D. Amarisiriwardena, R.M. Barnes, Appl. Spectrosc., 52 (1998) 205
[57] E.H. Larsen, S. Stürup, J. Anal. Atom. Spectrom., 9 (1994) 1099
[58] Krushevska, M. Kotrebai, A. Lasztity, R.M. Barnes, D. Amarasiriwardena, Fresen. J. Anal. Chem., 355 (1996) 793
[59] Klaue, D.B. Blum, Anal. Chem., 71 (1999) 1408
[60] Beauchemin, Current status of ICP-MS, in D. Beauchemin, D.C. Gregoire, D. Günther, V. Karanassios, J.-M. Mermet, T.J. Wood, Discrete sample introduction techniques for inductively coupled plasma-mass spectrometry, Barceló D. (Ed), Comprehensive analytical chemistry 34, Wilson & Wilson's, Elsevier, Amsterdam, The Netherlands, 2000, pp. 1-212
[61] S. Branch, L. Ebdon, M. Ford, M. Foulkes, P. O'Neill, J. Anal. Atom. Spectrom., 6 (1991) 151
[62] S.J. Hill, M.J. Ford, L. Ebdon, J. Anal. Atom. Spectrom., 7 (1992) 719
[63] Lasztity, A. Krushevska, M. Kotrebai, R.M. Barnes, D. Amarasiriwardena, J. Anal. Atom. Spectrom., 10 (1995) 505
[64] Laborda, M.J. Baxter, H.M. Crews, J. Dennis, J. Anal. Atom. Spectrom. 9 (1994) 727
[65] J.W.H. Lam, J.W. McLaren, B.A.J. Methven, J. Anal. Atom. Spectrom., 10 (1995) 551
[66] F. Cubadda, A. Raggi, E. Coni, Anal. Bioanal. Chem., 384 (2006) 887
[67] J.L.M. de Boer, W. Verweij, T. van der Velde-Koerts, W. Mennes, Water Res., 30 (1996) 190
[68] S. Lustig, S. Zang, B. Michalke, P. Schramel, W. Beck, Fresen. J. Anal. Chem., 357 (1997) 1157
[69] S. Wu, X. Feng, A. Wittmeier, J. Anal. Atom. Spectrom., 12 (1997) 797
[70] Stroh, P. Brückner, U. Völlkopf, Atom. Spectrosc., 15 (1994) 100
[71] B.L. Sharp, A.S. Bashammakh, C.M. Thung, J. Skilling, M. Baxter, J. Anal. Atom. Spectrom., 17 (2002) 459
[72] S. Sakao, H. Uchida, Anal. Chim. Acta, 382 (1999) 215
[73] G.M. Scelfo, A.R. Flegal, Environ. Health. Perspect., 108 (2000) 309
[74] J. Riondato, F. Vanhaecke, L. Moens, R. Dams, J. Anal. Atom. Spectrom., 15 (2000) 341
[75] E.H. Larsen, G.A. Pedersen, J.W. McLaren, J. Anal. Atom. Spectrom., 12 (1997) 963
[76] P. Klemens, K.G. Heumann, Fresen. J. Anal. Chem., 371 (2001) 758
[77] M. Barbaste, K. Robinson, S. Guilfoyle, B. Medina, R. Lobinski, J. Anal. Atom. Spectrom., 17 (2002) 135
[78] J. Goossens, T. De Smaele, L. Moens, R. Dams, Fresen. J. Anal. Chem., 347 (1993) 119
[79] X. Feng, S. Wu, A. Wharmby, A. Wittmeier, J. Anal. Atom. Spectrom., 14 (1999) 939
[80] J.A. Nobrega, Y. Gelinas, A. Krushevska, R. Barnes, J. Anal. Atom. Spectrom., 12 (1997) 1239
[81] W. Zhu, E.W.B. de Leer, M. Kennedy, G.J.F.R. Alaerts, Fresen. J. Anal. Chem., 360 (1998) 74

[82] H.M. Al-Swaidan, Anal. Lett., 21 (1988) 1469
[83] L.B. Rasmussen, E.H. Larsen, L. Ovesen, Eur. J. Clin. Nutr., 54 (2000) 57
[84] G.J. Martin, J.B. Fournier, P. Allain, Y. Mauras, Analusis, 25 (1997) 7
[85] M.J. Baxter, H.M. Crews, M.J. Dennis, I. Goodall, D. Anderson, Food Chem., 60 (1997) 443
[86] J.R. Dean, L. Ebdon, R.C. Massey, Food Addit. Contam., 7 (1990) 109
[87] J.D. Greenough, H.P. Longerich, S.E. Jackson, Can. J. Appl. Spectrosc., 41 (1996) 76
[88] S. Augagneur, B. Medina, J. Szpunar, R. Lobinski, J. Anal. Atom. Spectrom., 11 (1996) 713
[89] A.P. Packer, M.F. Giné, Spectrochim. Acta B, 56 (2001) 69
[90] M.S. Jimenez, R. Velarte, J. R. Castello, J. Anal. At. Spectrom., 18 (2003) 1154
[91] C.A. Ponce de Leon, M. Montes Bayon, J.A. Caruso, Anal. Bioanal. Chem., 374 (2002) 230
[92] J.R. Dean, L. Ebdon, R. Massey, J. Anal. Atom. Spectrom., 2 (1987) 369
[93] Nobrega, J. A. Gelinas, Y., Krushevska, A., & Barnes, R. (1997) J. Anal. Atom. Spectrom. 12, 1243
[94] F.A. Rivero Martino, M.L. Fernandez Sanchez, A. Sanz-Medel, Anal. Chim. Acta, 442 (2001) 191
[95] T. Alkanani, J.K. Friel, S.E. Jackson, H.P. Longerich, J. Agric. Food Chem., 42 (1994) 1965
[96] Amarasiriwardena, M. Kotrebai, A. Krushevska, R.M. Barnes, Can. J. Anal. Sci. Spect., 42 (1997) 69
[97] J.A. Moreton, H.T. Delves, J. Anal. Atom. Spectrom., 13 (1998) 659
[98] S. Cao, H. Chen, X. Zeng, J. Anal. Atom. Spectrom., 14 (1999) 1183
[99] J. Allibone, E. Fatemian, P.J. Walker, J. Anal. Atom. Spectrom., 14 (1999) 235
[100] Baxter, M.J., Crews, H.M., Robb, P., & Strutt, P.R. (1997) in Plasma source mass spectrometry – Developments and applications, G. Holland & S.D. Tanner (Eds), Spec. Publ. 202, The Royal Society of Chemistry, Cambridge, UK, pp 95-108
[101] J.K. Friel, C. Mercer, W.L. Andrews, B.R. Simmons, S.E. Jackson, H.P. Longerich, Biol. Trace Elem. Res., 54 (1996) 135
[102] Cubadda, M. Baldini, M. Carcea, L.A. Pasqui, A. Raggi, P. Stacchini, Food Addit. Contam., 18 (2001) 778
[103] H.M. Crews, M.J. Baxter, T. Bigwood, J.A. Burrell, L.M. Owen, C. Robinson, C. Wright, R.C. Massey, Food Addit. Contam., 9 (1992) 365
[104] G.B. Jiang, Q.F. Zhou, B. He, J.Y. Liu, Sci. China Ser. B-Chem., 43 (2000) 531
[105] W.A.C. Anderson, S.A. Thorpe, L.M. Owen, S.E. Anderson, H.M. Crews, S.L. Reynolds, Food Addit. Contam., 15 (1998) 288
[106] L. Castle, C.P. Offen, M.J. Baxter, J. Gilbert, Food Addit. Contam., 14 (1997) 35
[107] Skrzydlewska, M. Balcerzak, F. Vanhaecke, Anal. Chim. Acta, 479 (2003) 191
[108] P.J. Fordham, J.W. Gramshaw, H.M. Crews, L. Castle, Food Addit. Contam., 12 (1997) 651
[109] Z.-W. Zhang, S. Shimbo, N. Ochi, M. Eguchi, T. Watanabe, C.-S. Moon, , M. Ikeda, Sci. Total Environ., 205 (1997) 179
[110] S. Shimbo, Z.-W. Zhang, C.-S. Moon, T. Watanabe, H. Nakatsuka, N. Matsuda-Inoguchi, K. Higashikawa, M. Ikeda, Int. Arch. Occup. Environ. Health, 73 (2000) 163
[111] M. Ikeda, Z.-W. Zhang, S. Shimbo, T. Watanabe, H. Nakatsuka, C.-S. Moon, N. Matsuda-Inoguchi, K. Higashikawa, Sci. Total Environ., 249 (2000) 373
[112] R.A. Schoof, L.J. Yost, J. Eickhoff, E.A. Crecelius, D.W. Cragin, D.M. Meacher, D.B. Menzel, Food Chem. Toxicol., 37 (1999) 839
[113] T. Roychowdhury, T. Uchino, H. Tokunaga, M. Ando, Food Chem. Toxicol., 40 (2002) 1611

[114] J.M. Llobet, G. Falcó, C. Casas, A. Teixidó, J.L. Domingo, J. Agric. Food Chem., 51 (2003) 838
[115] K. Shiraishi, M. Yamamoto, J. Radioanal. Nucl. Ch., 196 (1995) 89
[116] M. Yukawa, Y. Watanabe, Y. Nishimura, Y. Guo, Z. Yongru, H., Lu, W. Zhang, L. Wei, Z. Tao, Fresen. J. Anal. Chem., 363 (1999) 760
[117] O. Wappelhorst, I. Kühn, H. Heidenreich, B. Markert, Nutrition, 18 (2002) 316
[118] Ysart, P. Miller, H. Crews, P. Robb, M. Baxter, C. de L'Argy, S. Lofthouse, C. Sargent, N. Harrison, Food Addit. Contam., 16 (1999) 391
[119] H.M. Crews, L.M. Owen, N. Langford, S.J. Fairweather-Tait, T.E. Fox, L. Hubbard, D. Phillips, Toxicol. Lett., 112-113 (2000) 201
[120] Medek, P., Pavlickova, J., Zbiral, J., Cizmarova, E., & Kuban, V. (2001) Int. J. Environ. Anal. Chem. 81, 207
[121] L. Samsøe-Petersen, E.H. Larsen, P.B. Larsen, P. Bruun, Environ. Sci. Technol., 36 (2002) 3057
[122] P. Robb, L.M.W. Owen, H.M. Crews, J. Anal. Atom. Spectrom., 10 (1995) 625
[123] S. Mounicou, J. Szpunar, D. Andrey, C.J. Blake, R. Lobinski, Food Addit. Contam., 20 (2003) 343
[124] Bibak, S. Stürup, V. Haahr, P. Gundersen, V. Gundersen, J. Agric. Food Chem., 47 (1999) 2678
[125] V. Gundersen, I.E. Bechmann, A. Behrens, S. Stürup, J Agric. Food Chem., 48 (2000) 6094
[126] S. Kelly, M. Baxter, S. Chapman, C. Rhodes, J. Dennis, P. Brereton, Eur. Food Res. Technol., 214 (2002) 72
[127] S. Branch, S. Burke, P. Evans, B. Fairman, C.S.J. Wolff Briche, J. Anal. Atom. Spectrom. 18, (2003) 17
[128] I.E. Bechmann, S. Stürup, L.V. Kristensen, Fresen. J. Anal. Chem., 368 (2000) 708
[129] F. Cubadda, A. Raggi, E. Marconi, Microchem. J., 79 (2005) 97
[130] M.J. Baxter, J.A. Burrell, H.M. Crews, A. Smith, R.C. Massey, Food Addit. Contam., 9 (1992) 225
[131] J.L. Fernandez-Turiel, J.F. Llorens, F. Lopez-Vera, C. Gomez-Artola, I. Morell, D. Gimeno, Fresen. J. Anal. Chem., 368 (2000) 601
[132] Reimann, K. Bjorvatn, B. Frengstad, Z. Melaku, R. Tekle-Haimanot, U. Siewers, Sci. Total Environ., 311 (2003) 65
[133] Misund, B. Frengstad, U. Siewers, C. Reimann, Sci. Total Environ. 244 (1999) 21
[134] R.W. Dabeka, H.B.S. Conacher, J.F. Lawrence, W.H. Newsome, A. McKenzie, H.P. Wagner, R.K.H. Chadha, K. Pepper, Food Addit. Contam., 19 (2002) 721
[135] C.J. Park, P.J. Oh, H.Y. Kim, D.S. Lee, J. Anal. Atom. Spectrom., 14 (1999) 223
[136] R.C. Richter, S.R. Koirtyohann, S.S. Jurisson, J. Anal. Atom. Spectrom., 12 (1997) 557
[137] Evans, E.H., Truscott, J.B., Bromley, L., Jones, P. Turner, J. & Fairman, B.E., in R.W. Morrow and J.S. Crain (eds.), Applications of inductively coupled plasma-mass spectrometry to radionuclide determinations, Vol. 2, ASTM Special Technical Publication, Vol. 1344, pp 79-88, 1998
[138] C.M.R. Almeida, M.T.S.D. Vasconcelos, Anal. Chim. Acta, 396 (1999) 45
[139] C.M. Almeida, M.T.S.D. Vasconcelos, J. Anal. Atom. Spectrom., 16 (2001) 607
[140] K.E. Ødegård, W. Lund, J. Anal. Atom. Spectrom., 12 (1997) 403
[141] Wyrzykowska, K. Szymczyk, H. Ichichashi, J. Falandysz, B. Skwarzec, S. Yamasaki, J. Agric. Food Chem., 49 (2001) 3425

[142] D.M. Templeton, F. Ariese, R. Cornelis, L.-G. Danielsson, H. Muntau, H.P Van Leeuwen, R. Łobiński, Pure Appl. Chem., 72 (2000) 1453
[143] K.L. Ackley, C. B'Hymer, K.L. Sutton, J.A. Caruso, J. Anal. Atom. Spectrom., 14 (1999) 845
[144] E.H. Larsen, M. Hansen, W. Goessler, Appl. Organomet. Chem., 12 (1998) 285
[145] R. Tukai, W.A. Maher, I.J. McNaught, M.J. Ellwood, Anal. Chim. Acta, 457 (2002) 173
[146] J.W. McKiernan, J.T. Creed, C.A. Brockhoff, J.A. Caruso, R.M. Lorenzana, J. Anal. Atom. Spectrom., 14 (1999) 607
[147] N.P. Vela, D.T. Heitkemper, K.R. Stewart, Analyst, 126 (2001) 1011
[148] S. Branch, L. Ebdon, P. O'Neill, J. Anal. Atom. Spectrom., 9 (1994) 33
[149] S.L. Cleland, L.K. Olson, J.A. Caruso, J.M. Carey, J. Anal. Atom. Spectrom., 9 (1994) 975
[150] U.T. Kumar, N.P. Vela, J.G. Dorsey, J.A. Caruso, J. Chromatogr. A, 655 (1993) 340
[151] N.P. Vela, J.A. Caruso, J. Anal. Atom. Spectrom., 11 (1996) 1129
[152] J.A. Brisbin, J.A. Caruso, Analyst, 127 (2002) 92
[153] J.A. Caruso, D.T. Heitkemper, C. B'Hymer, Analyst, 126 (2001) 136
[154] Kuehnelt, K.J. Irgolic, W. Goessler, Appl. Organomet. Chem., 15 (2001) 445
[155] Sanz, R. Muñoz-Olivas, C. Cámara, Anal. Chim. Acta, 535 (2005) 227
[156] Z. Mester, J. Lam, R. Sturgeon, J. Pawliszyn, J. Anal. Atom. Spectrom., 15 (2000) 837
[157] L. Yang, Z. Mester, R.E. Sturgeon, J. Anal. Atom. Spectrom., 18 (2003) 431
[158] S. Aguerre, G. Lespes, V. Desauziersb, M. Potin-Gautier, J. Anal. Atom. Spectrom., 16 (2001) 263
[159] J. Vercauteren, A. De Meester, De Smaele, T., Vanhaecke, F., Moens, L., Dams, R., & Sandra, P. (2000) J. Anal. Atom. Spectrom. 15, 651
[160] J. Szpunar, P. Pellerin, A. Makarov, T. Doco, P. Williams, B. Medina, R. Lobinski, J. Anal. Atom. Spectrom., 13 (1998) 749
[161] J. Meija, S. Mounicou, J.A. Caruso, J. AOAC Int., 87 (2004) 205
[162] T. Lindemann, A. Prange, W. Dannecker, B. Neidhart, Fresen. J. Anal. Chem., 368 (2000) 214
[163] Hintelmann, R. Falter, G. Ilgen, R.D. Evans, Fresen. J. Anal. Chem., 358 (1997) 363
[164] P. Rodrıguez-Gonzalez, J.M. Marchante-Gayon, J.I. Garcia Alonso, A. Sanz-Medel,. Spectrochim. Acta B, 60 (2005) 151
[165] E.H. Larsen, G. Pritzl, S.H. Hansen, J. Anal. Atom. Spectrom., 8 (1993) 1075
[166] E.H. Larsen, Fresen. J. Anal. Chem., 352 (1995) 582
[167] E.H. Larsen, Spectrochim. Acta B, 53 (1998) 253
[168] E.H. Larsen, J. Engman, J.J. Sloth, M. Hansen, L. Jorhem, Anal Bioanal Chem., 381 (2005) 339
[169] Shibata, Y., & Morita, M. (1989) Anal. Chem. 61, 2116
[170] S. McSheehy, J. Szpunar, J. Anal. Atom. Spectrom., 15 (2000) 79
[171] S. McSheehy, P. Pohl, R. Lobinski, J. Szpunar, Analyst, 126 (2001) 1055
[172] S. McSheehy, P. Pohl, R. Lobinski, J. Szpunar, Anal. Chim. Acta, 440 (2001) 3
[173] J.R. Dean, L. Ebdon, M.E. Foulkes, H.M. Crews, R.C. Massey, J. Anal. Atom. Spectrom., 9 (1994) 615
[174] Schmeisser, W. Goessler, N. Kienzl, K.A. Francesconi, Analyst, 130 (2005) 948
[175] M. Moldovan, M.M. Gomez, M.A Palacios, C. Cámara, Microchem. J., 59 (1998) 89
[176] Dudoit, S.A. Pergantis, J. Anal. Atom. Spectrom., 16 (2001) 575
[177] P.A. Gallagher, C.A. Schwegel, X.Y. Wei, J.T. Creed, J. Environ. Monit., 3 (2001) 37
[178] J.A. Day, M. Montes-Bayon, A.P. Vonderheide, J.A. Caruso, Anal. Bioanal. Chem., 373 (2002) 664
[179] P. Thomas, K. Sniatecki, J. Anal. Atom. Spectrom., 10 (1995) 615

[180] P. Thomas, K. Pereira, D. Koller, Analusis, 25 (1997) 19
[181] Shraim, N.C. Sekaran, C.D. Anuradha, S. Hirano, Appl. Organomet. Chem., 16 (2002) 202
[182] M.L. Magnuson, J.T. Creed, C.A. Brockhoff, J. Anal. Atom. Spectrom., 12 (1997) 689
[183] M.L. Magnuson, J.T. Creed, C.A. Brockhoff, Analyst, 122 (1997) 1057
[184] C.W. Huang, S.-J. Jiang, J. Anal. Atom. Spectrom., 8 (1993) 681
[185] R. Falter, G. Ilgen, Fresen. J. Anal. Chem., 358 (1997) 401
[186] D.S. Bushee, Analyst, 113 (1988) 1167
[187] C.C. Wan, C.S. Chen, S.-J. Jiang, J. Anal. Atom. Spectrom., 12 (1997) 683
[188] C.S. Chiou, S.-J. Jiang, K.S.K. Danadurai, Spectrochim. Acta B, 56 (2001) 1133
[189] Q. Tu, J. Qian, W. Frech, J. Anal. Atom. Spectrom., 15 (2000) 1583
[190] Hintelmann, R.D. Evans, Fresen. J. Anal. Chem., 358 (1997) 378
[191] Rosenkranz, G. O'Connor, E.H. Evans, J. Anal. Atom. Spectrom., 15 (2000) 7
[192] S. Slaets, F. Adams, I.R. Pereiro, R. Lobinski, J. Anal. Atom. Spectrom., 14 (1999) 851
[193] M.S. da Rocha, A.B. Soldado, E. Blanco, A. Sanz-Medel, J. Anal. Atom. Spectrom., 16 (2001) 951
[194] U.T. Kumar, J.G. Dorsey, J.A. Caruso, E.H. Evans, J. Chromatogr. A, 654 (1993) 261
[195] Szpunar, S. McSheehy, K. Polec, V. Vacchina, S. Mounicou, I. Rodriguez, R. Lobinski, Spectrochim. Acta B, 55 (2000) 779
[196] M. Monperrus, O. Zuloaga, E. Krupp, D. Amouroux, R. Wahlen, B. Fairman, O.F.X. Donard, J. Anal. Atom. Spectrom., 18 (2003) 247
[197] Vercauteren, D. Peres, C. Devos, P. Sandra, F. Vanhaecke, Anal. Chem., 73 (2001) 1509
[198] J.W. Waggoner, L.S. Milstein, M. Belkin, K.L. Sutton, J.A. Caruso, H.B. Fannin, J. Anal. Atom. Spectrom., 15 (2000) 13
[199] P. Vonderheide, J. Meija, M. Montes-Bayon, J. A. Caruso, J. Anal. At. Spectrom., 18 (2003) 1097
[200] V.V. Salov, J. Yoshinaga, Y. Shibata, M. Morita, Anal. Chem., 64 (1992) 2425
[201] Nowak, A. Seubert, Anal. Chim. Acta, 359 (1998) 193
[202] Seubert, M. Nowak, Fresen. J. Anal. Chem., 360 (1998) 777
[203] J.T. Creed, M.L. Magnuson, J.D. Pfaff, C. Brockhoff, J. Chromatogr. A, 753 (1996) 261
[204] J.T. Creed, C.A. Brockhoff, Anal. Chem., 71 (1999) 72
[205] M.J. Dennis, A. Burrell, K. Mathieson, P. Willetts, R.C. Massey, Food Addit. Contam., 11 (1994) 633
[206] D.T. Heitkemper, L.A. Kaine, D.S. Jackson, K.A. Wolnik, J. Chromatogr. A, 671 (1994) 101
[207] Sacher, B. Raue, J. Klinger, H.J. Brauch, Int. J. Environ. Anal. Chem., 74 (1999) 191
[208] S. Saverwyns, K. Van Hacke, F. Vanhaecke, L. Moens, R. Dams, Fresen. J. Anal. Chem., 363 (1999) 490
[209] Y.L. Chang, S.-J. Jiang, J. Anal. Atom. Spectrom., 16 (2001) 858
[210] H.M. Crews, J.R. Dean, L. Ebdon, R.C. Massey, Analyst, 114 (1989) 895
[211] Álvarez-Llamas, M.R. Fernandez de la Campa, A. Sanz-Medel, J. Anal. Atom. Spectrom., 18 (2003) 460
[212] C.N. Ferrarello, M.R. Fernandez de la Campa, J.F. Carrasco, A. Sanz-Medel, Anal. Chem., 72 (2000) 5874
[213] Rodrıguez-Cea, M.R. Fernandez de la Campa, E. Blanco Gonzalez, B. Andon Fernandez, A. Sanz-Medel, J. Anal. Atom. Spectrom., 18 (2003) 1357
[214] J. Szpunar, P. Pellerin, A. Makarov, T. Doco, P. Williams, R. Lobinski, J. Anal. Atom. Spectrom., 14 (1999) 639

[215] S. Mounicou, J. Szpunar, R. Lobinski, D. Andrey, C.J. Blake, (2002) J. Anal. Atom. Spectrom. 17, 880
[216] R. Koplik, H. Pavelkova, J. Cincibuchova, O. Mestek, F. Kvasnika, M. Suchanek, J. Chromatogr. B, 770 (2002) 261
[217] S.A. Baker, N.J. Miller-Ihli, Spectrochim. Acta B, 55 (2000) 1823
[218] E.G. Yanes, N.J. Miller-Ihli, Spectrochim. Acta B, 59 (2004) 883
[219] L.M.W. Owen, H.M. Crews, R.C. Massey, Chem. Spec. Bioavailab., 4 (1992) 89
[220] S.F. Boulyga, H.-J. Dietze, J.S. Becker, Mikrochim. Acta, 137 (2001) 93
[221] P.C. Li, S.-J. Jiang, Anal. Chim. Acta, 495 (2003) 143.
[222] D.T. Heitkemper, N.P. Vela, K.R. Stewart, C.S. Westphal, J. Anal. Atom. Spectrom., 16 (2001) 299
[223] N.P. Vela, D.T. Heitkemper, J. AOAC Int., 87 (2004) 244
[224] Polatajko, J. Szpunar, J. AOAC Int., 87 (2004) 233
[225] S. Wangkarn, S.A. Pergantis, J. Anal. Atom. Spectrom., 15 (2000) 627
[226] B.S. Sheppard, J.A. Caruso, D.T. Heitkemper, K.A. Wolnik, Analyst, 117 (1992) 971

Index