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EXTRACTION OF ORGANIC ANALYTES FROM *FOODS*

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Extraction of Organic Analytes from Foods

A Manual of Methods

RSC Food Analysis Monographs

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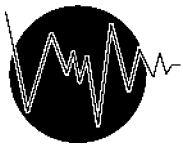

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Preface

A wide range of analytical protocols, including extraction procedures to measure the concentration of an analyte in stated food matrices, are published by the Association of Official Analytical Chemists (AOAC). These are kept up to date through their validation procedure, which differentiates between methods that are under development and those that have been approved through collaborative trials and other tests as statistically reliable. The chemical composition of a particular food can be found by consulting compilations, *e.g*. *The Composition of Foods* by McCance and Widdowson that provides an updated source of information on new and existing foods in common use, *via* regularly published supplements. Other monographs in this RSC series on food analysis have already dealt with *Quality in the Food Analysis Laboratory* (including sampling), *Dietary Fibre Analysis*, *Chromatography and Capillary Electrophoresis in Food Analysis*, *Mass spectrometry of Natural Substances in Foods*, and *The Maillard Reaction*. This contribution sets out to compile a laboratory manual of methods used for the preparation and extraction of organic chemical compounds from food sources.

Chapter 1, introducing extraction methodology, Chapter 2, compiling and differentiating sample preparation for extraction procedures, and the introductory sections of the subsequent chapters are pitched at the undergraduate level. Practising food analysts may find the compilation of extraction techniques into five physical groups: partition, solvation, distillation, adsorption and diffusion (Chapters 3–7, respectively) a useful structure and content for training programmes, and the applications (referenced in subject indices organised by commodity, method, chemical class and analyte) may provide useful examples from the literature to illustrate the historical development of the physical methods applied to food analyses.

It must be emphasised, however, that the examples have been chosen to illustrate the analytical processes and are not intended to be a comprehensive record of work, or even the major work, done using that process. One serious incursion into the literature on extraction methodology will highlight the enormity of the task in making such a record.

To some extent, the selection of extraction methods for separate study is arbitrary since the various stages of analysis cannot be always cleanly dissected

one from the other. This is apparent when the first chromatographic method in series with another can be seen as a microextraction (separation) process.

Sometimes, the relatively simple procedures involved in the extraction of target compounds may be mistakenly considered to be less of an intellectual challenge than the more sophisticated separation and detection techniques. But, because the extraction stage is often identified as the major source of error in the total analysis, there is justification for paying extra attention to this area, especially now that it is being put on-line in automated assays.

In the appendices, examples of methods that have been compared, combined or used in collaborative trails have been correlated and used to form the beginnings of a database.

Ironically, remote sensing methods of acquiring compositional information from foods are developing rapidly and making the classical "sampling" and the current "extraction for analysis" methods redundant! Fortuitously, the equally rapid development of on-line extraction and separation/detection methods requires easy access to existing information. This collation of methods and applications may be a handy reference for the developers of the "extractionless" methodology of the future.

Ron Self
Norwich, UK, 2005

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I wish to thank the authors of all the work referred to in the text, without which a collation of methods would not have been possible. Where more than a brief summary of the referenced method has been cited, permission has been obtained from the publisher or author, as appropriate. In the case of the six classical line illustrations (Figures 1.1, 1.2, 5.3, 5.4, 5.5, and 5.8), neither the Publisher (William Heinemann Ltd.) nor the author (Dr F. Sherwood Taylor) owned the copyright. Every effort was made to trace the owners of material used here, and anyone not contacted is invited to write to me.

Abbreviations

2-ME	2-mercaptoethanol
AC	affinity chromatography
AcCN	acetonitrile
AcD	acid distillation
AcE	acid extraction
ACE	angiotensin I converting enzyme
AcHyd	acid hydrolysis
AEC	anion-exchange chromatography
AEDA	aroma extract dilution analysis
Alc	alcohol
AlkHyd	alkaline hydrolysis
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric pressure chemical ionisation
Aq	Aqueous
ASE	Accelerated solvent extraction (see also PLE)
ATP	Adenosine triphosphate
B&D	Bligh and Dyer
BDC	buoyant density centrifugation
BE	Babcock extraction
BFR	brominated flame retardant
BHT	butylated hydroxy toluene
C_{18}	octadecyl silica
$C_{18\ ec}$	octadecyl end-capped silica
CC	column chromatography
CCC	countercurrent chromatography
CCD	countercurrent distribution
CCGC	capillary column gas chromatography
CC-SFE	countercurrent supercritical fluid extraction
COVB	carbowax divinylbenzene
CE	capillary electrophoresis
CEC	capillary electrochromatography
CF	continuous flow
CFFE	continuous free flow electrophoresis
CFS	continuous flow system
CGE	capillary gel electrophoresis
CI	chemical ionisation
CIC	capillary ion chromatography
CITP	capillary isotachophoresis
COI	compound of interest

Conc	concentrated
COP	cholesterol oxidation product
CPCB	coplanar PCB
C(PR)	colorimetric (pararosaniline)
CRM	certified reference material
CTAB	cetyltrimethylammoniom bromide
$(CS)_5$	collaborative study among 5 laboratories
CW-DVB	Carbowax-divinylbenzene
CW-TPR	Carbowax-templated Resin
CZE	capillary zone electrophoresis
Da	dalton
DAD	diode array detection
D&S	Dean and Stark
DC	dry column
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
DEC	disposable extraction cartridge
Dep	deproteinised
DF	dietary fibre
DH	dehydrated
DHPSE	dynamic high-pressure solvent extraction
D-HS	dynamic headspace
DI	direct injection
Dil	dilute
Dist	distillation
DMDS	dimethyl disulphide
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulphoxide
DMTS	dimethyl trisulphide
DPP	differential pulsed polarographic
DS	digestible starch
DVB	divinylbenzene
ec	end capped
EC	electron capture
ECD	electron capture detection
EDB	ethylene dibromide
EDC	ethylene dichloride
EDTA	ethylenediaminetetraacetic acid
EI	electron ionisation
ELISA	enzyme-linked immunosorbent assay
Enz	enzymatic
ENZ	enzymic method
EnzHyd	enzyme hydrolysis
EO	ethylene oxide
EOF	electro end osmotic flow
e-scheme	extraction scheme

ESE	enhanced solvent extraction (see also PLE)
ESI	electrospray ionisation
ES-ITMS	electrospray ion trap mass spectrometry
ESO	enzymatic sulphite oxidase
ESy	extracting syringe
EtOH	ethanol
EU	European Union
FA	fatty acid
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide
FAME	fatty acid methyl ester
FAPAS	Food Analysis Performance Assessment Scheme
FD	freeze drying
FDA	Food and Drugs Administration
FFA	free fatty acid
FFF	field flow fractionation
FI	flow injection
FIA	flow injection analysis
FID	flame ionisation detector
FLD	fluorescence detection
FMASE	focused microwave-assisted Soxhlet extraction
FMN	flavin mononucleotide
FMOC	9-fluorenylmethyl chloroformate
FS	flow switching
FSV	flow switching valve
GC	gas chromatography
GCO	gas chromatography-olfactometry
GE	gel electrophoresis
GLP	gas/liquid partition
GLPA	gas/liquid partition analysis
GPC	gel permeation chromatography
h	hour
H	height of a theoretical plate
HA	heterocyclic amines
HAA	heterocyclic aromatic amine
HBV	high-boiling volatile
HCB	hexachlorobenzene
Hex	hexane
HFB	heptafluorobutyrate
HLB	hydrophilic–lipophilic balance
HPIC	high-performance ion chromatography
HPTLC	high-performance thin-layer chromatography
HS	headspace
HSCCC	high-speed ccc
HSGC	high speed gas chromatography
HS-GC	headspace gas chromatography

HS-LC	headspace liquid chromatography
HS-SE	headspace sorptive extraction
HS-SPME	headspace solid-phase microextraction
HVD	high vacuum distillation
IAC	immunoaffinity chromatography
IAE	immunoaffinity extraction
IC	ion chromatography
ICM	Iodometric Committee Method (for sulphites)
IDF	insoluble dietary fibre
IE	ion exchange
IEC	ion exchange chromatography
IEF	isoelectric focusing
IexC	ion exclusion chromatography
IFJU	International Fruit Juice Union
IgG	immunoglobulin G
ILC	interlaboratory comparison
IMP	instant mashed potato
IPA	isopropyl alcohol
IPT	International Proficiency Test
IRMM	Institute for Reference Materials and Measurements
IRMS	isotope ratio mass spectrometry
ISO	International Standards Organisation
ITC	isothiocyanate
ITP	isotachophoresis
IUPAC	International Union of Pure and Applied Chemistry
$K_{A/W}$	air/water partition constant
$K_{O/A}$	octanol/air partition constant
$K_{O/W}$	octanol/water partition constant
$K_{oil/water}$	oil/water partition constant
$K_{S/M}$	stationary phase/mobile phase partition constant
LBV	low-boiling volatile
LC	liquid chromatography
LE	leading electrolyte
LLE	liquid/liquid extraction
LLLE	liquid/liquid/liquid extraction
LLP	liquid/liquid partition
LLP-E	liquid/liquid partition–extraction
LME	liquid membrane extraction
LMW	low molecular weight
L-N	Likens–Nickerson
LOD	limit of detection
LOQ	limit of quantification
LPME	liquid phase microextraction
LRM	laboratory reference material
LSE	liquid–solid extraction
LSLE	liquid/solid/liquid extraction

LSLP	liquid/solid/liquid partition
LTP	low temperature precipitation
LTVD	low temperature vacuum distillation
LVI	large volume injection
MA	microwave assisted
MAE	microwave-assisted extraction
MAH	monocyclic aromatic hydrocarbon
MALDI	matrix-assisted laser desorption ionisation
MASE	microwave assisted solvent extraction
MA-SOX	microwave-assisted Soxhlet extraction
MBSE	membrane-based solvent extraction
MCAC	metal chelate affinity chromatography
MD	membrane diffusion
MDE	microdiffusion extraction
MECC	micellar electrokinetic capillary chromatography
MEE	Mojonnier ether extraction
MEECC	microomulsion electrokinetic chromatography
MemASE	membrane-assisted solvent extraction
MeOH	methanol
MESI	membrane extraction with sorbent interface
MIP	molecularly-imprinted polymer
MISPE	molecularly-imprinted solid-phase extraction
MMLLE	microporous membrane liquid/liquid extraction
M(MT)M	methyl(methylthio)methyl
MMTSO	methylmethane thiosulphinate
Mod	modified
MOD	mineral oil distillation
ModMEE	modified Mojonnier ether extraction
MOPSO	OH-4-morpholinopropanesulphonic acid
MPa	mega Pascal
MRA	multiresidue analysis
MRC	Medical Research Council
MRL	maximum residue level
MRM	multiresidue method
MS	mass spectrometry
MS^n	multistage mass spectrometry
MS-MS	mass spectrometry-mass spectrometry (tandem method)
MSPD	matrix solid-phase dispersion
MTB	*S*-Methyl thiobutanoate
MW	molecular weight
MWCO	molecular weight cut-off
MWD	Monier-Williams distillation
N_2	nitrogen
NAA	neutron activation analysis
NBS	National Bureau of Standards
n.d.	not detected

NDMA	*N*-Nitrosodimethylamine
NFPA	National Food Processors Association
NIR	near-infrared
NP	normal phase
NPAH	nitrated polycyclic aromatic hydrocarbon
NPD	nitrogen phosphorus detector
NPLC	normal phase liquid chromatography
NPN	non-protein nitrogen
NPYR	*N*-Nitrosopyrrolidine
NSP	non-starch polysaccharides
NT	not tested
od	outside diameter
ODS	octadecylsilica
OEP	optimum extraction protocol
OM	official method
Op	optimised
OP	organophosphorus
OPP	organophosphorus pesticide
Org	organic
Pa	Pascal
PA	polyacrylate
PAC	polyaromatic compound
PAD	pulsed amperometric detection
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PCP	pentachlorophenol
PCR	polymerase Chain Reaction
PDMS	poly(dimethylsiloxane)
PDMS-DVB	polydimethylsiloxane-divinylbenzene
PEEK	polyetheretherketone
PEG	polyethyleneglycol
Pet. Ether	petroleum ether
PFE	pressurised fluid extraction (see also PLE)
PHWE	pressurised hot water extraction (see also SWE)
pI	isoelectric point
PLE	pressurised liquid extraction (also known as PFE, PSE, ASE, ESE)
PLM	particle-loaded membrane
POAE	press oil aroma extraction
pptn	precipitation
PS	phenylsilane
PSE	pressurised solvent extraction (see also PLE)

psi	pounds per square inch
PTFE	polytetrafluoroethylene
P & T	purge and trap
PVDF	polyvinyldifluoride
RAG	rapidly available glucose
RAM	restricted access media
RDS	rapidly digestible starch
R-G	Roese-Gottlieb
RIs	retention indices
RP	reversed phase
RP-IRLC	reversed phase ion pairing liquid chromatography
RP-LC	reversed phase liquid chromatography
RRI	relative retention Index
RRT	relative retention Time
RS	resistant starch
RSD	relative standard deviation
RT	retention time
RmT	room temperature
SAP	saponification
SAX	strong anion exchange
S-BSE	stir-bar sorptive extraction
SC	short-chain carbohydrate
SCoD	sweep co-distillation
SD	standard deviation
SDE	simultaneous steam–organic solvent distillation extraction
SDF	soluble dietary fibre
SDME	single drop microextraction
SDS	sodium dodecyl sulphate
SDSt	slowly digestible starch
S-DVB	styrene-divinylbenzene
SE	solvent extraction
SEC	size exclusion chromatography
SF	shake flask
SFE	supercritical fluid extraction
SGC	silica-gel chromatography
S-HS	static headspace
SIM	selected ion monitoring
SLE	solid/liquid extraction
SLM	supported liquid membrane
SLME	supported liquid membrane extraction
SLP	solid/liquid partition
s/n	signal-to-noise
SO	sulphite oxidase
SolD	solvent distillation
SOX	Soxhlet extraction
SPDE	solid-phase dynamic extraction

SPE	solid-phase extraction
SPE/HPLC	combined solid-phase extraction and high-performance liquid chromatography
SPME	solid-phase microextraction
SPR	surface plasmon resonance
SRMs	standard reference materials
Std	standard
StD	steam distillation
SWE	sub-critical water extraction (see also hot water extraction, PHWE, pressurised water extraction, high temperature water extraction, superheated water extraction, hot liquid water extraction)
μ-TAS	miniaturised total chemical analysis
TCA	trichloroacetic acid
TCB	trichlorobenzene
TCE	trichloroethylene
TCP	tetrachlorophenol
TD	thermal desorption
TDF	total dietary fibre
TE	terminating electrolyte
TEA	thermal energy analysis
TIC	total ion current
TMS	trimethylsilyl
TP	theoretical plate
TS	total starch
p-TSA	*p*-toluenesulphonamide
TVA	total volatile analysis
UAE	ultrasound-assisted extraction
UAMD	ultrasound-assisted microwave digestion
UASE	ultrasound-assisted soxhlet extraction
UDMH	unsymmetrical dimethylhydrazine
UHT	ultra-high temperature
USE	ultrasonic Extraction
USEPA	US Environmental Protection Agency
VC	vinyl chloride
VIS	visible spectrophotometry
VLE	vapour–liquid equlibrium
VOC	volatile organic compound
VSS	vacuum steam-stripping
ZRM	zero reference material

CHAPTER 1

Methodology and Proximate Analysis

1 Extraction of Organic Analytes

Opening Statement

The preparation for analysis of a small amount of material (the sample), representative of the bulk food, and normally supplied to the analyst, can be divided into the following stages:

1. The first stage for most food matrices is to prepare a weighed and calibrated aliquot – the sample for analysis – in preparation for quantitative extraction of the compounds of interest (analytes).
2. For some food samples, the material has to be rendered accessible to the extracting agent – preparation for analysis.
3. The next stage can then be either (a) removal of the analytes from the sample matrix or (b) removal of interferents from the matrix – the extraction. In each case, the analytes are in a form to be recognised and quantified unambiguously in subsequent examinations.
4. The final stage is to examine the extract, which will normally contain matrix components other than the target analytes, using various of chemical and physical methods to make qualitative or quantitative measurements of the analytes – the analysis.

One contemporary objective in the development of analytical methodology is to automate the whole assay, and there are two ways forward. The classical extraction procedure can be given over to robotic control, or the information about the chemical composition can be "extracted" directly from the sample matrix by remote sensing. Perversely, remote sensing makes extraction redundant. Thus, it is necessary at the outset to recognise that the future analysis may be an extractionless, remote sensing, robotic operation. Although considerable

progress has been made already towards these goals, as a "hands on instrumental analytical chemist" the *modus vivendi* for this monograph was to present classical and modern experiential and methodological data in ways that may be a helpful record and also serve as a transitional reference of methodology to facilitate the advancement of the era of robotic analytical workstations.

Food Sample for Analysis

Foods (and drinks) are nutrient-containing substances that can be metabolised into body tissue and into energy to sustain body tissue. In modern parlance foods are largely solid, and drinks are largely liquid. It is convenient to refer to all nutrient sources as food – the nutrient-carrying matrix – and to consider the removal of compounds from a sample of food as an extraction. However, the English language has many words to express the idea of removing something from the whole. In analytical chemistry, for example, there is no clear distinction between a separation method and an extraction method, and it gets worse because chemists also fractionate, purify, isolate, partition, disperse and distribute components of mixtures. Here, an extraction is thought of as an operation on a sample of food that concentrates the target components, normally by removing them from the bulk of the food sample, often in preparation for further examination such as chromatographic separation. In analytical chemistry, a separation is seldom carried out on the raw material (however, see Chapter 8, Section 2, direct injection), but on an extracted or cleaned up sample for analysis. In addition, there are many procedures associated with extraction that in themselves do not actually remove anything from the sample. These processes are dealt with in Chapter 2 (Sample Preparation for Extraction), and are treated as extraction aids.

The natural origins of human foods are biologically diverse, ranging widely in texture and composition – from nutmeg to oysters. The extremely complex endogenous composition of food is made even more complex in the modern environment where so many extrinsic, additional items – additives such as antioxidants, contaminants from agriculture such as herbicides and industrial adulterants such as hydrocarbons from petroleum – may also be present. This extends the quantitative range of analyses practised by food analysts from the gram amounts encountered in proximate analysis (Section 6) to picogram and even lower amounts of highly toxic contaminants *e.g.* PCBs. To cover more than 12 orders of magnitude requires an enormously diverse armoury of techniques.

Analysis of Foods

It is usually a concern over the chemical composition or contamination of food and the effect this has on its value to the consumer that generates the need for analysis. The quality of food is based on the natural composition, the balance between the nutrient and the anti-nutrient composition. The health and prosperity of early civilisations depended upon their ability to refine their food

supply in the short term by removing toxic materials using extraction methods, or in the long term through crop selection and plant breeding.

History of Food Extraction

Many extraction methods were invented to remove sufficient quantities of toxins (anti-nutrients) from the biological source to make the material acceptable and safe to eat. Notably, nature historically used toxins in sources of human and animal foods to maintain the balance between the survival of the browser and the browsed! These practices were incorporated into the culture of the technology employed in the early analytical laboratories.

The natural processes used to extract moisture in order to increase the "shelf-life" of food and the early uses of extraction methods to concentrate important components, *e.g.* essential oils, formed the bases for methods of analysis as the science of measurement began to develop. Historically, the extraction of bulk components from food made use of physical processes, such as pressure, to remove the juice or oil from the pulverised pulp. Warm air or sun drying of tomatoes or fish extracted sufficient water to reduce bacterial attack to an acceptable level in preparation for storage. Solvent extracts of essential oils from the pulverised plant, seed, or nut were concentrated by distillation in simple stills. Spicy and resinous plants were solvent refluxed in fractionation columns and valuable components separated and extracted in this way. The modern method of supercritical fluid extraction (SFE) uses ultrapure carbon dioxide as solvent, thus eliminating the fear of toxic residues in the extract. Cold-pressing methods are still used to produce high quality extracts of citrus fruits, and hydrodistillation, the steam distillation of an aqueous solution of the food matrix, was practised, especially on powders, from earliest times. There are many other examples where extraction from the bulk material was used to refine our food supplies.

Analytical data defining food quality and the methods used to obtain them have to be validated; several regulatory bodies oversee this process (FDA, FSA, AOAC, FAO, WHO, *etc.*). In 1963, the FAO and the WHO set up the Codex Alimentarius Commission to develop food standards, guidelines and codes of practice. Their web-site is Codex@fao.org. Ultimately, there are definitive methods that can be applied to analysis that provide an acceptable degree of confidence and that are universally recognised. For example, The Canadian Health Protection Branch, Health Canada published *The Compendium of Methods for Chemical Analysis of Foods*, which was extant till 1995 and is currently being updated. The e-mail contact is Xu-Liang Cao@hc-sc.gc.ca. Details of the extraction from the food matrix of the compounds of interest will be given in the method. These data are already correlated and readily available to the practising food analyst and only illustrative examples will be further discussed. It is the historical context, background principles, general practice, and the development of emerging and tentative experimental methods leading to the ultimate automated assay that form the major part of this study.

The ways in which the methodology has been used are illustrated in the examples from the scientific literature chosen to cover a range of commodities and analytes. Modern databases contain huge amounts of information on extraction methods for food analysis and the reader may wish to base further searches for information on keywords found in the appropriate sections of this manual of methods.

Stages in Food Analysis

Stages that may be required in the analysis of foods are:

1. Setting the protocol.
2. Sampling the food.
3. Preparing the sample in readiness for extraction of the chosen analyte or compound of interest (COI), including standardisation.
4. Extraction of the COI.
5. Separation from, or removal of, substances interfering with the detection of the COI in the extract.
6. Detection (recognition or visualisation) of the COI.
7. Identification and/or quantification of the COI.
8. Recording the information.

Items 3–5 are the subjects of this monograph.

Defining the Stages in Food Analysis

Protocol. It is important to have a clearly defined protocol and to adhere to it, so that the analysis can be reported unambiguously, verified by the analyst, and, if necessary, reproduced for verification by other analysts.

Sampling. This is the process of preparing a representative portion of the whole food for analysis. This sample is usually re-sampled by the analyst (the sample for analysis) and may need treatment before the target compound(s) can be extracted. If quantitative results are required, an internal standard (*e.g.* an isomer with similar chemical properties, but distinguishable from the analyte by GC retention time, or an isotope distinguishable by its mass spectrum) may be added to allow any subsequent losses to be compensated for during the analysis.

Preparation of the Sample for Extraction. The definition of sample preparation is ambiguous in the literature, often covering all processes up to and including the separation stage. The definition of sample preparation for extraction here is "the execution of procedures necessary to prepare the original sample for extraction." Such processes include grinding, digestion, and centrifugation. Occasionally, mainly for liquid foods, no preparation for extraction is required.

Extraction. There can be no hard and fast rule, but having entitled this monograph "extraction methods" the definition of an extraction process used in collecting together relevant subjects was "one where a part of the sample is removed from the whole starting sample." It may be the part containing the compounds of interest or it may be an unwanted part being discarded, leaving a less complex and usually more concentrated remainder for further study.

Direct Analysis without Extraction. The analyte can be in sufficient concentration, and free from interference from the matrix, usually in a liquid food, that no extraction stage is necessary. For example, a sample of the matrix can be injected directly into the separation stage, *e.g.* HPLC. This possibility becomes more feasible with the use of guard columns and as the resolving power of the separation and detection stages improve. The use of chromatography–MS and electrophoresis–MS, especially MS^n and HRMS methods means that many potential interferents can be circumvented without the need to remove them by extraction. Alternatively, a colorimetric reaction can visualise and quantify the analyte in a crude extract, as is the case when the biuret reaction is used to measure protein content.

Separation. The term separation is reserved for chromatographic and electrophoretic processes where the main objective is not to remove or extract something for further stages of analysis, but to finally resolve components of mixtures for detection and identification.

Exceptions are made in the case of preparative separation, which was the forerunner of two-dimensional separation, where the fractions are collected for manual transfer to a further stage of analysis. This can be seen as a preliminary extraction – and again with multistage chromatography, where each stage serves to fractionate the mixture, presenting one or every fraction extracted as the input to further stages of analysis. Multistage or two-dimensional chromatography is capable of extremely complex, on-line, automated separations and can be seen as a combined extraction and separation system.

Because the separation stage is not in the remit for this monograph, a more pedantic stance has been taken to draw a distinction between the two stages than is necessary when dealing with the analytical process *in toto*. Nevertheless, on several occasions the separation process has been viewed as a micropreparation process simply to raise the prospects for automated microanalytical methods to be developed.

Detection for Identification and/or Quantification. The signal recorded when a component of the sample is registered (recognised, standardised or calibrated) above a base line, and the signal content is converted into qualitative or quantitative information.

Direct Detection by Remote Sensing of the Food Sample. If the analyte can be recognised (detected) in the food sample prepared for analysis by a sensing

probe, then the required analytical data can be extracted directly without the need for physical, chemical, or biochemical intervention. This can be considered to be "virtual extraction or virtual removal of interference." Such methods have the potential to be rapid, economical in time and resources, and ideal for automation.

Recording the Information. For those wishing to refresh on recording and general good analytical chemical practice, a text such as *Fundamentals of Analytical Chemistry* by D.A. Skoog and D.M. West[1] provides a thorough treatment. Some further discussion of the stages of food analysis with relevance to the extraction techniques that form the major part of the work will follow.

2 Sampling

Introduction

Assuming that the strategic arguments have been addressed and the reason for undertaking the analysis defined, the first analytical procedure is to obtain a representative sample of the bulk material. If the sampling process is inaccurate, all the subsequent, and often expensive extraction, separation and identification stages will be in jeopardy. Sampling is not covered in this monograph, but a few general comments will serve to put into perspective the material upon which the extraction will be made.

There are several different problems that can beset the sampling for analysis procedure. For example, if a new cultivar of broccoli has been created in the greenhouse on a limited experimental scale, and there are only one or two small florets on each plant, it may be permissible only to use a small part of it for, say, glucosinolate analysis. Therefore, high efficiency is required of the extraction and high sensitivity of the analytical methods employed. Even so, the sample represents only a single plant and the results should not be expressed otherwise.

A sampling problem of a different kind is generated when it is necessary to choose a representative amount from a 30-ton truck loaded with carrots from the field; how does one ensure that the relatively small sample of material needed for pesticide analysis is representative of the whole assignment? Also, once the representative sample has been taken, how should it be stored so that no changes in its composition occur until it is analysed? These problems and many more are dealt with in textbooks on sampling and standardisation, for example from the ACOL series, Woodget and Cooper's, *Samples and Standards*.[2]

Standardisation and Validation of Methods

If known quantities of standard reference materials (SRMs) – ideally isomers of target compounds – are added and thoroughly mixed into food samples at the outset (standard addition), subsequent methodology can be calibrated against

losses occurring during handling, to provide quantitative measurements of composition that can lead to the validation of the analytical procedure.

Recovery, Sensitivity and Limit of Detection

Measures of method performance, such as recovery, the limit of detection (LOD), and quantification (LOQ), are generally based on the use of standard addition and on the assumption that the additional standard material behaves like the natural substance in any physical and chemical treatments employed.

As far as the extraction process is concerned, the total recovery specified for the whole analysis includes the efficiency of the adsorbing medium, *etc.*, but, like all other parts of the assay, any losses that do occur are compensated for by the standard addition process. In practise, losses during extraction should be kept to a minimum, and for high sensitivity to be achieved in trace component analysis it is important to have as near a loss-free system as possible. With modern treated surfaces in separation columns and measuring instruments, and with the use of bonded stationary phases, there is less unwanted (irreversible) adsorption. Once receptor molecules of the target compounds have filled all the active absorption sites, any remaining molecules can proceed to the detector. The limit of detection is expressed as the threshold sensitivity of the detector to the remaining molecules, and is given a signal-to-noise ratio, *e.g.* 3 : 1. The LOQ is the lowest concentration of an analyte that can be determined with an acceptable precision and accuracy.

Precision, Accuracy, Reproducibility and Repeatability

Measures of reliability include the extraction stage, and errors of analysis need to be accounted for. Replication of the sampling and standardisation of the procedure is normal when quantitative measurements are being made, and a statistical evaluation of the reliability of this stage will be an integral part of the precision, accuracy, reproducibility, and repeatability of the whole analytical procedure. Most analytical methods provide this information and, therefore, it is assumed here that extraction is one of the processes, but not necessarily the limiting process, represented in the values arrived at for the whole method.

Certified Reference Material (CRM)

The importance of the provenance of the reference material used in the validation process is recognised, for example, by the European Union in the Fifth Framework Programme – the Measurement and Testing Programme. Two recent projects in the food analysis area are DIFFERENCE – Production of high quality CRMs for dioxins analysis in food and feed, and SPECIFIC MIGRATION – CRMs for control of migration testing of plastics for food packaging.[3]

Many CRMs for food analysis are standard matrices for interlaboratory comparisons of candidate methods. FAPAS has been instrumental in running

several trials[4] for the standardisation of data from analytical laboratories worldwide. Trials have been made on pesticides, toxins, veterinary drug residues, trace and nutritional elements, food colours, preservatives, sweeteners, alcohol congeners, fatty acids, nitrate, and proximate analyses.

The preparation of a laboratory reference material (LRM) of beef extract for heterocyclic amines (HA) determination was described.[5] Three levels of HA from 10 to 75 ng g^{-1} were added to the material, which was dehydrated, ground, sieved, homogenised, bottled and labelled for testing for suitability as a CRM in interlaboratory trials.

Measurement Uncertainty

Sample preparation is estimated to be the major stage of an analytical chromatographic procedure and the extraction process can make the major contribution to the total uncertainty of the assay. Therefore, the reader is referred to the *Eurochem/CITAC Guide*[6] and to the "Sample Preparation Perspectives" column[7] for the details on these and the seven hints for analysts:

1. Use adequate working techniques.
2. Use large volumes.
3. Minimise the number of working steps.
4. Make sample and reference measurements in a close time proximity and use the same instrument.
5. Use an internal standard.
6. Prepare an artificial matrix or use a certified matrix reference material.
7. Perform multiple analyses.

Remote Sampling

A modern approach to the automation of the sampling process is given in the "Process Column" article on extractive and remote sampling.[8] Four categories of sampling are given:

1. Non-contact sampling
2. Remote sampling
3. Extractive loop sampling
4. Grab sampling (remote off-line analyser)

Based around optical process spectroscopy, methods 1–3 are realising the objective of turning the whole analysis over to automation. Obviously, when the information required to quantify the analyte can be "extracted" remotely from the starting sample, extraction methods are redundant! There are several examples, *e.g.* using NIR spectroscopy where this is already well established. The authors discuss the state of the art.

In the meantime, it may be helpful to introduce the approach to sampling and sample handling as a prelude to dealing with the extraction processes.

3 Preparation for Extraction (Resumé of Extraction Aids)

Introduction

The raw food material may have to be subjected to some pre-treatment before an extraction can be performed effectively. Some food components are distributed throughout the whole cellular and intracellular structure. Superficial use of an extraction method would be inefficient, and ways of penetrating to the encapsulated or occluded analyte are categorised as pre-treatments or extraction aids. Some analytes are to be found only in specialised tissues that might be dissected from the whole and bulked (concentrated) to form the sample. In general, the preparation is to render the sample easier to extract. The main extraction aids are listed here and amplified in Chapter 2.

Change of Volume

Dilution aids processes where there is plenty of material, but where particulate matter might block filters or membranes. Conversely, trace amounts of analytes may be concentrated to increase the chance of detection.

Removal by dissection, often under the microscope, can enable parts of the food rich in a particular component to be bulked and used as a sample of smaller volume. This is a useful means of pre-concentration of the analyte. Dissection is also employed when interest is focused on only a part of the foodstuff, *e.g.* the seeds of a fruit or the intermuscular fat of a cut of meat.

Change of pH

The isoelectric point (pI) of an ionisable compound is the point at which the anion and cation contents are in equilibrium. A mixture of ionisable compounds, *e.g.* zwitterionic proteins, at a particular pH will often contain positively charged (below their pI) and negatively charged (above their pI) components. Separation can be effected directly by electrophoresis. In general, changing the pH of a food sample can facilitate the release of selected analytes. As an aid to extraction, it is often a prerequisite of membrane methods that the analyte is neutral and therefore a pH change will facilitate the transport of an analyte across the membrane.

Change of Structure (Cell Disruption)

Disintegrate and Homogenise

It may be necessary to break down the bulk structure so that the target components are accessible to the extractant. Very dry and hard foods (<5% moisture) are ground to a powder, *e.g.* in a mortar. Dry foods (<15% moisture) may be comminuted (disintegrated) in a blender, and wet foods liquidised to a slurry or

pulp. Blending or liquidising is often sufficient to render the sample homogeneous on the scale required for the extraction to be complete and reproducible from sample to sample. It is unlikely that the disintegration will release all the analyte, and over-zealous handling may cause decomposition, so a compromise has to be struck.

Biochemical Release

Enzyme hydrolysis (digestion) can be employed to degrade the cellular structure in order to release analytes from the matrix to provide a greater yield. Technology built up for vitamin analysis assumes several different biological states of the vitamin exist, and details the chemical classes from which the compound of interest is to be targeted for release. Mild acid and alkaline hydrolyses are used to release classes of chemical compounds that may be bound to structures or occluded in a chemical bond.

Chemical Release

There are occasions when the whole food has to be totally chemically digested to release the analyte. For the proximate analysis of protein, the food is digested in concentrated H_2SO_4 and the resultant nitrogen (representing the original protein) is converted into $(NH_4)_2SO_4$, which on distillation with NaOH releases NH_3 for steam distillation into a chemical trap of 0.1 M H_2SO_4 for subsequent titration against an indicator.

Microwave-assisted Extraction (MAE)

MAE is a sample preparation step in which internal vibrational energy is provided to the food matrix to release components into the liquid state or at least to render components accessible to extraction, *e.g.* solvent extraction.

Ultrasound-assisted Extraction (UAE)

Ultrasonics is another way of providing internal energy into the bulk of the material to interact with the structure and aid the extraction of components that otherwise would remain immobilised.

Change of State

Some soluble constituents can be treated with a chemical coagulating reagent, causing them to precipitate. In analytical terms, the larger the particle size precipitated, the easier will be the separation by filtration extraction. Small particles block filter beds and extend the separation time. Centrifugation is an alternative means of separation and works well with certain two-phase systems. The two layers are separated by decanting the supernatant phase, leaving the

compounds of interest more concentrated as either the coagulant or the supernatant. If necessary, the reaction product may be converted back into the original compound.

Additional heating, stirring or adding an electrolyte will be required if colloidal suspensions are involved and often the precipitation process will not be simple if coprecipitation occurs, taking down normally soluble material occluded to the precipitating particles. Factors affecting precipitation include, as well as particle size, solubility of the precipitate in the medium, temperature, reactant concentrations, the rate of mixing of the reactants, and the relative supersaturation and the balance between nucleation and particle growth (Skoog and West, 1982).[1]

Simply heating a sample can cause evaporation and, thus, extraction of volatilisable material from the matrix. Evaporation to dryness and condensation of the vapour phase separates solids and liquids and is, if taken to completion, an effective extraction process for stable analytes.

Dissolution will extract solubles for further treatment. Water, as a solvent, is very effective in many assays of solid foods. Heating or cooling for solid/liquid or liquid/solid to change the state is useful for analytes close to their transition point.

Change of Chemical Composition

It is often efficacious to add a chemical reaction into the protocol to avoid interference between the analyte and other co-extracted material at a later stage in the assay. There are many examples of derivatisation to increase the volatility of compounds for headspace (HS) analysis, or to change the retention time (RT) in chromatographic separation.

Flow Switching and Automation

The employment of instrumental methods under computer control is viewed as an extraction aid since processes like on-line flow switching (FS) may be used to effect extractions by diverting unwanted fractions away from the final separation stage. Other automated processes can also aid the extraction, such as continuous flow workstations with robotic arms that carry out several routine sample preparation steps and provide an extract for further study.

Flow Switching for Analyte Extraction in On-line Analyses

Flow switching, also called column switching, is a technique used in chromatography to change the direction of the mobile phase flow, *e.g.* to fill a sample loop with an aliquot from an external flow and then transfer it into the mobile phase flow to the separation column. When FS is used with a pre-column technique, sample loading onto the pre-column can take place with the eluent venting to waste until, *e.g.*, unwanted components with a low affinity for the sorbent have been extracted to waste. Then, by switching the flow to a mobile

phase with greater solubility for the COI, the analytes can be transferred to the analytical column for separation in an automated process. It is possible to effect front- middle- and end-cutting of the adsorbed fraction in this way. These processes are seen as assisting in the extraction.

Automated Preparative-scale GC Injections and Fraction Collection

The use of carousels, automatic injection systems, and fraction collectors provide mechanical assistance in the preparation of samples for separation and fractionation–extraction. Dilution or chemical reaction, *e.g.* derivatisation, may be performed robotically on the sample for analysis and the extracted fractions subjected to further separation.

Miniaturisation

The introduction of benchtop mass spectrometers to replace the floor-standing instruments of the 1960s and 1970s started the move towards small footprint modules for complex, multiple compound analysis. The combination of GC with MS brought further reductions in the overall size of "benchtop" instrumentation. As the number of assays, and the number of analytical steps that are coupled together increases, the need for further miniaturisation continues. Nanotechnology on the molecular scale may be a future development in analytical methodology, but, for now, microchip instrumentation is moving apace, and examples of combined sample preparation, separation and detection on a chip using capillary electrophoresis (CE) technology are given.

4 General Approach to the Extraction of Analytes

Phase Separation

Many foods and food products are natural polyphasic systems and simple phase separation methods may remove unwanted fractions of the matrix. Alternatively, maceration can be used to produce a slurry that may be physically separated into solid and liquid fractions. The common use of an organic solvent to remove certain soluble components from the aqueous food matrix depends on the partition ratio (k) of the analyte between the two phases. If an analyte has a significantly different ratio from that of other constituents, then an extraction is practical. The greater the difference the more likely it is that a single step extraction will produce a clean enough sample for the separation stage. Components of a mixture that have only small differences in k require multiple extractions by the same, or different, methods.

Filter Bed

The simplest form of phase separation is filtration. If there has been a separation of phases so that some of the sample is in the liquid and some in the solid state

then providing that the particles of the solids are greater than the pore size of the filtration medium they will be retained on the filter bed. Filters are defined by their particle retention size and speed of filtration, and a wide range of papers from 2–30 μm, glass fibres from 0.5–2.5 μm and frits of approximately 70 μm, and membranes (nylon, PVDF, PTFE, *etc.* with pore sizes around 0.2–1.0 μm) with speeds between 20 and 2500 s per 100 ml are manufactured to accommodate the extraction. Losses will occur and either standardisation or exhaustive washing is required to retain a quantitative recovery.

Separating Funnel

The distribution of analytes with different partition constants between two immiscible liquid phases enables a physical separation. If, after a time for equilibration, the amount of the COI in one phase greatly exceeds the amount in the other then a single-stage extraction in a separating funnel might be sufficient to separate it from interferents. This applies especially to the mixing together of a liquid food and a solvent in a separating funnel. Careful choice of solvent can extract different chemical classes quickly and efficiently.

Filter Funnel

If solid has formed in a liquid food, or if a comminuted food matrix contains sufficient liquid phase, the use of a suitable porosity filter paper will extract the solid, and purify the liquid food, for further study. Filtration processes are involved in most of the sample preparation for extraction protocols encountered in food analysis. Compensation against analyte loss is necessary.

Büchner Funnel

A range of sizes and porosities of fixed glass frits, and the ability to add a medium such as Celite as a filtration aid, makes the Büchner funnel invaluable in food analysis.

Centrifuge

The soluble and insoluble components of a food matrix can be separated by centrifugation, after which an extraction can be made by decanting the supernatant liquor. Components of the sample may be deliberately precipitated and separated by filtration or centrifugation from the solubles in the supernatant liquor. Many food assays contain a centrifugation step, and an interesting application is in the preparation of bacterial cultures for polymerase chain reaction (PCR) analysis:

Buoyant Density Centrifugation (BDC). In food microbiology, BDC is used to prepare samples for PCR analysis. The density gradient was externally

calibrated using density marker beads (Pharmacia Biotech, Uppsala, Sweden) and the buoyant densities of bacterial strains and food homogenates were determined by centrifugation in a continuous density gradient. 1.7 ml 50% stock isotonic solution [100% stock isotonic solution: 100 ml BactXtractor™ (QRAB, Uppsala, Sweden), 850 ml NaCl, and 100 mg peptone] was placed in a 2.2 ml test tube and 0.5 ml of analyte layered on top. Alongside the analytical tube, the calibration tube was filled with 0.5 ml peptone water and 5 µl each of 7 different density marker beads placed on the gradient medium (50% stock isotonic solution) surface in place of the analyte. Tubes were centrifuged at 16200*g* for 7 min and buoyant densities determined against a calibration curve.[9] The method was optimised and, after centrifugation, the supernatant was removed, leaving the bacteria at the bottom of the tube. The tube was filled with phosphate buffer saline and the bacteria pelletised at 9500*g* for 5 min, the upper layer again removed and 75 µl volume containing the bacteria was taken for PCR analysis. During development of this method, processed brawn, raw beef and raw minced pork were used as samples. (Summarised from ref. 9 with permission from Elsevier)

Decanting

When centrifugation, precipitation, simple settling or sedimentation has separated the liquid and solid phases, the liquid phase can be decanted to extract the soluble components. When distribution ratios are less distinct, multiple extractions, multistage separations or more complex procedures such as countercurrent distribution are necessary.

Distinction between Separation and Extraction

Continuous partitioning from a mobile phase while it is passing over or through a stationary phase is a chromatographic separation in analytical parlance. Now that solid-phase extraction (SPE) methods are important in the preparation of samples for subsequent chromatographic separation, it is convenient to consider pre-separation methods as extraction methods and separation methods as those operated with on-line detection of the components (fractions) of the sample mixture. This is only a guide, since it would be feasible to couple a detector to some extraction methods, but the prime objective of an extraction is to simplify, or purify, a sample for further chromatographic and spectroscopic examination. The distinction is blurred by preparative-scale chromatography performed to concentrate and separate components of a complex mixture, the result being a number of distinct fractions for further study.

Most extraction methods employ some form of partitioning such that a component or components of the food are removed from the matrix. Processes such as distillation, solvent extraction, SPE and countercurrent distribution are partitioning processes. Normally, components that are extracted can also be concentrated, either by selective adsorption and extraction in a small volume of a different solvent or by solvent evaporation where the analytes are significantly less volatile than the solvent.

Consider the Resolution of the Total Assay

The objective for the extraction step is to remove as much of the bulk matrix as is necessary for the analytes to be recognised and/or quantified unambiguously in the subsequent steps in the analysis. At one time, this was a rigorous requirement, but as the separation and, particularly, the detection stage increased in resolving power there was less need for absolute purification at the extraction stage, and therefore it was necessary to evaluate the whole procedure in order to optimise the performance/analytical effort factor. Conversely, as the resolving power of sample preparation methods improves, less resolution is necessary at the later stages of the analysis, again requiring optimisation to avoid overkill. In designing a screening method for carbamate and organophosphate pesticides in food matrices, the use of an electrochemical bioassay meant that a lyophilised solvent extract of homogenised food could be used directly, whereas for GC and HPLC analysis an additional C_{18} SPE and a salted out organic extract was required.[10]

The use of ECD-GC and NPD-GC for pesticide analysis elicited the comment that a simple UAE with acetone–DCM over anhydrous NaCl was sufficient and no further clean-up was necessary (Navarro *et al.*, 2000, Chapter 2, ref. 16).

High-resolution Detection

High-resolution mass spectrometry detection can often provide additional resolving power for would-be interferents at the end of the assay. Small differences in the fractional mass of ions detected may be specific to the target compound and not to isobars (ions of the same nominal mass but of different atomic composition). In addition, using MS^n techniques provides "dry" ion separation analogous to "wet" chromatographic separations as on-line detection procedures. Therefore, it may be unnecessary and inefficient to spend time finessing the removal of potential interfering substances at the extraction stage, making it more important to design the assay as a whole. Optimisation of the corporate parts of an assay to obtain the most efficient use of resources can be a difficult and time-consuming operation. Consequently, where researchers are known to have gone to the effort to report their experiences at optimisation, they have been referenced here. It is a sinecure that time spent in successful optimisation, leading to a decrease in analysis time, is recovered handsomely in the repetitive routine assay.

Special Case of Labile Samples

When the sample is sensitive to light or heat, special extraction conditions have to be used. It is mandatory to work in the dark at reduced temperatures when handling, *e.g.*, carotenoid samples.

Special care is needed when analysing cooked foods containing labile compounds. Many of the nutrient, pigment, and vitamin values change during the cooking processes and, therefore, the state of the cooked food, or the details of

the cooking process, have to be added to the description of the analytical protocol. Additional problems occur, especially for nutritional assays, when oil is added during the cooking process.

Where carotenoids are concerned, in fresh fruits and vegetables, their biosynthesis continues during storage and can cause errors when raw and cooked foods are compared. For these and other precautions and methods of calculation for labile components, the paper by de Sá and Rodriguez-Amaya is recommended reading.[11] For carotenoid extraction from cooked foods, they preferred to disintegrate the sample with cold acetone in a mortar rather than in an electric blender, and for raw foods an acetone pre-treatment in an ultrasonic bath for 20 min was used.

Other stages in the extraction of carotenoids included processes listed here and explained in the appropriate chapters later:

1. Stir-fried material cooled in a freezer for 2 h to solidify the oil.
2. Filtered in freezer using cold glass-sintered funnel.
3. Partitioned with 10% ethyl ether in petroleum ether.
4. Saponified with equal volume of 10% KOH in MeOH, added to petroleum ether extract containing 0.1% BHT (mixed at room temperature in the dark).
5. Washed.
6. Concentrated in rotary evaporator.
7. Dried under N.
8. Redissolved in filtered acetone.

Classification of Plant Crops for Extraction

The *Codex Alimentarius Commission* has classified plant crops into 24 botanical types. This may be a useful record for the food analyst because it may help to categorise extraction methods by commodity.[12] This was addressed and 6 groups of plants have been recognised and classified.[13] Briefly, the classes are:

1. Root and bulb vegetables.
2. Low chlorophyll and oil content fruits and vegetables.
3. High chlorophyll plants and crops (excluding high oil content commodities).
4. Dried fruits of high sugar content.
5. Dried crops of low oil content (that can be powdered).
6. High oil content crops.

Classification of Foods for Pesticide Analysis

In the area of pesticide analysis, food materials have been classified according to the solvent system used for their extraction.[14] Groups I and II, vegetables, fresh fruits, whole milk, green cheese, eggs and meat are extracted in acetone, while groups III and IV, cheese, dried legumes, wheat meal, pasta, rice and bread, require acetone–water. For a more comprehensive review of this

classification system for pesticide analysis, consult Tekel' and Hatrík (1996) (Chapter 8, ref. 77).

5 Resumé of Extraction Methods

Introduction

Within the general principle of partition, four physical processes have been recognised in the extraction of analytes from foods: solvation, distillation, adsorption, and diffusion. All other associated processes: percolation, filtration, precipitation, microwave radiation, enzyme hydrolysis, *etc.*, which assist in the release or removal of components from the bulk material are considered to be extraction aids and are dealt with in Chapter 2.

Partition (Chapter 3)

Introduction

Partition is the fundamental process whereby a chemical compound in a food matrix transfers to an extractant. Partition constants quantify the efficacy of the extraction.

Partition–Extraction

If two compounds are soluble in two immiscible solvents to different extents, by mixing the two solvents containing the compounds, until a dynamic equilibrium is created, the compounds will be distributed between the solvents according to their partition or distribution constants. In practice, solvent pairs can be chosen so that, at equilibrium, the solutes (analytes) are substantially separated when the solvents are separated. This method of extraction is common in the food flavour industry and has general applications in food analysis.

Gas/Liquid, Liquid/Liquid, Solid/Liquid Partition

GLP, LLP and SLP are terms defining the states of matter involved in the distribution. The time taken to establish equilibrium between the two states varies considerably with the composition of the binary system.

Microdiffusion Extraction (MDE). Volatile components evaporate into the headspace around foods approximately according to their air/water partition constants. The temperature may be raised to increase the rate of (a) the formation of volatiles from involatile precursors and (b) the rate of their vaporisation from the liquid state. Volatiles are then concentrated by condensation at a small volume external site or trapping in-line chemically for subsequent controlled desorption. In a way, the natural evaporation process is a microdistillation, or a microdiffusion, of molecules that can enter the gas phase. If time is not important, microdiffusion as a method of extraction is effective and cheap.

Solvation (Chapter 4)

Solid/Liquid Extraction. Solvent extraction is a particular case of partition. If an immiscible solvent is added to a comminuted food sample and the sample shaken, first the particles swell by sorption and capillarity and then diffusion from the solid into the solvent occurs for any food components soluble in the solvent, and at equilibrium they will be present in the solvent in concentrations proportional to their partition constants. In favourable cases, most of the component can be extracted into the solvent. In special cases, hydrolysis will help to increase the release of otherwise unavailable components. The extraction process is governed by:

1. Nature of the solvent.
2. pH of the medium.
3. Particle dimensions.
4. Temperature.
5. Volume of solvent.
6. Number of extraction steps (3×20 ml rather than 1×60 ml).

Matrix Solid-phase Dispersion (MSPD). MSPD is a new approach to the optimisation of the extraction and clean up, *e.g.* for multi-residue methods (MRMs) in pesticide analysis. The finely dispersed food matrix is placed in a column and mixed with a solid-phase adsorbent such as Florisil, and the target compounds eluted selectively from the dispersed sample with organic solvents.

Sub-critical Fluid Extraction. Solvents raised to temperatures and/or pressures near to the critical region exhibit properties conducive to efficient solute extraction. Several methods are available for food analysis:

1. Pressurised Liquid Extraction (PLE). The efficiency of the extraction increases as the temperature and/or pressure of the liquid extractant approaches the supercritical region.
2. Subcritical water extraction (SWE). Hot-water extracted analytes mix under pressure with an organic solvent. The mixture is then cooled rapidly and the water enters a polar, sub-critical state and partitions with the organic solvent.

A closely related method is,

3. Pressurised Hot-water Extraction (PHWE). The dielectric constant of water decreases as the temperature increases, and non-polar analytes dissolve easier in low dielectric constant solvents. This method is finding uses for contaminant analyses.

Supercritical Fluid Extraction (SFE). A supercritical fluid is similar in properties to a dense gas. The use of non-toxic CO_2 as the extractant has been in use for many years on commercial and analytical scales.

Distillation (Chapter 5)

If food constituents can be volatilised without decomposition, then they can be concentrated by condensation into an extract. This extraction is effected in practice through the process of distillation. Historically, volatilisation, distillation and fractionation are associated with the production of food essences. More recently, the analysis of food flavour compounds has used these processes, and the culture of chromatographic separation is founded on these principles. The Theoretical Plate (TP) is a concept of separation efficiency in which a TP is considered to be a volume in a fractionating column, or a chromatographic column, large enough in which to achieve equilibrium between the mobile and the stationary phases. At equilibrium, solutes will be distributed between the two phases according to their partition constants. This volume reduces as the efficiency of the column increases. A reflux fractionation process can be used to separate substances that have sufficiently different boiling points and therefore condense at different places (heights) in the reflux condenser. The width of the band occupied by the condensed material constitutes a plate, and the more efficient the column the narrower the plate. Hence the concept of the height equivalent of a theoretical plate, (HETP, or simply *H*).

Steam Distillation (StD). Since many foods contain water, and many more are prepared for eating by cooking in water, steam distillation is a very important process in food science. In food analysis, it serves to extract volatile materials to an external collector for further analysis.

Organic Solvent Distillation–Extraction. Mainly used for the extraction of water from food samples, but, in principle, a higher boiling non-polar solvent volatilises a lower boiling polar solvent, carrying it over during a distillation. If the polar solvent is the more dense then it can be collected below the non-polar distilling solvent and collected into a burette for quantitative measurement. [see Dean and Stark distillation].

Simultaneous Steam and Organic Solvent Distillation–Extraction (SDE). The Likens–Nickerson steam distillation/solvent distillation–extraction method has found many applications in flavour analysis. A steam distillate of the volatile material from a food sample co-condenses with the vapour of an organic solvent. The mixed condensates separate, with the denser material below, and return to their respective boiling flasks. Thus, after an hour or two the condensable, organic soluble volatiles are transferred continuously from the water vapour to the organic vapour and concentrated in the organic solvent distillation flask.

Countercurrent Distribution. Continuous agitation of a binary phase system (two immiscible solvents), such that one solvent moves in a direction opposed to the flow of the other (countercurrent), will enable equilibria to be established

frequently between the solvents, allowing solutes to be distributed effectively in concentrations proportional to their partition constants.

Sweep Co-distillation (SCoD). An inert gas at high temperature is used to sweep out volatilisable material for downstream condensation from a sample mixed with a solid packing in a glass tube.

Adsorption (Chapter 6)

A very useful physical phenomenon is the adsorption of molecules to solid (or liquid) immobilised particles. The reversibility of the adsorption defines the type of extraction or separation that can be effected. A one-off irreversible extraction can remove unwanted material so that a less strongly adsorbed analyte can be eluted for further treatment. However, if the analyte and the interferents have different affinities for the adsorbent then reversibly adsorbed compounds can be eluted sequentially in an extraction protocol. The chromatographic technique is the vehicle used in modern analytical chemistry to achieve multiple equilibration steps – and hence separations – according to the distribution constants of the solutes. In solid/liquid chromatography, the distribution constant (K) is the ratio of the concentration of analyte in the stationary phase (C_s) to that in the mobile phase (C_m).

$$K = \frac{C_s}{C_m} \tag{1.1}$$

The value of K for analytes determines their order of elution. Low K analytes elute earlier than those with higher values. In terms of an extraction protocol, a large difference between the K values of compounds in a mixture may be used to design a separation/extraction strategy.

Solid-phase Extraction (SPE). The rules of liquid chromatography apply to SPE. A tube filled with an adsorbent powder is wetted with a solvent and then a sample in the same solvent is applied to the top of the column. Either the compound of interest washes through with the solvent and the contaminants are retained on the column or the COI is retained on the solid phase while some contaminants elute with the solvent. Changing the nature of the solvent may be used to elute selectively other contaminants adsorbed by the solid phase. Finally, the COI may be concentrated by being eluted in a small volume of a suitable solvent. There are six general categories or modes of extraction.

1. Adsorption
2. Bonded phase partition
3. Normal phase
4. Reversed phase
5. Ion-pairing
6. Ion-exchange

Liquid foods may be applied directly to the adsorbent phase and washed through to leave the analytes adsorbed for subsequent elution (Holland *et al.* reference 38 in Chapter 6).

Solid-phase Microextraction (SPME). Utilises the same principles as SPE, but in practice is a microprobe bearing a support-coated liquid phase on a fused silica fibre in a stainless steel sheath (needle), allowing the retracted microprobe to be injected into the heated inlet zone of a gas chromatograph. Fibres can be immersed in some liquids and suspended in most headspace environments above liquid and solid samples. Volatiles and semi-volatile substances are adsorbed on the bonded stationary phase and desorbed by heating, normally in the GC inlet, or eluted with a solvent, normally in the LC inlet, where they will be separated for further analysis. SPME is a solventless extraction technique providing on-line transfer of the extract to the separation stage.

Multidimensional Solid-phase Chromatography in the Extraction Mode. Most solid-phase chromatography techniques can act as extraction systems:

1. When on-line dual chromatography is in use, the first separator acts as an extractor for the second stage process.
2. As off-line separators when combined with fraction collection of the eluent.
3. When a guard column is used to protect the main analytical column. The guard column extracts unwanted components of the sample or the mobile phase.

Chromatographers will be familiar with "heartcutting" of a fraction from one column and transfer to a second column, usually with a different polarity phase, to take full advantage of the two-dimensional separation. For many years the 2D approach was accomplished through stainless steel switching valves, but problems of dead volume and extra path length limited the resolution of the on-line system. Off-line methods were slow and liable to sample degradation during the transfer. The valveless switch was introduced in 1968 that used a pressurised auxiliary gas flow to balance and, when required, re-direct the carrier-gas flow.[15] Recent applications use this principle and apply modern pressure control and sensing systems. The outcome is the automated transfer of selected fractions from column 1 on to column 2 for final separation and detection. Together with modern software-controlled decision making, 2D analytical methods are feasible for automated assays.

Special Case of Preparative-scale Chromatography. Large-scale chromatography has always been used in conjunction with fraction collectors to separate components for further study. With modern detectors having higher sensitivity, smaller amounts of purified material that are sufficient for subsequent high sensitivity separation stages can be made available.

High Concentration Capacity Microextractions. Methods such as stir-bar sorptive extraction (S-BSE) increase the capacity of adsorbent over that used in SPME to extract analytes from aqueous food matrices. Like SPME they are solventless and capable of on-line operation. However, the increased amount of extract requires longer to desorb than is possible during thermal desorption in the inlet to a GC, if high resolution is to be maintained. Therefore, purpose-built desorption units are commercially available. Desorbed material is cold trapped and re-concentrated for injection into the chromatographic system.

Diffusion (Chapter 7)

Introduction. The diffusion of molecules or ions from a point of high concentration to one of lower concentration governs the movement of analytes across membranes in processes like dialysis, permeation, *etc.* Fick's law governing the mass transfer states (Equation 1.2):

$$M_s = -DA\frac{dn}{dx} \tag{1.2}$$

where, M_s is the mass of analyte carried across an area A of a surface normal to the direction of diffusion per second, and n is the analyte concentration at an arbitrary point x. Classically, membrane separations were viewed as diffusion processes, and have been segregated here under this heading.

However, in practice, a complex physical relationship exists between aqueous food matrices and membranes that permit the passage of certain components through their structure. Membranes of porous impregnated or non-porous structures are used to separate two liquid phases, and therefore adsorption and solvation processes will also affect the mass transfer. In general, the molecular size determines the permeation selectivity of the membrane.

Permeation and percolation processes involving solution and adsorption can be used to great advantage in the extraction of chosen components. Dialysis, which involves both molecular size and ionic charge, can be valuable when coupled to ion separation methods such as CE[16] and capillary ion chromatography (CIC).

Microporous Membrane Liquid/Liquid Extraction (MMLLE). If a thin hydrophobic microporous membrane separates two immiscible liquid phases, *e.g.* one aqueous and one organic phase, solutes can undergo mass transfer.

Membrane-assisted Solvent Extraction (MemASE). The technique uses a sample vial into which a membrane bag attached to a steel funnel insert is fitted under a septum-carrying metallic crimp cap such that a few microlitres of solvent may be placed in the membrane bag and the bag immersed in a volume (*e.g.* 15 ml) of aqueous sample. Diffusion of hydrophobic analytes into the organic solvent is sampled by hypodermic syringe for large volume injection (LVI) into a GC.

Sorbent Impregnated Membranes. Chemically modified membrane surfaces are "tailored" to suit the extraction. A specific chemical group can be impregnated to provide a high degree of specificity to the transport across the membrane. Ion exchange membranes have positively or negatively charged groups covalently attached to the polymer.

Supported Liquid Membrane Extraction (SLME). SLME is a three-phase extraction technique where two aqueous phases are separated by a thin, porous hydrophobic membrane carrying an organic liquid by capillary action.

Pervaporation. The donor liquid flow may be a fruit juice, and pervaporation involves the diffusion of gaseous substances dissolved in the aqueous phase across a hydrophobic membrane into the acceptor stream.

Dialysis. Dialysis separates sample components depending largely on their size relative to the pore size of the membrane, but other factors relating to the donor and acceptor liquids and physical properties of the analytes will need to be considered. In practice, various modes of operation have been described.

Osmosis. Osmosis is the passage of analyte molecules from a less concentrated to a more concentrated solution through a semipermeable membrane until both solutions are at the same concentration. Osmotic pressure is the pressure necessary to prevent the movement of molecules across a semipermeable membrane to establish equal concentrations on both sides. Typically, water molecules are extracted from liquid foods into concentrated brine solution.

Filtration. The most common method of liquid/solid phase separation effecting an extraction of soluble from insoluble constituents of a food matrix is filtration. The matrix may contain a natural binary system or the food can be comminuted to form two phases. In chemical analysis there are many materials that are permeable to liquids that act as barriers to solids. In practice the porosity of the barrier defines the completeness of the phase separation, and suspended particulate material may inadvertently pass through high porosity filters. Conversely, in practice, low porosity filters block too quickly to be effective. Modern membranes have been developed to produce a working compromise for many analytical purposes.

6 Proximate Analysis of the Major Food Components

Introduction

Proximate analysis of a food sample determines the total protein, fat, carbohydrate, ash, and moisture reported as the percentage composition of the product. There are food composition tables that contain proximate analyses for a large

number of established foods, and as new food items are added to our shopping baskets their proximate compositions are added to the database, periodically, in supplements.

Data contained in food composition tables and the analytical methods used to produce these data are continually under review[17] and regulatory bodies publish modifications as they reach a level of general acceptance and reliability. The quality of the assays and the definition of the composition (*i.e.* which components are included in the measurement) vary. The diverse range of analytical methods used introduces small differences among the compositional values that require the source to be identified and RSDs to be reported with the data. Extraction methods used in proximate analysis of the major constituents of foods and food products are outlined here and developments are discussed in subsequent chapters. Preparation methods used in conjunction with proximate analyses are described in Chapter 2.

The subject is approached from the classical viewpoint and updated from the recent literature.

Total Protein

The total protein content of a food sample is estimated as total nitrogen (*e.g.* the Kjeldahl method) after digestion, salt neutralisation and titration of the ammonia released against standard acid. A conversion factor is applied to calculate the total protein. Some functional groups, $-NO_2$ and $-N{=}N-$, do not react and need further treatment if their omission will make a significant difference. Even the classical Dumas' method gives different figures for some foods compared to the Kjeldahl method. There are now many modifications that have been developed to cope with a wider range of food matrices.

Total Carbohydrate

There are disputes about what should be included in the calculation of carbohydrate content. The chief difference lies in the reporting of "total" carbohydrates, made up of monosaccharides (sugars) and polysaccharides (starch and cellulose, including soluble and insoluble fibre). Should fibre be included or not? Some analysts report fibre separately and others include it along with the available sugars and starches to give total carbohydrate. Current qualitative and quantitative interest in fibre for nutritional marketing and food labelling requires a separate figure to be available anyway.

A further anomaly lies in the practice of reporting total carbohydrate as the difference, after summing the quantities of the other components. The analytical implications of these uncertainties are discussed in relation to extraction methods.

Total Lipid (Fat)

Total lipid (fat) content may be calculated simply as the material extracted into diethyl ether. However, there are concerns over the availability of the many

chemically different forms of fat and at least a digestion of the protein and carbohydrate would ensure the efficient release of fat from the tissue. Modern solvent-extraction methods are employed to improve the reproducibility, but there are still discussions about the nature of the lipids extracted under the different conditions. In addition, food labelling requirements entail further separation into saturated, polyunsaturated and monounsaturated fractions. Furthermore, recently, the omega 3 fatty acids have been reported separately owing to their importance in healthy foods.

Moisture Content and Total Solids (Ash)

Moisture is measured as a mass difference after dehydration, and total solids or ash is recorded as the material remaining after the removal of all vaporisable material by high temperature combustion in a furnace (*e.g.* at 500 ºC). Moisture may also be determined by chemical reaction. Again, the debate continues over the definition of extractable (or reactable) water.

Water Content – Direct Methods

Azeotropic Solvent Distillation

Water is the major component of most fresh foods and, therefore, the determination of moisture content defines the dry matter composition, a more quantitative starting point in the measurement of the nutritional value of the food. The Dean and Stark azeotropic solvent distillation–extraction method has been in use for many years (Chapter 5). The water is co-distilled by the solvent, *e.g.* toluene, and condensed into a side arm for volumetric estimation. The use of large amounts of organic solvents and the long time taken to reach a constant reading of the water distilled over has motivated the development of alternative methods.

Mass Difference

Evaporative Heating (Dehydration). A simple method of measuring moisture content is to heat the food to dryness by evaporating the water into the atmosphere (oven method) – a direct form of total volatile extraction – and measure the loss in mass. The temperature of evaporation has to be carefully chosen so that thermal decomposition of labile substances is minimised to avoid adding to the volatile loss, assumed to be water. The long time taken to reach a constant residual weight has stimulated the search for other methods.

Carter-Simon Moisture Meter. The Carter–Simon moisture meter is operated at 150 ºC. Approximately 7 g finely ground sample are heated in an oven for 20 min, followed by cooling in a desiccator for 10 min. The loss in weight is "calibrated".

Rapid Radiant Heating. Infrared and microwave drying methods are faster and more reliable since modern automated instruments remove inconsistency.

Desiccation

Another simple method is to place the wet sample in a desiccator with a strong desiccant, such as P_2O_5, and weigh the sample at intervals until equilibrium is reached.

Chemical Reaction and Volumetric Titration

Karl Fischer Titration. The classical Karl Fischer titration method was developed to answer some of the above criticisms.[18] MeOH reacts with SO_2 in the presence of a base to give the methyl sulphonic acid anion, which is then oxidised by I_2 if free water is present (Equation 1.3).

$$3B + MeOH + I_2 + SO_2 + H_2O = 3BH^+ + MeOSO_3^- + 2I^- \quad (1.3)$$

The amount of iodine consumed is measured by coulometry or volumetric titration and related quantitatively to the amount of free water present. The method is totally dependent upon the comminution of the sample matrix and modern high-speed mixers are used to disintegrate the cellular structure. Some help can be gained from solvent mixtures to dissolve certain foods. A whole book of recipes has become available to cope with multi-component foods containing starch, fat, and protein in different proportions and in different structured forms.

Modern development has been to find less toxic reagents[19] and to automate the sample preparation and titration. The Mettler-Toledo DL 38 Karl Fischer Titrator is an example.[20]

Combinations of Direct Methods

Evaporation and Titration. If the water vapour released during heating is directed through a Karl Fischer titration cell the moisture content may be calculated from the titre.

Evaporation, Hydration and Electrolysis. If the water vapour released during evaporation is passed through a tube of P_2O_5, the phosphoric acid hydration product may be electrolysed and the H_2 and O_2 measured.

Water Content – Indirect Methods

Rapid and Remote Sensors

NMR Spectroscopy. Free water (the H nucleus) is detected and the relaxation time is related to the physical environment of the nuclei. Again, a calibration is necessary for every food matrix.

NIR Spectroscopy. The use of NIR/IR spectroscopic methods introduces the additional capability to measure other components too. The "reading" has to be calibrated for every food matrix. FT-IR and chemometrics enable remote sensors to measure water and other parameters in "at-line" mode in food processing plants.

Microwave Spectroscopy. The wavelength shift and amplitude attenuation depend upon the water content. Averaged multiple wavelength measurements improve the precision.

Total Solid

Evaporative Methods

The residue after the extractable water has been evaporated is called the total solids. It is an essential part of the proximate analysis. Traditionally, oven evaporation methods have been used. By way of example, direct forced-air oven-drying methods were described in a very detailed and well-controlled collaborative study using AOAC method 990.20 for milk and were published in 1989. After collaborative studies over several years a report was made on the performance of the trials.[21] Repeatability and reproducibility improved over time.

In-line Sensors

In the food industry, in-line sensor methods have been developed for the measurement of total solids/moisture content, *e.g.* in continuous fruit juice processing.[22] NIR, guided microwaves and the Maselli refractometer were evaluated against the Abbe refractometer for the continuous processing of apple, grape, pear, apple-cherry and apple-banana juices. The automated methods gave good results over prolonged use without problems of deposit build up.

Total Lipid

Acid Digestion

A simple method of measuring the total fat content of food is to digest a sample in concentrated H_2SO_4 and measure the remaining lipid layer in a graduated tube. The fat content of tree nuts, peanuts, sunflower seeds, avocado and olives was determined using this method,[23] which is conveniently performed using the Gerber Tube method developed for milk lipids (see Solvent Extraction Methods for Lipids in Milk and Cream below). The results agreed with the Soxhlet extraction method.

SFE and Enzyme Transesterification

The rapid measurement of total nutritional fat content of meat used SFE at 12.16 MPa and 50 °C. The extract was transesterified with MeOH and catalysed

by an immobilised lipase. SFC was used to monitor the conversion of the triglycerides into FAMEs and the FAs were analysed by GC. Total fat, saturated fat and monounsaturated fat contents were calculated from the GC data.[24]

Solvent Extraction Methods for Lipids in Milk and Cream

Regularly used extraction methods for lipid analyses of milk are:

1. Soxhlet extraction (hot method)
2. Soxhlet extraction (standard method)
3. Bligh and Dyer extraction
4. Modified Bligh and Dyer method
5. Roese–Gottlieb extraction
6. Gerber method
7. Fleet and Linzell centrifugation
8. Creamatocrit method
9. Babcock method (milk)
10. Babcock method (cream)
11. Modified cream Babcock method
12. Mojonnier method (cream)
13. Modified Mojonnier method

Total lipid determination was the subject of an article comparing five solvent extraction methods, two solvent distillation and three liquid/liquid extraction (LLE) methods of analysis (1–5) above (Table 1.1) for five commodities.[25] These methods are all discussed under the appropriate extraction principle.

The Gerber digestion extraction (method 6) for measuring fat in raw and processed milks was the subject of a collaborative study in 2001.[26] The method is simple and rapid, but the volume of sample used seems to vary. A collaborative study was conducted to determine whether test portions by weight or by volume (11.13 g or 10.77 ml) were better. Eleven laboratories participated in the evaluation of aliquot addition by weight and ten laboratories participated in the evaluation of aliquot addition by volume. The Mojonnier ether extraction (MEE) method was used as the reference method. The fat content of milk samples ranged from 0.96 to 5.48%.

The Fleet and Linzell method (7)[27] simply separates (extracts) the fat from the aqueous layer of the milk by centrifugation, and the Creamatocrit method (8)[28] uses a capillary tube as a microcentrifuge tube, filled with milk, sealed, and spun at 15000 rpm for 5 min. The fatty phase length was measured as a % of the tube length occupied by the total sample. The method was compared to the Gerber method ($R^2 = 0.968$), and was preferred because of the danger associated with the concentrated acid digestion involved in the Gerber method.

The complex nature of the total lipid composition of foods, from non-polar glycerides to polar phospholipids, means that solvent extraction – which is the most common method – has to be effective across a range of polarities. This is made more difficult because lipids bind to proteins (lipoproteins) and sugars

Table 1.1 *Five total lipid determination methods compared for five commodities*
(Reprinted with modification from the *Journal of Food Composition and Analysis*, vol. 14, P. Manirakiza, A. Covaci and P. Schepens, "Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh and Dyer, and Modified Bligh and Dyer Extraction Methods", pp. 93–100, © 2001, with permission from Elsevier)

Method	*Measurement*	*Chocolate powder*	*Milk powder*	*Liquid milk*	*Margarine*	*Eggs*
	Producers content (mg g^{-1})	32	5	36	362	98
Bligh & Dyer	Mean	33	3	27	317	95.5
	RSD (%)	2.0	2.2	3.3	0.4	2.6
Modified	Mean	33.4	4.9	32	282	91.5
Bligh & Dyer	RSD (%)	2.3	0.4	0.5	0.4	1.1
Roese–Gottlieb	Mean	25.6	4.1	11.2	337	41
	RSD (%)	2.5	1.1	1.4	0.7	2.4
Standard	Mean	31	4.1	14.5	349	11(30[b])
Soxhlet[a]	RSD (%)	1.4	2.7	1.5	0.5	1.7(1.5)
Hot Soxhlet[b]	Mean	34.7	4.8	17.4	362	12.5(39[b])
	RSD (%)	2.1	1.7	2.3	0.5	1.2(2.0)

[a] Acetone–hexane (1:4). [b] DCM–hexane (1:4).

(glycolipids) on cell membranes, requiring a particularly polar solvent to remove them. Over the years several extraction methods have been devised, but the above methods represent the main usage in food analysis.

A collaborative study (11 laboratories) was carried out in 1988[29] using the Babcock method with the MEE method as standard for measuring the fat content of milk. Ten laboratories used the modified Mojonnier ether extraction (ModMEE) method and 10 used the Babcock extraction (BE) method. The ModMEE method gave consistently better within- and between-laboratory agreement. The overall mean test value for the BE method was significantly higher (0.021% fat) than that for ether extraction. The modifications of the AOAC Babcock method and the ModMEE method have been approved interim official First Action by the AOAC.

A collaborative study[30] in 1996 was set up to rationalise the analytical methods for the fat content of cream. The ModMEE method for the fat content of cream was developed along the lines of the method for milk (AOAC, OM 989.05). The cream Babcock method (AOAC, OM 920.111B-C) was modified to harmonise with the milk Babcock method (AOAC, OM 989.04) and also to clarify procedural details. Ten laboratories tested 9 pairs of blind duplicate heat-treated cream samples with a fat range of 30–45% using both methods. The ModMEE and Babcock methods for fat in cream have been adopted by the

AOAC. The new Babcock method replaced the AOAC Official Method 920.111B-C.

Recently, the same team published further collaborative studies on a modified Babcock method aligned with the MEE method[31] (Appendix 3).

Total Protein

The methods depend upon the combustion/digestion of the organic matter of the sample to release N for chemical reaction and volumetric estimation.

Lassaigne Test

The Lassaigne Test was devised for the qualitative measurement of N, S, and the halogens. Nitrogen detection is based on the digestion/reaction of the sample with heated Na. If N is present, NaCN is formed. This is reacted with $FeSO_4$ to form the hydroxide, which is precipitated. Heating to near boiling allows the ferrocyanide to form, which with acidified $FeCl_3$ forms the familiar blue precipitate, Prussian blue (Equation 1.4).

$$6NaCN + Fe(OH)_2 = Na_4Fe(CN)_6 + 2NaOH$$

$$4FeCl_3 + 3Na_4Fe(CN)_6 = \underset{\text{Prussian blue}}{Fe_4[Fe(CN)_6]_3} + 12NaCl \qquad (1.4)$$

A modified Lassaigne method was developed for N determination in meat products and baby food.[32] The modified method converts the cyanide into thiocyanate with $(NH_4)_2S_2$ in the presence of an excess of ferric ions in acidic medium for colorimetric measurement. The developers state that the simple spectrophotometric method requires a digestion time of only 15 min compared to the lengthy classical digestion of the Kjeldahl method.

Kjeldahl Method

The Kjeldahl N determination, developed in 1883, depends upon the fact that most organic N compounds are converted into $(NH_4)_2SO_4$ when heated with concentrated H_2SO_4; the exceptions are $–NO_2$ and –N=N– groups, which if present in any quantity should be previously reduced to the amine. Digestion is carried out slowly over a microburner in a loosely stoppered digestion flask to avoid losses by splashing. Figure 1.1 shows the specially shaped Kjeldahl digestion flask and the steam distillation apparatus. This classical apparatus, and others shown later, have been reproduced from a school textbook of organic chemistry to show clearly the principles of the method. The digestion products are then quantitatively rinsed into flask C, treated with excess alkali added from funnel B, and the ammonia generated is distilled over into flask D. From there it is steam distilled and condensed *via* the water-cooled condenser E into standard acid in flask F and in "U" trap G. The combined acid from F and G is titrated

to ascertain the yield of ammonia from which the $\%N_2$ (Equation 1.5) and hence the %protein (Equation 1.6) can be calculated, where x = mls standard acid, and w = the weight of sample.

$$\%\text{N} = \frac{x}{w} \times \text{molarity} \tag{1.5}$$

and

$$\%\text{Protein} = \frac{x \times 6.25}{w} \times \text{molarity} \tag{1.6}$$

With the objective of including the "true" protein level on the label, potentially available protein was calculated from N determined by the Kjeldahl method in an experiment to calculate the true protein content and assess the "*in vitro*" protein digestibility of milk-based starting formulae.[33] True protein was calculated as (total N − non-protein N) × 6.25. Non-protein nitrogen (NPN) was determined in the soluble fraction after the protein had been precipitated with trichloroacetic acid (TCA) and centrifuged. Digestibility was measured by direct enzyme digestion (pepsin and pancreatin) and defined as the increase in NPN after enzyme digestion.

Development of the Kjeldahl Method

New methods have been developed to replace the Kjeldahl method because of the necessity of carrying out two assays to find the difference between NPN and

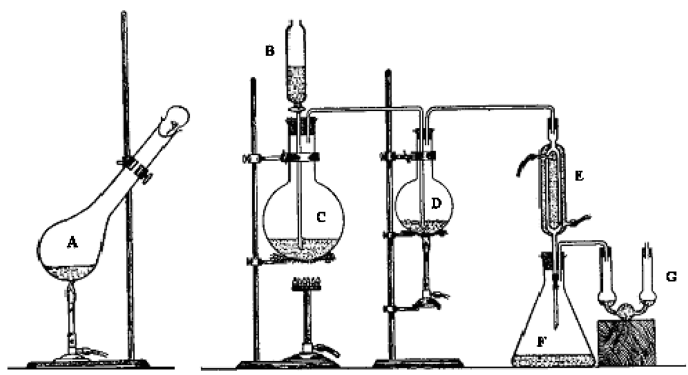

Figure 1.1 *Classical Kjeldahl apparatus.* A. *Digestion flask with a loose fitting stopper over a microburner,* B. *Tap funnel to dispense the 50% NaOH solution into the boiling flask* C *containing the cooled digestion mixture from* A. D. *Steam generator to distil the ammonia.* E. *Water-cooled condenser,* F. *Distillate collection flask containing standard acid, and* G. *"U" trap containing standard acid to retain any airborne ammonia*
(Reproduced from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd, London, 1949, (first published 1933), (see acknowledgements))

total protein N, and the long time taken by the Kjeldahl digestion, distillation and titration analysis.

A partially automated analyser was described which combined the Kjeldahl digestion with the Berthelot reaction for determining N in biological samples.[34] Any deviation in the results was attributed to the high temperature digestion. The introduction of ion chromatography to determine N as the ammonium ion, to replace the distillation and titration stages of the Kjeldahl method, and to speed up the assay, was developed for food and environmental samples,[35] and the use of microwaves for the efficient digestion of food matrices in an open vessel microwave system was developed by Feinberg *et al.* (1993) to reduce the sample preparation time.[36]

A colorimetric method for measuring N in Kjeldahl digests circumvented the distillation and titration stages and reduced the analysis time.[37] The method was used for nutritional studies on dairy products, dry cereals, cereal-based products, legumes, and cooked food mixtures. The need to analyse large numbers of samples also prompted Yasuhara and Nokihara to develop a colorimetric method for ammonia as an alternative to the Kjeldahl method, which was seen as uneconomical and environmentally unfriendly.[38] The Kjeldahl digest was taken as the starting point for a spectrophotometric method of determining total N, and a sampling rate of 14 per hour was achieved in this case.[39]

Kjeldahl Method Compared to Other Methods

In 1987 the Kjeldahl method was compared to the Hach and Kjeltec methods for a wide variety of samples.[40] The results for total N were in the order of Kjeldahl method > Hach method > Kjeltec method. The Hach method was more sensitive than the others to changes in N content, The Lowry method[41] compared well with the Kjeldahl method for wheat protein determination.[42]

The degree of enzymatic hydrolysis of pea protein using trypsin was controlled using the pH-stat method. The solubility of the hydrolysate was tested at 9 pH values, ranging from 2 to 10, and protein content was determined by three methods, the classical Kjeldahl volumetric method, the Lowry and the modified Lowry methods. There was no agreement between the Kjeldahl and modified Lowry method and no agreement between the Lowry and the modified Lowry method.[43]

The protein content of beans (*Phaseolus vulgaris*) was measured by the micro Kjeldahl, Lowry, Bradford, and biuret methods and the results compared.[44] The micro Kjeldahl method gave different values from the other three methods.

The Kjeldahl method and several spectrophotometric methods have been compared for the determination of total proteins in a range of dried milk powders (listed in Table 1.2 in the order of increasing sensitivity), which might replace the acid digestion and volumetric titration of released NH_3 of the Kjeldahl method.[45]

Spectrophotometric methods are said to be quicker and simpler than the Kjeldahl method, but most of them require a preliminary solvent (lipid) extraction and filtration stage. Samples of milk powder (2 g) were shaken with

Table 1.2 *Range of concentration covered by seven spectrophotometric methods of measuring protein in solvent extracts*
(Reprinted with modification from the *Journal of Food Composition and Analysis*, vol. 16, N.K.K. Kamizake, M.M. Gonçalves, C.T.B.V. Zaia and D.A.M. Zaia, "Determination of Total Proteins in Cow Milk Powder Samples: A Comparative Study between Kjeldahl Method and Spectrophotometric Methods", pp. 507–516, © 2003, with permission from Elsevier)

Method	*Concentration range ($\mu g\ ml^{-1}$)*
1. Biuret-340 nm	2000–10000
2. Biuret-550 nm	2000–10000
3. UV-280 nm	200–1000
4. *p*-Chloranil	30–120
5. Lowry	20–60
6. UV-220 nm	9–40
7. Bradford	1–5

18 ml (2:1 v/v) $CHCl_3$–MeOH for 5 min and the resultant solution filtered. The solvent was discarded and the filtrate re-extracted (shaken) for 5 min with 6 ml $CHCl_3$ and 6 ml H_2O, and filtered. If the solvent layer showed a negative biuret test then the solid was dried in preparation for the spectrophotometric measurement of total protein. The Bradford method[46] gave comparable results to the Kjeldahl method for total proteins in skimmed milk and whole milk powders, without the extraction of lipids. The Bradford method for proteins is based on the reaction with the dye BG-250 since peptides and amino acids do not react.

Collaborative Study of the Kjeldahl Method

The Kjeldahl total N method, AOAC Method 991.20 for total N in milk, was published in 1990 and monitored for 5 more years *via* a multi-laboratory quality assurance program (Lynch *et al.*, 1997).

Dumas Method

Dumas' method is based on the decomposition of compounds to CO_2, H_2O, and gaseous N by heated CuO and a bright Cu spiral, the nitrogen being collected over a solution of KOH. Again the diagram from Sherwood Taylor's *Organic Chemistry* illustrates the method (Figure 1.2)

The organic sample is mixed with an excess of fine CuO and inserted into the 1 m long combustion tube, together with a packing of coarse CuO and a spiral of bright metallic Cu gauze – to decompose oxides of N. The nitrometer contained

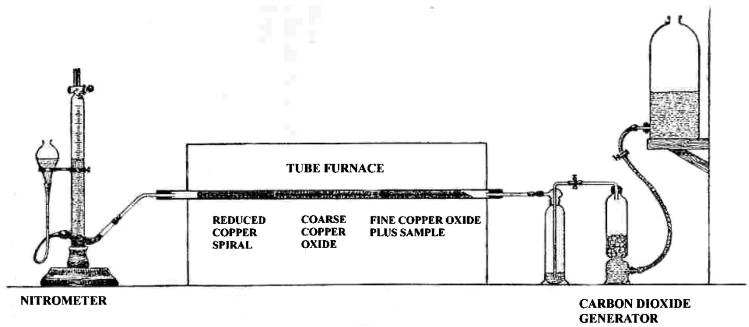

Figure 1.2 *Dumas' method for determining nitrogen. The CO_2 generator is coupled to the inlet of the combustion tube and the outlet from the tube goes to the nitrometer* (Reproduced from F. Sherwood Taylor, *Organic Chemistry*, William Heinemann Ltd, London, 1949. (First published in 1933), (see acknowledgements))

a 40% solution of KOH. CO_2 was used to purge the tube of air while the furnace was heated. Water and CO_2 produced by the combustion of the organic sample were adsorbed or condensed and the N gas was collected in the manometer and the volume recorded and corrected for NTP.

Modern Automated Analysers. The Dumas oxidative combustion system (Foss/Heraeus by Foss Electric (UK) Ltd.) is shown in Figure 1.3, and the schematic form in Figure 1.4.

The ground sample is placed into a small steel crucible, weighed on-line and the crucible placed on the sample chain for insertion midway in the combustion tube. Corundum balls are used as a spacer and the sample is heated by (a) a mobile furnace at 1020 °C traversing from the top of the tube to the sample position for a pre-set time before returning and (b) a static heater at 850 °C at the bottom. The CO_2 carrier gas is supplied at 1.35 bar. The oxygen supply is adjusted (from experience) to suit the composition of the sample and flows directly over the sample while it is being heated. The emerging gaseous mixture is passed through several furnace tubes containing adsorbents and reactants to remove sulphur oxides ($PbCrO_4$), provide post-combustion (CuO/Cu_2O + Pt/Al_2O_3) to complete the oxidation of gaseous products, hydrogen halides and halogens, reduce with Cu any oxides of nitrogen to N, and to remove of any residual oxygen (Cu – reduction tube) and moisture (P_2O_5).

The CO_2/N_2 mixture is measured in Chamber 1 of a thermal conductivity bridge against a reference flow of CO_2 in Chamber 2. The nitrogen content is given by difference.

The LECO FP-428 Dumas combustion analyser was used to measure total N in milk.[47] Suggestions were made for improvements. However, for milk, the Dumas N and Kjeldahl N methods showed good correlation.

The modified automatic Dumas and the traditional Kjeldahl methods were compared for the determination of nitrogen in infant food.[48] The results were

Figure 1.3 *Dumas method instrumentation manufactured by Foss Electric (UK) Ltd.*

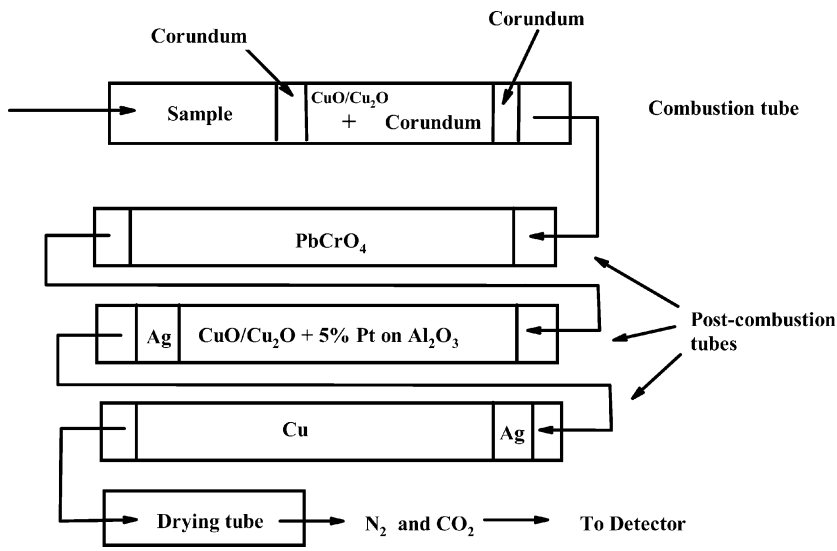

Figure 1.4 *Schematic drawing of the Macro N analyser*
(Configured from the analysis sheets of the Rowett Research Institute, Bucksburn, Aberdeen, Scotland, with permission)

similar but the Dumas method was, *inter alia*, much faster. The two methods were also compared for the application to foodstuffs in general.[49]

Collaborative Studies of Total Nitrogen Methodology

An interlaboratory study (11 laboratories) was conducted to compare the Kjeldahl and Dumas methods for routine analysis of proteins in dairy products (milk, skim milk powder, whole milk powder, whey protein concentrate, infant formula, casein, caseinate, two reference compounds (glycine and EDTA), and a secondary reference (skim milk powder).[50] The two methods gave similar values. The Dumas relative repeatability and reproducibility standard deviations were consistently about 0.35 and 0.75%, while Kjeldahl values declined generally with N content and were significantly larger. The conclusion was that Dumas' method needed Codex Alimentarius status as a recognised test method.

Interlaboratory (15 laboratories) performance was measured for a modified and optimised version of the Kjeldahl method (AOAC 920.123) for the total N determination in different textures of cheese.[51] Crude protein ($N \times 6.38$), g per 100 g levels from 18 to 36% were tested and material homogeneity, size and transfer of sample, recoveries and the modified AOAC method 991.20 were considered. As a result of the statistical data collected on 991.20, the trial directors recommended that the modified method be adopted First Action to replace 920.123 for hard, semi-hard and processed cheese.

Biuret Reaction for Colorimetric Measurement

Biuret (Figure 1.5) is formed from substances containing two or more –NH-CO- groups.

Biuret in the presence of dilute $CuSO_4$ gives a characteristic pink that can be quantified. Proteins and related compounds made strongly alkaline with NaOH, in the presence of dilute $CuSO_4$, give this reaction. In a recent example of the application of the biuret reaction (Arogundade *et al.* 2004)[52] examined the effect of salt type and concentration on the solubility of proteins in defatted *Colocynthis citrullus L.* seed flour, using the biuret method described by Gornall *et al.* (1949).[53]

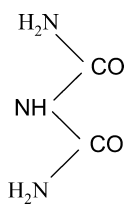

Figure 1.5 *Structure of biuret*

Neutron Activation Analysis (NAA)

NAA has been chosen as an example of a non-destructive remote sensing analyser (Section 3, Andrews and Dallin, 2003). The interest here is in the possible use of this analyser for the virtual extraction or visualisation of N directly in a ground sample in the presence of interference. Early application of NAA to N analysis of corn products experienced problems with activities from other elements in gluten interfering with the induced N activity.[54] Successful separation of the interfering activity led to a method with accuracies equal to the Kjeldahl method. In a recent report of the application of NAA to the measurement of the protein content of ground, oven-dried, homogenised Nigerian foods (soya beans and rice varieties), the nuclear reaction $^{14}N(n,2n)^{13}N$ was used to determine N (and hence protein content) by 511 keV gamma rays.[55] Many other elements can contribute to the intensity of the annihilation peak, and it has not hitherto been considered in the quantitative analysis of the N content of foods. The chief interferences are $^{79}Br(n,2n)^{78}Br$, $^{31}P(n,2n)^{30}P$, $^{31}P(n,\alpha)^{28}Al$, and $^{39}K(n,2n)^{38}K$, and proton recoil reactions from C and O. Once the contributions to the peak at 511 keV had been determined, corrections were applied for each individual reaction, and the total protein data were comparable with those obtained by the Kjeldahl method.

Non-protein Nitrogen

Often it is useful to estimate non-protein N and the Kjeldahl method has been used to measure the nitrogen content of fractions made from foods by various extraction techniques. NPN in pooled sweet and acid wheys was estimated by membrane dialysis at different MW cut-offs, and TCA/phosphotungstic acid soluble N fractions.[56] NPN values varied with membrane porosity, and dialysable N was generally lower than acid soluble N.

Total Carbohydrate

Introduction

Total carbohydrate consists of sugars (mono and oligosaccharides) and polysaccharides (starch and the non-starch polysaccharides; pectin, soluble and insoluble dietary fibre, *e.g.* cellulose and hemicellulose). Total starch (TS) is sub-divided into digestible starch (DS), resistant starch (RS) and dietary fibre (DF). An excellent text on the chemistry of food components covers these topics concisely.[57] The debate about the actual composition of the carbohydrate fraction, the main contention being between the Englyst[58] (enzymatic–chemical) and Prosky (Prosky *et al.*, 1988, Chapter 2, ref. 78) (enzymatic–gravimetric) methods, is about what should and should not be included. This continues,[59,60] and therefore, there is less evidence in the literature of routine carbohydrate analysis and more research and development of analytical protocols, which is discussed under the methods sections elsewhere, *e.g.* biochemical release of dietary fibre, Chapter 2.

Carbohydrate Content by Difference in Proximate Analysis

In a 1990 report, total carbohydrate was calculated as the residue by difference from the total of fat, protein, moisture/solids, ash, and fibre values. A review of collaborative studies of these parameters was made to determine the likely precision of the process. The procedure was judged as having poor precision among laboratories and high variability.[61] Even so, the "by difference" method was used in 2002 for the proximate analysis of Nigerian oil seed,[62] and Menezes *et al*., 2004 averred that most composition databases contain total carbohydrate data calculated by the difference method.

Saccharide Content by GC-MS

Introduction. The food is hydrolysed to prepare a sample for GC-MS analysis. The preparation of the alditol acetate derivative of the neutral mono- and oligosaccharide is popular, but for acidic residues the TMS-methyl derivative is preferred for GC-MS analysis.[63]

Monosaccharides and Oligosaccharides. The use of GC-MS to detect and quantify the volatile derivatives of the hydrolysed saccharides has been the method of choice for many years. A very instructive account of the sample preparation and analysis of alditol acetates by EIMS and CIMS was given by Kamerling and Vliegenthart in 1989.[64] Written from a mass spectrometric point of view, the chapter contains most of the mass spectra identifying the individual sugars and it also explains the fragmentation processes used diagnostically in the interpretation.

The sample preparation for the TMS derivative analysis by GC-MS of honey carbohydrates was to dilute, transfer to the autosampler vial and freeze dry for 4 h.[65]

Polysaccharides. Food grade polysaccharide gums, tragacanth, karaya, ghatti, carob, guar, arabic and xanthan were hydrolysed with TFA and the neutral monosaccharides derivatised for GC-MS. Sample preparation included defatting, starch degradation and protein precipitation.[66]

Starch Content

Introduction. Collaborative research studies by colleagues from CSIC and the Nutrition and Analytical Chemistry Faculties in Madrid have produced valuable working protocols for the analysis of starch fractions.[67,68] The inaccuracy of the "by difference" estimation of carbohydrates in food composition tables prompted them to develop methods for TS and RS.

Total Starch. 2 M KOH was used to dissolve the RS and the total starch was hydrolysed with amyloglucosidase. The released glucose was determined and the TS calculated as glucose $\times 0.9$. Total starch was measured on bread,

spaghetti, rice, biscuits, lentils, chickpeas, beans, frozen peas, boiled potatoes and crisps.

Resistant Starch. The method for RS published as an appendix to reference 68 contains the complete details to perform the assay. Briefly, it involved milling, defatting, and homogenisation as required by the sample matrix. The aliquot for analysis was then buffered (pH 1.5), digested with pepsin under constant shaking to remove protein, re-buffered to pH 6.9, digested with α-amylase to hydrolyse DS, centrifuged and washed (repeat centrifuge and wash) discarding the supernatants; RS was dispersed with KOH with constant shaking, adjusted to pH 4.75 and enzymically hydrolysed to glucose with amyloglucosidase, centrifuged and the supernatants collected, made up to volume, washed and re-buffered. The glucose released was quantified from a standard curve. After a collaborative trial among three laboratories the RS method was applied to rice, spaghetti biscuit, white bread, crispbread, pea flour, lentil flour, corn flakes and All Bran.

Digestible Starch. DS was calculated as the difference between TS and RS.

Applications. An excellent example of the application of modern proximate analytical methods (references 65 and 66) to the improvement in the measurement of carbohydrate fractions (starches) was reported by Rosin *et al.*, 2002.[69] They recognised rapidly digestible starch (RDS), slowly digestible starch (SDSt) and RS, and present methods for the analysis of TS and RS. DS was calculated by difference (TS – RS). RDS and SDSt were calculated from the percentage of 30 and 60 min hydrolysed starch after aliquots taken every 30 min (0 to 180 min) from the α-amylase digestion, further hydrolysed by amyloglucosidase, were used to construct the hydrolysis index.

RS analysis involved removal of protein by incubation with pepsin (40 °C, 1 h at pH 1.5), incubation with α-amylase (37 °C, 16 h at pH 6.9), suspending the sample in 2 M KOH and shaking for 30 min, and incubating with 1 ml (300 U ml^{-1}) amyloglucosidase at 60 °C and pH 4.75 for 45 min. TS, DS and RS values were obtained for whole rice, polished rice, corn, polenta, white spaghetti, white bread, potatoes, peas, beans, lentils and chickpeas.

The same 11 test foods were used in a study by the same group working with Brazilian colleagues to improve the quality of carbohydrate data for nutritional work on Brazilian foods.[70] The nomenclature from the nutritional viewpoint was the indigestible fraction being composed of total, soluble and insoluble fractions. (Summarised from ref. 69 with permission from Elsevier)

Non-starch Polysaccharide Content

Many nutritional groups use the phrase non-starch polysaccharides (NSP) to include all the components known as dietary fibre. In a study of the NSP of Mexican Foods, Sánchez-Castillo *et al.*, 1999,[71] used the method of Englyst *et al.* (1994, Chapter 2, ref. 81).

Figure 1.6 shows a flow diagram of the Englyst method for the release of neutral sugars for GC analysis.

Sample
|
Add 2 mL DMSO; 30 min at 100°C
|
Add 8 mL heat-stable amylase (Termamyl) solution; 10 min at 100°C
|
Add 0.5 mL pancreatin/pullulanase solution
|
30 min at 50°C; 10 min at 100°C
|
Cool to 0°C and add 0.15 mL HCl, 5 mol/L
|
Add 40 mL acidified ethanol; 30 min at 0°C
|
Centrifuge, wash with acidified 85% ethanol;
wash with ethanol; dry with acetone
|
Add 5 mL H_2SO_4 12 mol/L; 30 min at 35°C
|
Add 25 mL water; 1 h at 100°C
|
Hydrolysate → Portion for uronic acids
|
Gas–Liquid chromatography for neutral sugars
|
Add to 1 mL; 0.5 mL internal standard 0.4 mL ammonia solution,
12.5 mol/L 5 µL octan-2-ol; 0.1 mL sodium tetrahydroborate solution
|
30 min at 40°C
|
Add 0.2 mL acetic acid
|
Add to 0.5 mL;
0.5 mL l-methylimidazole; 5 mL acetic anhydride
|
Leave for 10 min
|
Add 0.9 mL ethanol
|
Leave for 5 min
|
Add 10 mL water; 2 × 5 mL KOH, 7.5 mol/L
|
Use top phase for GLC measurement of individual constituent neutral sugars

Figure 1.6 *Flow diagram of the procedure to release neutral sugars for dietary fibre measurement by the Englyst method*
(Reprinted from the *Journal of Food Composition and Analysis*, vol. 12, C.P. Sánchez-Castillo, H.N. Englyst, G.J. Hudson, J.J. Lara, M. de Lourdes Solano, J.L. Munguía and W.P.T. James, "The Non-starch Polysaccharide Content of Mexican Foods", pp. 293–314, © 1999, with permission from Elsevier)

Total Carbohydrate from the Acid Hydrolysate

Total carbohydrate of Nigerian spices was measured by absorbance at 420 nm on the sulphuric acid hydrolysate[72] using the method of Baker and Somers.[73]

Ultrasonics for Non-destructive Proximate Analysis

Mecozzi *et al.* (1999, Chapter 2, ref. 8) described the use of ultrasound-assisted extraction (UAE) at ambient temperature for the hydrolysis of polysaccharides to monosaccharides in the assay for total carbohydrates, and improved upon it by reducing the hydrolysis time further in 2002 (Chapter 2, Mecozzi *et al.*, 2002, ref. 9).

On-line Dialysis/HPLC

When the sugar concentration in a liquid food matrix is at the g l^{-1} level, a rapid automated analysis of individual sugars (and organic acids) using dialysis coupled *via* a flow-switching valve and injection loop to the HPLC has been developed for grape juice, red and white wine, apple juice, and cider (Vérette *et al.*, 1995, Chapter 7, ref. 51). Sensitivity in the g l^{-1} range is readily attainable from these liquid food matrices without the need for additional concentration by SPE.

Automation and Multicomponent Analysis

Remote Sensing

Ultrasonic Velocity Measurement. The notion of extracting compositional information non-destructively from the initial sample was taken a step further with the assessment of fat, moisture, and protein, plus other constituents directly from samples of raw meat mixtures.[74] Ultrasonic velocity measurements were found to increase with temperature in lean tissue and decrease with temperature in fatty tissues. From these values it was possible to predict fat and moisture contents (Figures 1.7 and 1.8).

Under the heading of proximate analysis, the use of ultrasonic velocity measurements to extract information remotely from a ground sample of dry fermented sausage exemplifies the trend towards automation and the circumvention of "wet" chemical methods of extraction. For the food processing industry, such remote data acquisition stations are replacing the need to send samples to the laboratory for analysis.

C, H, N Autoanalyser for Proximate Assays

The measurement of daily nutrient intake requires a method of obtaining proximate analysis on large numbers of samples. The estimation of dietary intake from C, H, N, autoanalyser data has been described.[75] The novel use of the autoanalyser for total fat content was correlated with Soxhlet ether extraction data ($n = 50$; $R = 0.979$; $Y = 0.941x - 0.43$; $p < 0.001$).

NIR for Remote Analysis

Total Lipids and Total Protein. NIR as a rapid and non-destructive method to determine total fat and protein in mixed, homogenised and freeze-dried human

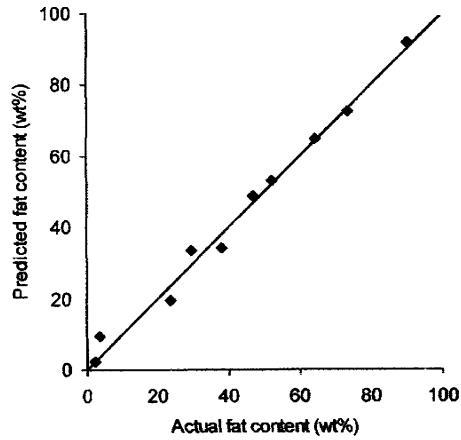

Figure 1.7 *Fat content predicted by ultrasonic velocity measurements against solvent extraction method values*
(Reprinted from *Meat Science*, vol. 57, J. Benedito, J.A. Carcel, C. Rossello and A. Mulet. "Composition Assessment of Raw Meat Mixtures using Ultrasonics", pp. 365–370, © 2001, with permission from Elsevier)

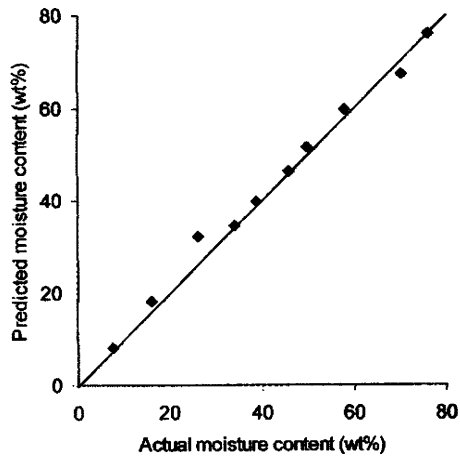

Figure 1.8 *Moisture content predicted by ultrasonic velocity measurements against oven drying method values*
(Reprinted from *Meat Science*, vol. 57, J. Benedito, J.A. Carcel, C. Rossello and A. Mulet. "Composition Assessment of Raw Meat Mixtures using Ultrasonics", pp. 365–370, © 2001, with permission from Elsevier)

diets was described by Almendigen *et al.*, (2000).[76] The new method was compared with the Kjeldahl and Folch methods for total protein and total fat content of student diets. A correlation coefficient of 0.99 against the Folch method for fat was seen as highly accurate and 0.81 for protein against the Kjeldahl method as acceptable; such that both assays for their nutritional work could be replaced

by the quicker and less expensive NIR probe. The method worked well for fat, but accuracy for protein was less convincing.

NIR for Moisture, Oil and Protein Analysis

The GRAINSPEC NIR whole grain analyser (Foss Electric Multispec Division, York, UK), calibrated by the manufacturer, was used to obtain proximate values for moisture, oil and protein content of the initial soybean grain samples in a study (interlaboratory test) of soymilk and tofu.[77]

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CHAPTER 2

Sample Preparation for Extraction

1 Introduction

For most assays the weight of sample taken for analysis and the amount of an internal standard added for quantitative analyses has to be known. Then, some preparative operations are likely to be needed before an extraction can be performed.

In the literature there are some techniques listed as extraction methods that are strictly extraction aids since they do not actually remove anything or fractionate anything from the sample. These techniques make the complete extraction easier, and may be grouped under six headings:

1. Change of volume
2. Change of pH
3. Change of structure
4. Change of state
5. Change of chemical composition
6. Automation.

This last classification is included to collect together the robotic assistance, including flow-switching that the workstation provides in the management of the sample preparation stage. Ultimately, as remote sensing methods improve, the extraction process, even the automated extraction, will become redundant.

2 Change of Volume

Dilution

Seldom is it necessary to dilute a sample because the analyte is too concentrated, but the bulk sample is diluted prior to homogenisation, for example, to create the right consistency for structural decomposition (Appendix 4 Scheme A4.2). Again, the texture may respond better to vortexing if it is moistened a little. Water is added to reconstitute dehydrated samples, and particulate or powdered

matter may be suspended or dissolved in liquid in preparation for stirring or homogenisation. Water may be added, *e.g.* to infant formulae samples, to extract analytes by simply filtering the aqueous suspension (solution). Dilution may help avoid premature blocking in filtration processes.

Evaporation

Evaporation and concentration are common operations in food analysis, especially following partitioning, solvent extraction, or adsorption. The Kuderna-Danish concentrator is typical of the apparatus designed to gently evaporate solvent so that the decreasing volume of solvent collects in a small volume tube attached to the bottom of the main evaporator flask. Heat may be applied in the form of boiling water and steam around the lower part of the flask. Application of heat to reduce volatile solvent volumes may be a source of gravimetric error and, certainly, thermal decomposition has to be avoided. Vacuum evaporation is valuable in reducing the excesses of oxidation during volume reduction. Strictly, evaporation to dryness is an extraction of the liquid and volatile substances from the residual solid.

Evaporation for Capillary Electrophoresis (CE)

Organic solvent extraction is a common preliminary step in food analysis. Unfortunately, many organic solvents are incompatible with the aqueous buffers used in CE. Therefore, the evaporation of the organic solvent and redissolution in a solvent compatible with CE is necessary. For example, samples extracted from corn in alkali solution were evaporated and the tosylated polyamines redissolved.[1]

Condensation

Low temperature condensation can concentrate volatiles from a gas stream and, providing the volume of the cooled area is kept low (cryofocusing), a useful concentrate can be obtained. Freezing aqueous homogenates and decanting or centrifuging the liquor from the ice produces a concentrate.

3 Change of pH

The change of charge on the sample molecule (ionisation) caused by a change of pH provides a useful method of separating analytes by electrophoresis. Acid and alkali fractions can be moved in opposite directions towards the anode and cathode and neutrals can be extracted from the middle ground.

Usually, pH change is introduced to facilitate a chemical conversion into a more stable or manageable structure. A melanin-like pigment was extracted from tea leaves washed with hot water at pH 10.5, followed by acid hydrolysis at pH 2.5 (Sava *et al.*, 2001).

Conversely, intrinsic changes in pH occurring in biochemical reactions can be monitored as a method of detection. ATP coupled reactions carried out at, or slightly above, the neutral point acidify the reaction mixture. Milli pH changes were measured in a constant buffering capacity solution to detect glucose, fructose, glycerol and gluconic acid.[2] In the determination of asparagine, pH changes in the acceptor flow caused by diffused NH_3 produced from asparagine at immobilised asparaginase were monitored.[3] The reduction of trimethylamine oxide to trimethylamine as an indicator of stored fish quality, using the measurement of pH change in the cultured medium,[4] correlated well with the standard total volatile base nitrogen method.

pH Measurement

The use of glass and polymeric membrane electrodes for the measurement of pH in milk and cheese exemplifies the need to be careful with measurements when neutral lipophilic compounds or hydrophobic peptides might be extracted into solvent polymeric membranes, causing loss of selectivity for monovalent cations when exposed to cheese.[5]

4 Change of Structure

Ultrasound

Introduction

Ultrasound is operated at frequencies of 20 kHz–1 MHz. Ultrasound as a common laboratory cleaning aid, and the ultrasound extraction of bioactive components from plant materials has been described.[6] The ultrasound vibrational energy is transmitted through the food matrix (cf. microwaves), causing alternate expansion and contraction cycles. During the expansion cycle, negative pressure causes cavitation and ultimately implosion at high temperatures and pressures.

Applications

Tea Solids. Tea solids have been extracted with water assisted by ultrasound.[7] The effect of temperature, irradiation time and power were studied.

Endosulfan Insecticides. Ethyl acetate extraction of endosulfan insecticides from tomato juice was enhanced by ultrasound applied during the matrix solid-phase dispersion (MSPD) process. (Albero *et al.* 2003, Chapter 4, ref. 79).

*Total Carbohydrate*s. Total carbohydrate analysis requires a hydrolysis step and ultrasound-assisted extraction (UAE) was proposed as an extraction aid.[8,9] Shorter hydrolysis times and improved accuracy were achieved than with the conventional unassisted extraction methods.

Xylans. The xylan component of corncobs in polymeric form was extracted both with and without the use of UAE. Ultrasonic extraction took less time at a lower extraction temperature and a lower concentration of alkali. The sugar composition and main structural features of the fractions obtained by extraction in 5% NaOH with and without application of ultrasound were similar.[10]

Saponins. UAE was simpler and more effective than conventional extraction methods for the isolation of ginsenosides (saponins) from various types of ginseng.[11] Different solvents were used for either direct sonication by an ultrasound probe horn or indirect sonication in an ultrasound cleaning bath and compared to refluxing boiling solvents in a Soxhlet extractor. Sonication-assisted extraction of ginseng saponins was about three times faster, and at a lower temperature, than the traditional extraction method.

Hemicellulose. An investigation of hemicellulose release from buckwheat hulls found increased yields using UAE compared to classical methods.[12] The new method retained the structural, molecular and immunomodulatory properties and, therefore, endorsed the potential of UAE for the extraction of industrially important polysaccharides from different tissues of plant materials.

Fumigant Residues in Wheat. MeBr, PH_3, COS, and CS_2 were solvent extracted in a gas-tight bottle with either UAE or heating at 50 °C. The volatile fumigants were extracted into the headspace in 2 h (UAE) or 7–20 h for 50 °C heating, compared to 8–35 h for ambient extraction.[13]

Volatile Compounds. The extraction efficiency at 60 °C with sonication approached that at 100 °C without. UAE of the volatile compounds from citrus flowers and citrus honey used n-pentane–diethyl ether mixture as solvent.[14]

N-Methylcarbamates. UAE-SPE-Chromatography-post column derivatisation-fluorescence detection has been fully automated for six *n*-methylcarbamates (oxamyl, dioxacarb, metolcarb, carbofuran, carbaryl and isoprocarb) in spiked apple samples.[15] The dramatic decrease in analysis time from 4 h to 2 min was noted. The LOD was 12 ng g^{-1}, the LOQ was 40 ng g^{-1}, and the repeatability and within laboratory reproducibility RSDs were 3.1 and 7.5% respectively.

Developments

UAE Methods. 17 fungicides were extracted from must and wine samples by sonication in acetone–DCM over anhydrous NaCl. The filtrate through phase separator paper (Whatman 2100150 1 PS) was rotary evaporated to dryness and taken up in isooctane–toluene (1:1, v/v) for GC-ECD and GC-NPD analysis (with GC-MS confirmation), giving recoveries between 78 and 107%.[16] Because of the high selectivity of both detection methods, no clean up was necessary.

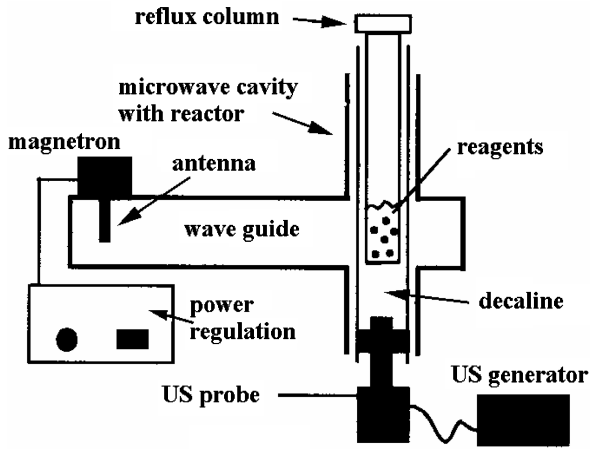


Figure 2.1 *Single mode microwave system combined with ultrasound (US)* (Reprinted from *Ultrasonics Sonochemistry*, vol. 11, S. Chemat, A. Lagha, H.A. Amar and F. Chemat, "Ultrasound Assisted Microwave Digestion", pp. 5–8, © 2004, with permission from Elsevier)

Ultrasound-assisted Microwave Digestion (UAMD). By combining microwave and ultrasound irradiation into a simultaneous extraction (Figure 2.1), a new technique for digesting solid and liquid food samples was proposed.[17] It decreases digestion time, and it has been used so far to determine copper in edible oils and for total Kjeldahl nitrogen in powdered milk, cow's milk, rice, corn, flour, beef, corned beef, and chickpea. (Chapter 1, Section 6).

Dynamic Ultrasound-assisted Extraction. Three dynamic flowing solvent UAE methods were evaluated as alternatives to supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), focused microwave-assisted Soxhlet extraction (FMASE), or IR spectroscopy, using the Soxhlet method as standard for the extraction of fat from bakery products.[18] The dynamic flow methods were:

1. Flow in one direction.
2. Forward and backward flows.
3. Forward and backward flows, changing the extractant batch-wise.

The extraction efficiency improved in the order 1 to 3, and all three methods dramatically reduced the extraction time (from 5–8 times) compared with the Soxhlet extraction, with which the new method agreed impressively for % fat extracted at 100% extraction efficiency.

Ultrasound-assisted Soxhlet Extraction (UASE). The application of UAE to the classical Soxhlet extraction for total fat from oleaginous seeds is dealt with in Chapter 5, Section 3.

Figure 2.2 *Schematic representation of the paired dipole motion in an electromagnetic field causing vibrational heating*

Comparison with other Extraction Techniques. UAE has been compared with Soxhlet, microwave-assisted extraction (MAE) and SFE.[19] Additionally, a scheme for putting UAE on-line with SPE (concentration), derivatisation, or detection was outlined.

Microwaves

Introduction

Microwave heating is based on the energising rotation of dipole pairs (Figure 2.2) and the movement of ions electrophoretically in an electromagnetic field. The schematic example uses water molecules as the most likely dipoles to be significant in food analysis.

In the microwave frequency range of 100 MHz to 3 GHz the alignment and relaxation of the dipoles at up to 5×10^9 times per second, and the electrophoretic motion through the cellular structure, causes vibrational and frictional heating, respectively, on the molecular scale. Polar liquids with their high dielectric constants absorb microwave energy strongly while apolar solvents such as hexane are not heated to any extent. In general, some constituents will be heated more than others, giving rise to the superheating phenomenon in MAE. Ionised liquids with their permanent dipoles also absorb strongly, so acids heat more than neutral compounds. Polar organic solvents, *e.g.* alcohols, have higher heat dissipation factors than water, making them ideal for closed vessel MAE, where they can be heated above their normal boiling points. The food matrix will be composed of lower dielectric constant material, which will remain at a lower temperature. This is ideal for thermally labile components such as vitamins.

Development

Development of Solvent Systems. The actual composition of the extraction solvent mixture for MAE should take account not only of the microwave absorption properties but also of the way that the solvent interacts with the food matrix and the solubility of the analytes therein. Sparr Eskilsson and Björklund (2000) (Chapter 8, ref. 6) recognise three mechanisms by which the extraction heating process might be categorised:

1. Use a solvent or solvent mixture that absorbs microwave energy strongly.
2. Use a mixture of solvents in various proportions, with both high and low dielectric loss components.
3. Use an apolar solvent system on a high dielectric loss sample matrix.

This provides a range of options in the choice of solvent "tailored" to suit the type of analyte and matrix. They discuss many solvent systems, including, under mechanism 3, the value of thermal disintegration of the cellular structure of high moisture material releasing ingredients for solvation in the transparent solvent.[20]

Pre-treatment. Food samples are homogenised and suspended in organic solvents and irradiated in an open or closed vessel in a microwave oven, without causing the sample to boil. The process can be repeated several times to complete the extraction of the target compounds. It may be advantageous to add a drying agent to a high moisture sample, and anhydrous sodium sulphate was used for grape juice.[21] If all that is required after the extraction is to centrifuge the sample and decant the supernatant layer for chromatographic separation, then this method is rapid, simple and suitable for multiple sample batches.

Optimisation. The parameters to consider are:

1. Solvent composition
2. Solvent volume
3. Moisture content
4. Extraction time
5. Extraction temperature.

Resonant Microwave Sensors. The need for remote at-line sensing of moisture in food processing was addressed in the study of open microwave resonators and their application to the monitoring of rolled oats, whole grains of oats, barley and wheat.[22]

Applications

General Approach. An early example of the use of MAE in food analysis required the sample to be ground and then extracted with MeOH or MeOH–H_2O for polar compounds, or hexane for non-polar compounds, as solvents. The mixture was irradiated for 30 min without boiling. The procedure was repeated several times and the samples centrifuged to obtain the supernatant.[23] It was developed as a rapid, general extraction method for large numbers of samples.

Dithiocarbamate Fungicides. Dithiocarbamate fungicides from peaches were extracted and acid hydrolysed to CS_2 in a single step using MAE.[24] The evolved

CS_2 was trapped in supernatant iso-octane for GC analysis. The LOD was 0.005 mg kg^{-1} for thiram and ziram.

Fumigant Residues in Grain. MAE was used to release fumigants from foodstuffs by placing, *e.g.*, wheat in gas-tight Erlenmeyer flasks and irradiating in a domestic oven.[25] Microwave power was optimised for the release of CH_3Br, PH_3, and CS_2, and COS into the headspace. The low LOQ (<1 ng g^{-1}) was put down to the absence of solvent-based interferents.

Pigments from Paprika. Thirty solvent mixtures were compared for MAE of pigments from paprika powder. The dielectric constant of the extractant was an important parameter.[26]

Trichlorobenzenes in Fish. Saponification followed by LLE and MAE were compared using n-pentane as solvent for both methods.[27] No difference was found between the methods for recoveries, but MAE was quicker and used less solvent.

MAE and Headspace-Solid-phase Microextraction (HS-SPME)

MAE was used to prepare an aqueous extract of pesticides (dichlorvos) from chopped vegetables from which the headspace was sampled by HS-SPME for electron capture (EC)-GC analysis.[28] 10% aqueous ethylene glycol at pH 5 and medium microwave power for 10 min was found to be optimal.

Microwave-assisted Soxhlet Extraction

The MA-SOX method was developed for the hydrolysis and extraction of fat in cheese.[29] Dramatic economies in time (from 7 h to <1 h) and significant savings through solvent recovery were reported. The combination was used to extract fat from fried meat and fish samples. Microwave power, number of cycles, and microwave irradiation times were optimised, and a faster and cleaner extraction was reported.[30]

Divide into Parts

Dissect

It is normal in food analysis to separate botanically different parts of an item of food by dissection. The florets, stems and leaves of broccoli were sorted after cutting apart (Bertelli *et al.*, 1998, Scheme A4.1, Appendix 4). To extract pectic polysaccharides from primary cell walls the parenchyma of vegetables[31] or the mesophyll of grasses[32] are dissected from the organ. The full treatment of cell walls to release polysaccharides is discussed by Selvendran and Ryden[33] and O'Neill *et al.*[34]

Table 2.1 *Composition (% total mean weight) of raw pork cuts derived by dissection (n = 4)*
(Reprinted from the *Journal of Food Composition and Analysis*, volume 16, I. Clausen, J. Jakobsen, T. Leth and L. Ovesen, "Vitamin D_3 and 25-hydroxyvitamin D_3 in Raw and Cooked Pork Cuts", pp. 575–585, © 2003, with permission from Elsevier)

Cut	*Lean meat*	*Lard*	*Intermuscular fat*	*Rind*	*Mixed part*[a]
Loin	53	18		8	21
Leg	100				
Thin belly	32	13	8	6	40
Neck	64		8		28

[a] Connective tissue with adhering meat and fat.

Dissect and Manually Separate

Typically, meat carcasses are separated into offal and "cuts" of meat. Therefore, the analyst has access to specific organs, muscles and other tissues from which to take samples for analysis.[35] In this case raw and cooked pork cuts also were dissected into lean meat, lard, intermuscular fat, rind, and a mixed part of connective tissue with adhering meat and fat and the % total weight measured (Table 2.1). The samples were used to study the distribution of vitamin D_3 and 25-hydroxyvitamin D_3. Multiple extractions were required prior to HPLC separation (Appendix 2).

Lysis

Chemical and Biochemical Release

Release as an Extraction Aid. The quantitative yield of analyte from a food is the calibrated measurement of the amount of "free" material in the sample taken for analysis. If the preparation process does not release all the analyte from the food matrix, it is not possible to quantify the total content of the analyte in the food by the chosen method. When biochemical release is invoked it is expected beforehand that a complex exists in the food, which may not release the analyte without it being disintegrated first. In practice, the technology built up for, say, vitamin analysis assumes the existence of several different biological states and declares in advance the chemical classes that are to be targeted for the release of the compound of interest. In other words, there will be other states of complexation of the analyte with the matrix – present in only small amounts – from which the analyte will not be released.

Ill-defined Release. Another scenario is where a small change in the protocol may release an additional, related class of compounds. An interesting example of this is the measurement of dietary fibre. There are several different chemical

classes that, according to the conditions set up for the release, may or may not be included in the hydrolysate. These must be declared in the assay.

Inadvertent Hydrolysis. The steam distillate of the essential oil of *Alpina galangal* (*L.*) swartz did not contain galangal acetate, a pungent principle found as a major volatile by headspace GC, because the acetate was hydrolysed/isomerised in the aqueous solutions (Yang and Eilerman, 1999, Chapter 5, ref. 26).

Acid Hydrolysis

Amino Acids. Solvent extraction of free *o*-tyrosine, present in unirradiated chicken, preceded acid hydrolysis to release bound *o*-tyrosine from the proteinaceous residue of irradiated chicken.[36]

Carbohydrates. Acid hydrolysis is used extensively in carbohydrate analysis to cleave glycosidic bonds. Degrees of acid hydrolysis can be used to hydrolyse bonds of a certain dissociation energy, and therefore the process is used in structure elucidation experiments. Here, we are concerned with its use as an extraction aid.

The structure of the cell wall material of potatoes,[37] wheat bran,[38] wheat arabinoxylans[39] and the pectic polysaccharides of cabbage[40] was aided by different acid hydrolysis treatments. The carbohydrate content of wheat glutens was determined after acid hydrolysis.[41] Mild acid hydrolysis cleaved the phosphate diester linkages of the cell wall polysaccharides of food protein yeast.[42] Oligosaccharides of five bean cultivars were characterised by FAB-MS after complete acid hydrolysis.[43]

Florfenicol Amine. U.S. FDA guidelines were used in the determination of the veterinary antibiotic florfenicol in channel catfish muscle.[44] Acid hydrolysis had previously (in other species) been demonstrated to convert florfenicol and its known metabolites into florfenicol amine and to release a significant amount of FFA from non-extractable florfenicol residues. Acid hydrolysis before solvent extraction should yield a more accurate estimate of the total florfenicol-related residue.

Lipids. Acid hydrolysis of oat starch assisted the release of total lipids that were extracted in $CHCl_3$–MeOH (2:1 v/v) at ambient temperature (free lipids), followed by n-propanol–water (3:1 v/v) at 90–100 °C (free and bound lipids).[45–47] In a comparison of four extraction methods, the use of acid hydrolysis with ether extraction was better for total crude fat estimation than solvent extraction alone, SFE or SPE.[48]

Miscellaneous. Acid hydrolysis helped to release paralytic poisons from shellfish.[49] The difference between free and bound β-damascenone in red grapes

and wine was determined using acid hydrolysis.[50] The flavanol content (quercetin, myricetin, kaempferol) of lingonberry, blackcurrant, bilberry, strawberry and raspberry was determined after acid hydrolysis of the glycosides.[51]

Nucleic Acids. Acid hydrolysis with TFA and formic acid in a pressurised digestion vessel for 15 min at 240 °C liberated purine bases (pyrimidine bases required 45 min) from nucleic acids, nucleotides and nucleosides in carbohydrate-rich foods. A DCM extraction removed artefacts before cation exchange chromatography.[52] The work was continued with SPE of the hydrolysates.[53]

Vitamins. With minerals and vitamins, the presence of fats, proteins, and carbohydrates, in other words the bulk of the sample, has to be considered as a source of loss of analyte. When concentrated mineral acids are employed to digest the matrix, *e.g.* for inorganic elements, the method has removed any likely bonding sites and, if chemical degradation has been ruled out, the analyte is largely interference free.

Solvent Extraction followed by Acid Hydrolysis

Isoflavanones. Phytoestrogens were MeOH extracted from Japanese foods and converted into aglycones by acid hydrolysis for total isoflavone analysis of glycosides and free aglycones by HPLC.[54]

Method Development for Steroidal Glycosides and Saponins. Several combinations of solvent extraction and acid hydrolysis were tested on artefacts of diosgenin and spirostadiene in fenugreek seeds.[55] After an extraction using 80% EtOH, a solution of 1 M H_2SO_4 in water containing 70% 2-propanol at 100 °C for 2 h gave the highest recovery of diosgenin and reduced diene artefact formation compared to extraction with aqueous HCl. A further petroleum ether extraction to defat 10 mg sub-samples of crushed seed, and drying at 60 °C before hydrolysis, was reported.

Alkali Hydrolysis or Saponification

α-Tocopherol. An early example of the move towards automation was reported for the analysis of α-tocopherol.[56] In a continuous-flow method, the unsaponifiable material is extracted into isooctane and extractions are inserted between selective reaction steps.

Cholesterol Oxidation Products (COPs). *COPs* were released from tallow using three saponification methods and transesterification.[57] The flow diagram of the experimental protocol for the comparison of the four COP release methods: (1) Cold saponification, 1 M KOH–95% EtOH or (2) with KOH–MeOH,[58] (3) hot saponification, KOH–95% EtOH,[59] and (4) transesterification[60]

is shown in Figure 2.3. Released compounds were concentrated by SPE, the non-polar fraction eluted with hexane-based solvents and the polar fraction with acetone-based solvents.

Carotenoids. Analysis of carotenoids in vegetables and fruits is often accompanied by a saponification step to remove interference, *e.g.* from chlorophylls and lipids, and also to hydrolyse esterified forms of carotenoids. An extraction in hexane–ethanol–acetone preceded saponification with 10% methanolic KOH

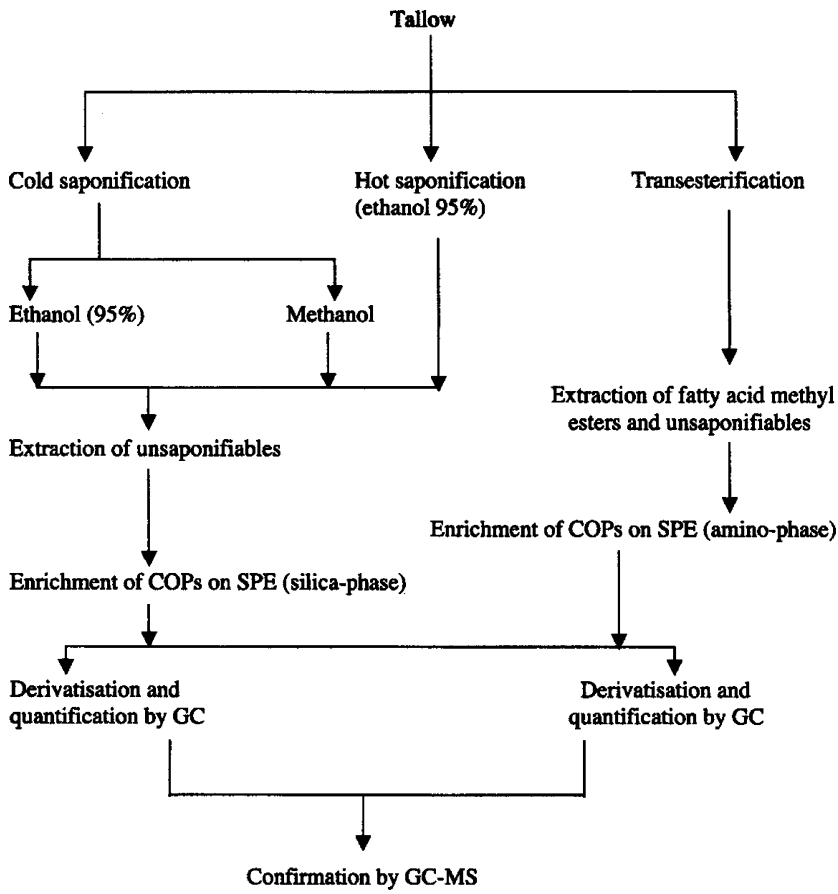


Figure 2.3 *Experimental protocol for the comparison of four COP release methods. (1) Cold saponification 1 M KOH–95% EtOH or (2) with KOH–MeOH, (3) hot saponification KOH–95% EtOH and (4) transesterification. The unsaponifiable material was concentrated and fractionated by SPE and the COPs converted into the TMS-ethers for GC and GC-MS*
(Reprinted from *Food Chemistry*, vol. 84, S.J.K.A. Ubhayasekera, T. Verleyen, and P.C. Dutta, "Evaluation of GC and GC-MS Methods for the Analysis of Cholesterol Oxidation Products", pp. 149–157, © 2004, with permission from Elsevier)

for the preparation of carotenoids from new sweet orange (Earlygold) for separation by HPLC.[61] Some 25 carotenoids were analysed in 40 min on a C_{30} reversed phase (RP) column.

A low-cost saponification protocol was developed for the extraction of carotenoids from fruit and vegetables to replace the conventional time-consuming method. The two protocols are shown in Scheme A4.4 of Appendix 4. Miniaturisation (8 times reduction in the sample size), a change of extraction solvent, and a reduction in the time the carotenoids spend exposed to the alkali conditions provided the improvements.[62]

Recently, some unusual carotenoid esters of mango have been studied using saponification release (30% (w/v) KOH in diethyl ether) and LC-APcI-MS.[63] Figure 2.4 shows the separation of carotenoids before and after saponification. However, saponification did not release the native carotenoid esters, and alternative extractions were used for 64 fruit and vegetable samples.[64]

Isocoumarin. 6-Methoxymellein is a bitter principle found in carrots. Its formation on processing was studied by saponification of carrot tissue to solubilise the lactone structure into an aqueous phase for subsequent solvent extraction. The concentration of isocoumarin accumulated in root tips treated with ethylene, and increased with wounding. The latter observation allowed various processing procedures to be compared, with the reduction in bitterness in mind.[65]

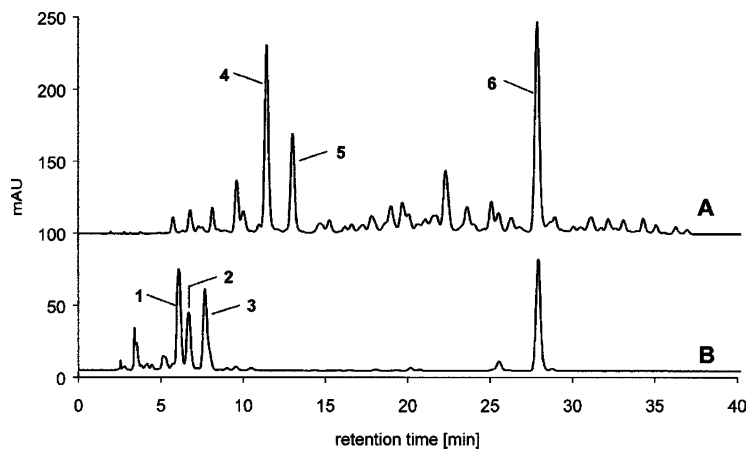


Figure 2.4 *HPLC chromatograms of a normal extract (A) and an extract after saponification (B). The numbered peaks are 1. violaxanthin, 2. Neochrom luteoxanthin (tentative identity), 3. unidentified free xanthophyll (m/z 601), 4. violaxanthin dibutyrate, 5. unidentified esterified xanthophyll, 6. β-carotene*
(Reprinted from *Phytochemistry*, vol. 64, I. Pott, D.E. Breithaupt and R. Carle, "Detection of Unusual Carotenoid Esters in Fresh Mango (Mangifera indica L. cv. 'Kent')", pp. 825–829, © 2003, with permission from Elsevier)

Enzyme Hydrolysis

Peptides. Several different enzymes with specific peptide bond activity have been used to release peptides from proteins, the most common being trypsin with its specificity for leaving arginine or lysine residues at the C-terminus of the peptide. The release of bioactive proteins and peptides for nutraceutical applications brought a plea for more work on the automation of peptide recovery from food sources.[66] Bioactive peptides are often resistant to digestion peptidases. Example sources of bioactive peptides are

1. Angiotensin I Converting Enzyme (ACE) inhibitors from milk, corn and fish.
2. Opioids from wheat gluten and casein.
3. Exorphins and opioids from wheat and milk.
4. Immunomodulatory peptides derived from rice and soybean.

Vitamins. Vitamins are compounds essential to health that cannot be synthesised and have to be ingested from the food supply. Two analytically distinct classes of vitamins are the water-soluble and fat-soluble substances. Vitamins complex with proteins and form phosphates and glycosides with carbohydrates. Enzyme and acid hydrolysis have been used to release them from the matrix.

The HPLC method for biotin used a papain hydrolysis for various foods, adding takadiastase for high starch foods. H_2SO_4 hydrolysis degraded the vitamin and the enzyme method was preferred because it also gave information on the bioavailable forms.[67]

Carotenoids. The recovery of essential nutrients from non-food sources is gaining importance. Marigold flowers were traditionally extracted with solvents to remove carotenoids. Recent extractions were assisted by simultaneous enzyme release.[68] Further studies were conducted on the enzymic hydrolysis of marigold flowers as a source of lutein, and paprika as a source of capsanthin.[69]

Acid Hydrolysis followed by Enzyme Hydrolysis

Vitamins. The convention of using acid hydrolysis, to convert insoluble starch into soluble sugars and to denature protein to start the release of vitamins for enzyme hydrolysis, was challenged by Ndaw *et al.*,[70] arguing that many of the diastases have protease as well as phosphatase activity. They carried out an investigation on cereals (wheat flour, porridge oats and rice), vegetables (carrots, peas), orange juice, powdered milk, meats (pork and veal escalopes), fish (mackerel fillet) and yeast to test the hypotheses that acid hydrolysis was not needed, and that a mixture of enzymes was as efficient as the most efficient diastase, and was more reproducible for use in a reference analytical protocol.

An optimised cocktail of enzymes, 10 mg α-amylase, 100 mg papain, 20 mg acid phosphatase and optionally 20 mg β-glucosidase, capable of extracting B_1, B_2, and B_6 vitamins in a single step from free, phosphorylated, and the protein-bound forms was described. The paper gives a wealth of information – and the

source references – on the extraction protocols for the three vitamins over the past twenty years.

Nevertheless, for the extraction of vitamins, the use of acid hydrolysis to release the protein-bound portion is popular. Methods describe the optimum strength of the acid, *e.g.*, 0.1 M HCl, the amount of enzyme to give >95% hydrolysis of the phosphorylated form [mg of activity (U)], and the temperature of the reaction (*e.g.* in a water bath at 100 °C).[70] Sulphuric acid was used to dephosphorylate foods for the measurement of free vitamin B_6.[71] Others have used an acid phosphatase.[72]

A combination of acid hydrolysis followed by enzyme hydrolysis was used to assist in the assay of vitamin B_1 in food. The digest was extracted on a weak acid cation exchange column (Amberlite CG 50).[73]

Biochemical Release for Dietary Fibre Analysis

The measurement of dietary fibre is given a section of its own because the development, in the 1990s, of an accurate and rapid assay was important when the function of the "cell-wall remnants" of our foods had become a multifarious health issue. The remnants making up dietary fibre are

1. Hemicelluloses
2. Celluloses
3. Lignin
4. Pectins
5. Gums
6. Waxes.

All these chemical classes are precipitated in 78% EtOH and therefore recorded in the measurement of dietary fibre. The dietary fibre content of food affects colonic and coronary heart conditions, cholesterol level, glucose metabolism, blood lipids, and many more modern concerns.

Englyst Method. In early reports of the analysis of carbohydrates from foods, Southgate distinguished between available[74] and unavailable[75] carbohydrates. His methods have been refined by, among others, the team at the MRC Dunn Clinical Nutrition Centre in Cambridge, UK, so that total, soluble and insoluble dietary fibre fractions can be measured as NSP in plant foods.[76] Starch is digested enzymatically and then acid hydrolysis of the residue releases NSP calculated as the sum of the constituent sugars analysed by GC as the alditol acetates.

Prosky Method. Independently, the FDA team in Washington developed an enzyme–gravimetric method (*J. AOAC*, 1984, **67**, 1044) for TDF (AOAC, 985.29) and tested it on soy isolate, white wheat flour, rye bread, potatoes, rice,

wheat bran, oats, corn bran, and whole wheat flour in a collaborative study by 9 laboratories (Appendix 3).[77]

The method entails the enzymic hydrolysis of starch and the gravimetric measurement of the residue, which, after correction for the protein (total $N \times 6.25$) and ash, gives the dietary fibre content. The method was later extended to measure insoluble dietary fibre (IDF) (AOAC, 991.42) and soluble dietary fibre (SDF),[78] and this modified method was used in a collaborative study on soy isolate, white wheat flour, rye bread, potatoes, rice, corn bran, oats, Fabulous Fiber, wheat bran, and a high fibre cereal, undertaken by 13 laboratories (Appendix 3). A second collaborative study was made of SDF in which 13 laboratories used the enzymatic–gravimetric method on apricots, carrots, chickpeas, onions, raisins, and sugar beet fibre (Fibrex).[79]

International Survey on Dietary Fibre (1995). An excerpt is printed from the abstract.[80]

> An international survey was conducted to get the views of 147 professionals in the field on the definition of dietary fibre. The survey also solicited opinions on analytical methods for nutrition labelling, quality control and nutrition research. The survey finds that dietary fibre is generally defined as polysaccharides and lignin that are not hydrolysed by human alimentary enzymes. Support is strong for expansion of the definition to include oligosaccharides that are resistant to hydrolysis by human alimentary enzymes.

The Prosky method was preferred for food labelling and quality control, and the Englyst method was thought to be more appropriate for nutrition research.

Recent Developments. In a recent determination of dietary fibre, Englyst and co-workers used DMSO to ensure that all the starch was dispersed before being released by enzyme hydrolysis, and the NSP was precipitated in EtOH. Uronic acids were measured colorimetrically, but lignin, a minor component of human plant foods was not measured. Acid hydrolysis of the NSP residue released the constituent sugars, which were derivatised to the volatile alditol acetates ready for separation by GC, HPLC or spectrometry.[81] Good agreement was obtained for a wide range of foods. After modification, the rapid spectrophotometric method was ideal for food labelling and quality control. The criticism of the Prosky method was that the starch dispersal step was inefficient. There are a great many applications of the method in the literature of interest to nutritionists, the food industry, and the medical profession. A valuable contribution was the measurement of the NSP content of 228 Mexican foods (Sánchez-Castillo *et al.*, 1999, Chapter 1, ref. 71).

Uronic Acid Constituents of NSP. Two sets of hydrolysis conditions were tested for the release of uronic acids:

1. Prolonged treatment with H_2SO_4 alone.
2. Hydrolysate obtained by the Englyst procedure is buffered to pH 3.5–4.0 and polymers containing uronic acids hydrolysed enzymatically.

The two methods were optimised for fruit, vegetable and cereal products.[82]

Inulin and Oligofructose. Some oligo- and polysaccharides (fructans) do not precipitate from 78% EtOH and, therefore, they are not normally recorded in the dietary fibre content of food. An AOAC method was described for fructans, which involves enzyme hydrolysis with amyloglucosidase and then fructozyme to release sugars for separation by anion-exchange chromatography (AEC).[83] Later that year a collaborative study among 9 laboratories used the method on low fat spread, cheese spread, chocolate, wine gum, dry ice mix powder and biscuits.[84] The method was accepted as an AOAC official first action.

Classification of Dietary Carbohydrates. The Englyst team set out their preference for the enzyme–chemical measurement of dietary carbohydrates including dietary fibre[85] based on the fact that food processing does not change the values. They also define short-chain carbohydrates (SCs), rapidly available glucose (RAG), and review the various types of starch:

1. Rapidly digestible starch
2. Slowly digestible starch
3. Resistant starch.

Englyst and Prosky Methods for Dietary Fibre Analysis Compared. The Englyst method was compared to the AOAC Prosky method in a study of 17 individual Hungarian foods and 10 whole meals.[86] However, the comparison was qualified by statements about the nature of the extract from each method. The Englyst enzymic–chemical method extracts plant cell wall NSP, while the Prosky enzymic–gravimetric method extracts indigestible polysaccharides and lignin (Table 2.2), the difference between the two extraction protocols creates problems in calculating dietary fibre intake.

Comparison of Biochemical Release Methods

Strength of Acid in Acid Hydrolysis. In a comparison of HPLC and microbiological assays, Rose-Sallin *et al.* found that the lack of specificity of the *L. plantarum* and the stronger acid hydrolysis associated with the microbiological assay released more niacin than the milder acid hydrolysis used with the HPLC method. This was attributed to part of the non-bioavailable niacin being released in the latter case. They also evaluated various combinations of hydrolysis for the release of niacin (nicotinic acid and nicotinamide) from cereal-based food products for HPLC analysis.[87] With fluorescence detection, higher specificity and sensitivity, regardless of the need for the extra step of

Table 2.2 *Individual foods analysed in duplicate by the Englyst (NSP) and the Prosky method. In most cases, the Prosky method released more material than the NSP method*
(Reprinted from *Food Chemistry*, vol. 64, M. Kontraszti, G.J. Hudson and H.N. Englyst, "Dietary Fibre in Hungarian Foods Measured by the Englyst NSP Procedure and the AOAC Prosky Procedure: A Comparison Study", pp. 445–450, © 1999, with permission from Elsevier)

Food	*NSP* (%fresh)	*Prosky* (% fresh)	*Difference* (*Prosky – NSP*)
Beetroot	2.1	3.3	1.2
Carrot	2.0	3.1	1.1
Cucumber	0.8	1.3	0.5
Dill	3.2	6.3	3.1
Garlic	3.0	6.4	3.4
Gherkin	0.6	0.5	–0.1
Green peas	3.2	4.8	1.6
Kohlrabi	1.3	1.9	0.6
Lettuce	0.8	1.5	0.7
Onion	2.1	2.4	0.3
Paprika	1.3	2.1	0.8
Parsley	3.6	5.6	2.0
Potato	1.1	1.6	0.5
Puffed rice	0.9	6.1	5.2
Radish	1.1	L3	0.3
Rye bread	3.1	7.4	4.3
Tomato	1.0	1.8	0.8
Mean	1.8	3.4	1.6
SD	1.0	2.2	1.5
Min	0.6	0.5	–0.1
Max	3.6	7.4	5.2

post-column derivatisation, aided in the simplification of the preparation for separation. With this detection system, alkaline hydrolysis of the literature-recommended acid followed by alkaline hydrolysis, was not necessary. Furthermore, the use of enzyme digestion, either before or after acid hydrolysis, was not necessary. The full protocol for the release of niacin in these experiments is shown in Scheme A4.3, Appendix 4.

Hydrolysis with lipase type VII from *Candida rugosa* released carotenoid esters from red pepper extracts in a non-quantitative way, compared to saponification.[88] But the replacement of alkali hydrolysis by enzyme hydrolysis was preferred in the food industry as being more "natural". Later, the use of saponification for the release of carotenoid esters was questioned,[64] and for work on eight cultivars of potato the team from the Universität Hohenheim chose SPE and lipase enzyme hydrolysis[89] of residual triglycerides, in conjunction with LC-APcI-MS. The result of these experiments showed the quantitative importance of the esters of carotenoids (41–131 μg per 100 g) compared with the carotenoids themselves (175 μg per 100 g).[90]

Combined Enzyme Hydrolysis and Solvent Extraction

The release of carotenoids from marigold flowers was improved from around 50% to 97% by adding a simultaneous enzyme release stage along with the solvent extraction.[68]

The effect of enzyme digestion as an aid to the solvent extraction of chloramphenicol from animal tissues was tested.[91] Glucuronidase digestion allowed a ten-fold increase in the amount of chloramphenicol extracted, while neither protease digestion nor ultrasound treatment had any effect. With oxytetracycline, direct aqueous, organic solvent, enzyme digestion, and sonication gave similar results, interpreted as showing there was no binding of the analyte to the tissue (beef kidney).[92]

Autolysis

To find out whether acid hydrolysis was necessary in the extraction of sulforaphane from broccoli, samples were divided into two equal parts; one part was autolysed at room temperature for 24 h while the second part was treated by acid hydrolysis (concentrated HCl at room temperature for 24 h).[93] The full protocol for the extraction is given in Appendix 4 Scheme A4.1.

Development of Biochemical Release Applications

Melanin-like Pigment. Alkaline hydrolysis followed by acid hydrolysis and repeated precipitation were used in the extraction of the melanin-like pigment from black tea leaves (Figure 2.5).[94]

Phenolic Acids. To optimise the extraction of phenolic acids (benzoic acid, *p*-hydroxybenzoic, vanillic, and protocatechuic acids) and cinnamic acid derivatives (coumaric, caffeic, ferulic, and chlorogenic acids) from barley varieties by HPLC, a number of biochemical release strategies were tested:[95]

1. Simple hot water.
2. Extraction after acid hydrolysis.
3. Acid plus alpha-amylase hydrolysis.
4. Acid plus alpha-amylase plus cellulase hydrolysis.

The three-step process (4) was preferred.

Further work was carried out to optimise the extraction of phenolic acids (*m*-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, syringic, *o*-coumaric, *m*-coumaric, *p*-coumaric, caffeic, ferulic, sinapic, chlorogenic, and ellagic) from plant foods.[96] Free phenolic acids were extracted directly with MeOH and 10% acetic acid. Bound phenolic acids were released first with alkaline hydrolysis and then with acid hydrolysis before extraction with diethyl ether–ethyl acetate (1:1). Ellagic acid required a long hydrolysis (20 h).

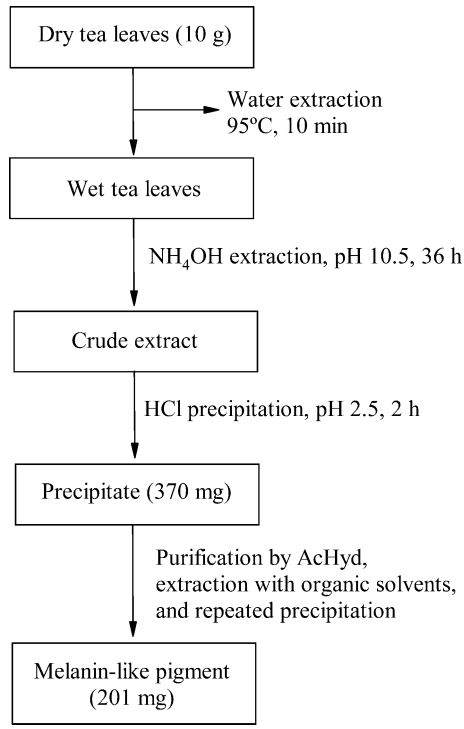


Figure 2.5 *Scheme for the extraction of melanin-like pigment from black tea leaves. pH regulated alkali extraction of wet tea leaves after water-soluble material had been removed gave an extract for acid hydrolysis, and repeated precipitation yielded the melanin-like pigment*
(Redrawn from *Food Research International*, vol. 34, V.M. Sava, B.N. Galkin, M-Y. Hong, P-C. Yang and G.S. Huang, "A Novel Melanin-like Pigment Derived from Black Tea Leaves with Immuno-stimulating Activity", pp. 337–343, © 2001, with permission from Elsevier)

Sterols. Interest in the effect of dietary cholesterol on serum levels, and a general interest in plant sterols and their conjugation in food sources required attention to the chemical and biochemical efficacy of the extraction methods in use. Toivo *et al.* made a valuable contribution by studying the recovery of free, esterified, and glycosidic sterols (brassicasterol, campesterol, campestanol, stigmasterol, sitosterol, Δ^5-avanasterol) from whole wheat flour, a diet composite, rapeseed oil, sunflower kernel, corn meal and dried onion.[97,98] The structures of free sterol and some conjugates are shown in Figure 2.6.

In a detailed account, the analytical steps required to follow the recommended sample preparation for GC analysis are:
A. Acid hydrolysis

1. Weigh sample into tube
2. Add solvent (1 ml absolute ethanol)
3. Shake vigorously

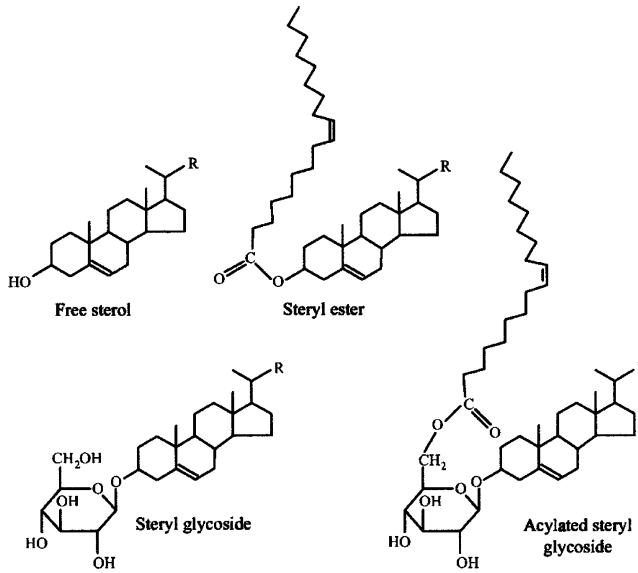


Figure 2.6 *Structures of free sterol and some conjugates found in plant foods. R varies with compound*
(Reprinted from *Food Composition and Analysis*, vol. 14, J. Toivo, K. Phillips, A-M. Lampi and V. Piironen, "Determination of Sterols in Foods: Recovery of Free, Esterified and Glycosidic Sterols", pp. 631–643, © 2001, with permission from Elsevier)

4. Add IS (50 μg dihydrocholesterol in 2.5 ml absolute ethanol)
5. Mix
6. Acid hydrolysis (5 ml 6 M HCl)
7. Shake vigorously
8. Heat to 80 °C for 60 min
9. Shake every 10 min
10. Cool bottom of tube
11. Add solvent (5 ml absolute ethanol)
12. Shake well
13. Solvent extraction (hexane–diethyl ether, 1:1, v/v)
14. Shake on rocker for 10 min
15. Centrifuge at 500 rpm for 10 min
16. Pipette 15 ml organic phase to new tube
17. Evaporate to dryness at 40 °C under N.

B. Saponification

18. Dissolve in 8 ml pyrogallol–EtOH (3%, w/v)
19. Add 0.5 ml 1.3% (w/v) aqueous KOH
20. Heat to 80 °C for 10 min
21. Shake vigorously every 2 min
22. Cool bottom of tube

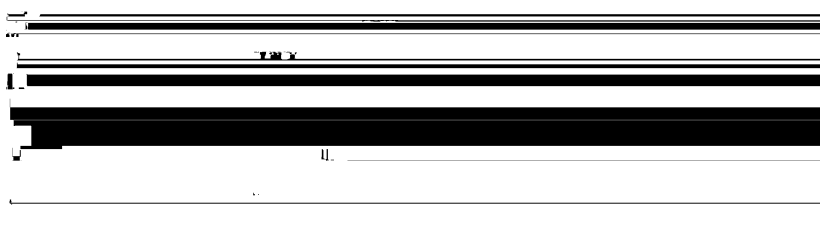
23. Add 20 ml cyclohexane and 12 ml deionised water
24. Shake on rocker for 10 min
25. Centrifuge at 500 rpm for 10 min
26. Pipette 15 ml organic phase to new tube
27. Evaporate to dryness at 40 °C under N.

Sample size, internal standard content, and hydrolysis time were optimised. For cholesterol the results suggested that the acid hydrolysis was not required. The paper is recommended as an example of the thorough evaluation and validation of a new method. (Summarised from ref. 98 with permission from Elsevier)

Extraction Aid for Unsaponifiable Fraction. Saponification with alcoholic KOH prepared samples of pork for extraction of the unsaponifiable fraction with diethyl ether – 40–60 °C petroleum ether (1:1) (Clausen *et al.*[35]).

Development of Biochemical Release Methods

Miniaturisation of On-line Protein Digestion and Separation. A μ-enzyme reactor was built on the lab-on-a-chip principle. A PVDF membrane containing immobilised trypsin was sandwiched between PDMS plates with micro grooves (or embedded capillary tubing) to form a low volume chamber with capillary inlet and outlet on opposite sides of the membrane. Using a Valco flow-switching valve, this reactor was coupled to a device with two PVDF membrane surfaces for μ-membrane chromatographic separation.[99] With denatured and reconstituted horse heart cytochrome *c* protein as a test material, a 250 μl aliquot was injected into the digestion reactor at a flow rate of 0.1 μl min^{-1} with the flow-switching valve in the loading position. The digest was then switched to the membrane chromatography–ESI-MS for separation and detection (Figure 2.7).



1. Load 2. Inject

Figure 2.7 *Schematic diagram of the 2-position/4-port Valco switching valve. Left, the loading position and, right, the injection position. The arrow points to the in-line ports. 1. Sample of digestion mixture loaded from the micro reactor into the loop of the valve. 2. Loop contents transferred to the micro-membrane chromatography system*
(Redrawn from the *Journal of Chromatography A*, vol. 924, Y. Jiang and C.S. Lee, "On-line Coupling of Micro-enzyme Reactor with Micro-membrane Chromatography for Protein Digestion, Peptide Separation, and Protein Identification using Electrospray Ionisation Mass Spectrometry", pp. 315–322, © 2001, with permission from Elsevier)

Homogenise

Grind

A pestle and mortar is used to grind small amounts of hard, dry food samples. If larger amounts are to be handled then disc grinding and knife grinding mills are used (Rostagno *et al.*, 2002, Chapter 4, ref. 157)

Blend

The Waring blender is well known in the food laboratory. It was used in the determination of cholesterol in ready-to-serve foods. Freeze-dried samples were either extracted in a Soxhlet distillation with 40–60 °C petroleum ether or with $CHCl_3$–MeOH–H_2O in a Waring blender.[100] It was employed in the extraction of toxic cyanogens from cassava flour using an orthophosphoric acid medium (see Total Cyanogens as HCN in Cassava Flour, Chapter 3).

In the development of a method to optimise the extraction of fresh cassava roots in dilute orthophosphoric acid, a new blender was designed to minimise processing time in field studies and tested using four varieties of cassava roots. The authors provide 29 literature references in a thorough review of the extraction of plant material (cassava) by wet and dry milling (pinmills and hammermills), sharp knife (Waring) and rotor-stator (Ultra turrax) homogenisers, extruders, and stomachers.[101]

Vortex

A fine dispersion is obtained by vortexing a solid sample (Cooper *et al.*, 1998, Chapter 6, ref. 81).

Stir to Mix

Stirring is one of the most frequently used operations in the homogenisation of liquid samples. Until recently, it was simply that, but with the advent of stir-bar sorptive extraction (SBSE) the modest (and, in the teaching laboratory, elusive!) stir bar is now an in-sample extraction aid. Flow injection analysis (FIA) systems often include an in-line mixing coil, which brings full automation closer (Azevedo *et al.*, 1999, Chapter 5, ref. 65).

5 Change of State

Vaporise (Volatilise)

The change of state of gaseous products from solid and liquid precursors when thermal, chemical, and enzymatic processes release volatile aroma and flavour compounds into the headspace is exploited in flavour chemistry. In food analysis, many volatile components are extracted by vaporisation (followed by condensation).

Dissolve or Liquify

A simple operation is to add water to a comminuted solid food, agitate for a while and then remove liquifiable fractions from the solid food matrix by filtration, centrifugation or decanting.

Samples like powdered infant formulae are dissolved in water, or, for example, 0.5 g in 10 ml 50% (v/v) EtOH,[102] in preparation for homogenisation, sonication and stir-bar mixing. The dissolved powder solution is then centrifuged and filtered to provide a supernatant liquid for analysis.

SPE of vitamin A and β-carotene from emulsified nutritional supplements required the sample to be dissolved in 5% (w/v) aqueous Na_2SO_4 containing 1 mM EDTA. Because the samples were light sensitive, this process was carried out in brown volumetric flasks.[103]

Slurry

Moist foods are homogenised into a slurry directly and low-to-medium moisture foods are mixed with water and then homogenised into a slurry. Slurrying can release bound analytes.[104] Dry ground pistachio nuts were compared with wet slurried samples for aflatoxin content, and the slurry released the mycotoxin for extraction.

Solidify

Solidification is associated with the change of state brought about by a temperature change, as in the solidification of butter. In analysis, it may be used to separate the solids phase from aqueous liquid whey.

Precipitate

In liquid foods, a precipitate may form naturally on standing, or precipitation can be induced by a chemical reaction to form a product of limited solubility. It may be possible to precipitate the compound and leave the impurities in solution, or *vice versa*. In food analysis, the separation of aqueous and lipid phases is a practical first step with many foods. (Adahchour *et al.*, 1999, Chapter 6, ref. 57). Precipitation is normally accompanied by filtration or centrifugation in chemical analysis.

Application of Precipitation Methods

In the determination of water-soluble vitamins in liquid and powdered infant milk,[105] protein was extracted effectively after precipitation with trichloroacetic acid (TCA), followed by centrifugation to separate the two phases. Recoveries were measured from two standard additions and values of >96% for nicotinamide, pyridoxal, pyridoxine, pyridoxamine and riboflavin, >88% for thiamin and > 76% for cyanocobalamin were recorded.

An ethanol precipitation step in the extraction of dietary fibre removes solubilised components, leaving low molecular weight (LMW) carbohydrates in solution.[106]

Comparison of Precipitation with Other Extraction Methods

TCA precipitation was compared to the reference dialysis method for the concentration protocol used in the evaluation of enterotoxin in dairy products, *e.g.* raw goat milk Camembert-type cheeses.[107] The TCA precipitation method for staphylococcal enterotoxins was quicker and easier and gave excellent recovery from dairy products.

Coagulate

Coagulation of proteins with TCA is well documented in food analysis. For example, proteins were extracted from coconut with water, salt solutions of different concentrations, HCl, NaOH, and HCl and NaOH at different pH. 1 M NaCl (100:75, coconut–salt solution) gave the best extraction and the protein extract was coagulated with TCA and re-extracted with water for amino acid analysis.[108] Saponins were extracted from white and green alfalfa leaf protein fractions by coagulating and washing the protein at pH 8.5. This removed four times more saponin than coagulation at pH 6.0 and washing at pH 4.5.[109]

6 Change of Chemical Composition

Derivative formation is well rehearsed for GC and GC-MS analyses where less-volatile compounds can be made volatile enough for equilibration between the gas and liquid phases. Additionally, the retention times of, say, free acids can be changed for identity checking, or the resolving of a complex region on the chromatogram. This approach is also useful in the extraction stage. Involatile fatty acids can be derivatised *in situ* releasing them into the HS from the liquid phase for GC analysis. In protein turnover studies phenylalanine was converted by enzymatic decarboxylation into phenylethylamine to avoid background interference with ions of the COI, and the heptafluorobutylamine derivative was made to facilitate GC-MS analysis of the headspace.[110]

React

Hydrolysis Reactions

Many hydrolytic reactions in acid, neutral, and basic environments are used in food analysis to render the complex starting material amenable to chromatographic separation as the aglycone. In a review of phenolic acids the preparation stages are reported to include hydrolytic cleavage reactions (Robbins, 2003, Chapter 8, ref. 52).

Enzyme Reactor

Oxalate Content. A rapid, mild extraction was required for soluble and total oxalate in foods because of its possible generation from ascorbic acid during extraction. Some 150 foods were tested for soluble and total oxalate content using an HPLC–enzyme reactor method that employed immobilised oxalate oxidase to convert oxalate into hydrogen peroxide. After enzymatic conversion in the reactor, H_2O_2 was estimated amperometrically after HPLC separation. The extraction process required homogenised samples to be suspended in 2N HCl (total oxalate) and distilled water (soluble oxalate) at various temperatures. Carrots, cherries, strawberries and cherry juice were used during the optimisation.[111]

Cholesterol Content. The immobilised cholesterol oxidase enzyme reactor was used to measure total (free and bound) cholesterol. Automatic FIA was used after the non-saponifiable fraction was dissolved in the water phase detergent, sodium cholate. H_2O_2 was measured photometrically.[112]

Chemical Labelling

1-Anthroylnitrile was found to react with T-2 toxin extracted from wheat, corn, barley, oats, rice, and sorghum using MeOH–water (80:20 v/v) and immunoaffinity chromatography (IAC).[113] The T-2 toxin reaction product was selectively analysed by HPLC fluorescence detection with a LOD of 0.005 μg g^{-1}.

Polymerase Chain Reaction (PCR)

The critical steps in the sample preparation for the detection of genetically modified crops in foods have been discussed by Terry *et al.*, 2002.[114] The production of purified DNA or protein, required for PCR techniques and immunodiagnostics, involves several cleanup steps, the optimisation of which was discussed.

Selected Reaction Monitoring

Using tandem mass spectrometry techniques, the change in mass between the analyte ions and its reaction product ions can be "set up". Any fragmentation process, during which specific mass is lost, will be recorded. Normally, the loss of a small neutral molecule, *e.g.* HCN or H_2O, is monitored, but specific losses related to a known reaction occurring in a chemical class, *e.g.* folate vitamers[115] or isoflavones and lignans, can be used.[116] As a result of using the highly selective reaction monitoring, simple extraction methods may be employed; in the latter case, one SPE step was sufficient and a LOD of the order of 10 pg ml^{-1} was achieved.

Defat

By far the most frequently used solvent for defatting food samples for analysis is n-hexane. In monitoring the migration of benzophenone, a volatile from UV-cured inks used in printing on food packages, foods in contact were defatted with n-hexane for analysis by GC-MS.[117] Whole coconut meal,[118] dry and 24 h imbibed rapeseeds and sesame seeds[119] were defatted with n-hexane. As were acidic chloroform extracts of wheat and oats in the extraction of ochratoxin A,[120] and the acetonitrile–water extracts of rice cultures in the analysis of zearalenone (A.K. Shrivastava and A.A. Ansari, 1992, Chapter 4, ref. 37)

Dehydrate or Lyophilise

Many of our foods, especially vegetables, contain high percentages of water, and the act of drying has been used for the preservation of food for centuries. The extraction of water to obtain the dry matter content of food is covered in the section on proximate analysis in Chapter 1. In food analysis various processes aid the drying of food samples.

Desiccate

The removal of moisture by absorption into a desiccant is used to reduce the level of moisture, or maintain samples moisture-free in the laboratory desiccator. Phosphorus pentoxide, anhydrous sodium sulphate, and silica gel are commonly used desiccants. The handling of hygroscopic substances requires desiccation.

Inactivate

Glucosinolates coexist with myrosinase enzyme in the plant. Therefore, any attempt to comminute the cellular structure of the sample for analysis will start a rapid enzyme hydrolysis. Samples can be completely dry (oven or freeze-dried) or frozen in liquid N. The use of aqueous MeOH and high temperatures is recommended to inactivate myrosinase.[121] To extract intact sinigrin, mustard seed was heated in an autoclave at 121 ºC for 10 min to inactivate the enzyme, and then ground in a food processor for 2 min. The seed meal was heated and boiling phosphate buffer was added, mixed, and shaken for 10 min in a bath at 100 ºC, cooled on ice and the suspension centrifuged ready for further processing.[122]

Deproteinise

The steps to inactivate the enzyme for glucosinolate analysis described in the previous section may be followed by deproteinisation with a 1:1 solution of barium and lead acetate (0.5 M each) (Jen *et al.*[122]). An alternative treatment for infant milk formula was to use acetic acid and sodium acetate.[123] In the

determination of nitrate and nitrite in 24 h diet samples, they were diluted with water and deproteinised with Carrez reagent.[124]

7 Automation and Miniaturisation

All the procedures that mechanise the sample preparation steps are included in this section. On-line separation and detection has been with us since the late 1950s, when GC was coupled to MS.[125] Many more combinations of separation and detection methods are available, including the successful linking of HPLC to MS. At the same time, the use of robotics to handle the more routine operations in sample preparation is providing commercial, stand-alone workstations that pipette solutions, transfer aliquots of reagents, wash syringes, evaporate, dilute, shake, stir and generally take over the manual processes of sample preparation from the analyst. These instruments are themselves being combined into "laboratories" capable of carrying out most of the stages identified here as the preparation for extraction preliminaries in food analysis.

The use of the flow-switching valve (FSV), a technique common in liquid chromatography, has enabled developers to link up preparative steps, so that the fully automated chemical analysis workstation is closer. Even the simple operation of FS during sample loading from a pre-column to the main column is an extraction aid, when an unwanted fraction of the sample is diverted away from the fraction containing the COI.

CE in its many forms is ideal for miniaturisation. The extremely small volumes of the fused silica tubing, with ids of <100 μm, mean that plumbing techniques like those used in the construction of analytical microchips are provided with connecting pipework for coupling new pre-separation devices to separation columns and their detection systems for automated analyses.

Immobilisation of enzymes and analyte conjugates as sensors may be miniaturised onto a chip. A deoxynivalenol conjugate with casein was immobilised on a sensor chip.[126] Competition for antibody binding between the sensor and free deoxynivalenol molecules in the test solution was measured. Three antibodies were compared. Because of the specificity of the sensor, a simple acetonitrile extraction and 10-fold dilution was all that was required in preparation for the surface plasmon resonance inhibition assay.

Flow Switching of Flowing Food Matrices

Pre-column as an Extractor

A simple FS protocol is shown in schematic form in Figure 2.8, where the pre-column is loaded from the food matrix, venting unadsorbed components to waste. After FS into the mobile phase the extracted (adsorbed) compounds are injected onto the analytical column. If higher resolution is required, the desorption of the analytes from the pre-column is arranged to be in the back flush mode. By so doing, any band broadening that occurs while loading the sample onto the pre-column will be reversed for higher resolution injection onto the analytical column.

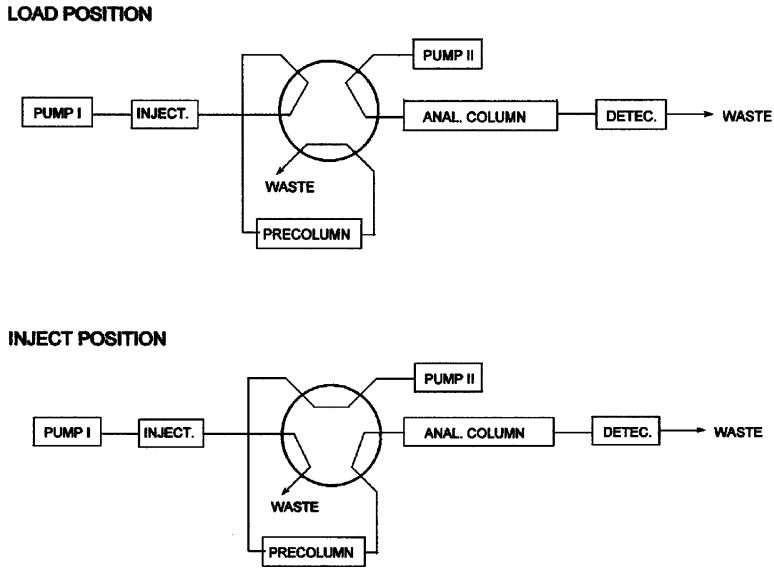


Figure 2.8 *Load and inject positions of a column switching system for the extraction of unwanted fractions, either of low affinity in liquid phase 1 (pump 1) or of selected affinity in liquid phase 2 (pump 2)*
(Reprinted from the *Journal of Chromatography A*, vol. 880, L. Bovanová and E. Brandšteterová, " Direct Analysis of Food Samples by High-Performance Liquid Chromatography", pp. 149–168, © 2000, with permission from Elsevier)

Application of Flow-switching for On-line Extraction–Separation

Gel Permeation Chromatography–Gas Chromatography (GPC-GC). An example of a flowing food matrix sampled on-line is the use of a GPC column, normally used for molecular weight order separations in its own right, put on-line with GC, under the control of a FSV, where it can serve as a pre-column extractor. Vreuls *et al.* used GPC to separate LMW organophosphorus pesticides from higher MW fat constituents of a sample of olive oil.[127] The GPC mobile phase containing the pesticide fraction was transferred directly to the GC and evaporation of the solvent was arranged *via* a solvent vapour exit before the sample entered the GC column.

Automation of the GC Injection

In GC-MS, *i.e.* automated separation and detection, the gas chromatograph has been on-line to the mass spectrometer for almost fifty years (Holmes and Morrell, 1957[125]) and, therefore, the further automation of the analytical system started with the automation of the GC injection process. Automatic injectors and sample handling carousels have also been around for many years, so the bottleneck for multi-sample operation is now in the "memory" of the liner in the

injection port. Koning *et al.* (2002) have described a liner exchange method[128] that inserts each sample in a μ-vial in an exchangeable liner, allowing the GC to be used continuously. The team continued the development programme with the publication of robotic handling of some sample preparation procedures, solvation and LLE, derivatisation, and the syringe maintenance associated with these operations. A method of difficult-matrix introduction for GC-MS was described that removes much of the manual clean up normally required to prepare samples for GC injection by using the OPTIC 3 (ATLAS GL International, Veldhoven, The Netherlands). Commercial ethyl acetate extracts of grape and pineapple spiked with pesticides were injected into a μ-vial held in a liner.[129] Automated sample preparation with the FOCUS XYZ, and direct thermal desorption injection (both from ATLAS GL International) put GC injection under the control of the FOCUS XYZ.

Automation of Coupled Sample Preparation and Separation

Miniaturisation of Sample Preparation and "Separation on a Chip"

The synergism of using fused silica tubing to devise and couple up miniaturised sample preparation techniques with CE methodology is seen as the way forward.

Introduction

Electrophoresis is the movement of charged particles in a liquid under the influence of an applied electric field. Electrophoresis can be carried out in fused silica tubing of 50 μm (and less) internal diameter and a length of a few centimetres, and columns of this size can be incorporated easily into "the laboratory on a chip". Connecting channels can be cut into the plate to construct "T" junctions for flow splitting and other "plumbing" necessary to couple electrochemical detectors, driving electrodes, injectors, *etc.* to these micro volume columns for on-line multiple-stage electrophoresis for extraction and separation of both charged and uncharged analytes. There are several discrete methods employing capillary electrophoresis that might be considered to operate in tandem for on-line, miniaturised sample preparation, extraction and separation. A brief résumé of CE techniques and some examples of tandem CE being used for preparation/separation is given.

Capillary Electrophoresis

Capillary electrophoresis is the family name of several electrophoretic separation techniques using narrow bore fused silica capillary columns. The inner walls of the capillary support a layer of readily ionised silanol functional groups. At a pH above 1 they are negatively charged, attracting a layer of cations from the buffer solution to form an electrical double layer (known as the Stern layer), which sets up a potential across the tube walls known as the *zeta potential* (Equation 2.1),

$$\zeta = \frac{4\pi\eta\mu_{eo}}{\varepsilon} \tag{2.1}$$

where η is the viscosity of the solution, ε is its dielectric constant and μ_{eo} is the coefficient of electroendosmotic flow. This double layer repels other cations, so that when a high voltage is applied they will be free to migrate to the negative electrode. As a result the bulk solution experiences a net flow toward the cathode. This phenomenon is known as electroendosmotic flow (EOF), which being the result of electrical attraction beyond a point very close to the capillary surface, the shear plane, causes all of the bulk electrolyte (buffer) solution to move with the same net velocity, creating a flow profile very close to the ideal "plug flow" model (Figure 2.9). This can be contrasted with the Poiseuille (laminar) flow profile exhibited by pressure-driven systems such as HPLC, which is caused by frictional forces.

As a result, the band broadening associated with HPLC pumping is eliminated in CE and separation efficiencies are much higher – several hundred thousand theoretical plates is common. EOF rate can affect efficiency and resolution indirectly as it influences the solute migration time. The overall rate of flow is given by Equation (2.2),

$$v_{eo} = \frac{\varepsilon\zeta E}{4\pi\eta} \tag{2.2}$$

where E is field strength (applied voltage/capillary length).

EOF is also essential to the practicable operation of CE analyses. When a mixture of analytes is introduced at the positive electrode, only the positively charged species will move away when the voltage is applied. However, the EOF is strong enough to sweep all analytes towards the grounded electrode eventually, and thus all components – cationic, neutral and anionic – can be determined in the same run. The order of expected elution is described diagramatically in Figure 2.10 and incorporates the ideas of both electroendosmotic flow and simple electrophoretic mobility.

This technique for separating ions according to their electrophoretic mobility has been applied to the separation of ionisable food components and was reviewed in 1996.[130] In 2000, the originator of MECC reviewed the use of CE

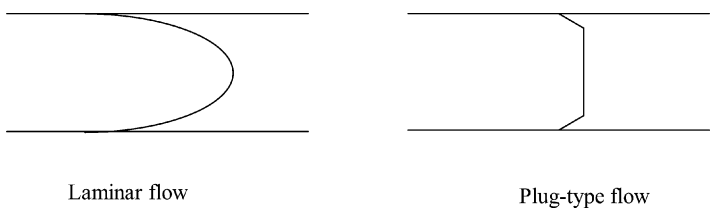


Figure 2.9 *Electrophoretic "plug-type" flow has a narrower front than the laminar flow associated with chromatographic separations*

for food pigment separation.[131] The capillary separating columns are 375 μm od and have an id from 100 μm down to 5 μm and are typically 50 μm for analytical separations. Now that capillary electrochromatography (CEC) is established, even neutral compounds may be separated in these low volume columns, – ideal for miniaturisation.

Capillary Zone Electrophoresis (CZE)

In free solution electrophoresis the sample is separated in a tube filled with buffered liquid and an electric field is applied *via* electrodes located at each end. Charged particles will migrate toward the anode (anions) or the cathode (cations) and separation occurs as a result of differences in rates of this *electrophoretic migration*; these rates are in turn directly related to the charge-to-mass ratios of the species in solution. The free solution method was developed and applied to protein separation by Tiselius, for which he was awarded the 1948 Nobel Prize. The general principle of ion migration is summarised in Figure 2.10.

Flow Injection – CZE. Anionic exchange resin micro-column SPE of *myo*-inositol phosphates was put on-line to CZE with an FI system for the analysis of food samples.[132]

Micellar Electrokinetic Capillary Chromatography (MECC)

Principles and Practise. Terabe introduced the technique of MECC in 1984. Its defining characteristic is the inclusion of a surfactant modifier in sufficient

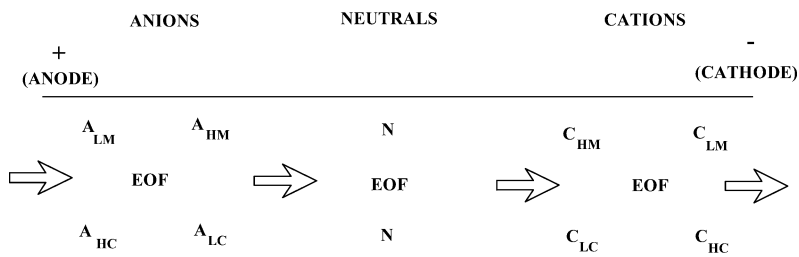


Figure 2.10 *Order of elution of analytes in CZE. The direction of the EOF in an untreated column will be from the anode to the cathode. Neutral components will travel in an unseparated zone in the electrolyte at the speed of the EOF. Anions A will have an electrophoretic mobility towards the anode but will be carried by the electroosmotic current towards the cathode and therefore their elution will be retarded compared to the neutrals. Conversely, cations will experience mobility in the same direction as the EOF and, therefore, will move ahead of the neutrals. Within each group, it is possible to add the effect of the mass-to-charge ratio on individual ions. The order of elution from the column will be low mass (LM) and high charge (HC) cations followed by high mass (HM) and low charge (LC) cations; neutrals; HM and LC anions; and LM and HC anions*

concentration to promote the formation of micelles in the electrolyte. Conditions are selected to ensure that the migration of the charged micelles opposes the general EOF, but the EOF must be strong enough to sweep the micelles in the direction of flow so that they will eventually move past an on-line detector. MECC essentially introduces an additional partitioning factor into the separation, which is dependent upon the hydrophobicity of the analytes. The less polar species will tend to have a higher residence time within the organic micelle "capsule" and so will be further slowed in their migration as they migrate against the EOF within the surfactant moiety (Figure 2.11).

The micelles are often referred to as a pseudostationary chromatographic phase. The formation and dissolution of micelles is a rapid, dynamic process – each micelle has a lifetime measured in microseconds.

The great strength of MECC is that mixtures of neutral compounds can be separated, as the separation mechanism is no longer entirely dependent upon the charge-to-mass ratio. The technique also enhances the separation of components that are similar in chemical nature if the combination of their electrophoretic and chromatographic "mobilities" are different.

The most common additive is the anionic surfactant sodium dodecyl sulphate (SDS). The influence of the surfactant can be much more significant with cationic additives. The most documented species are the alkyl ammonium halide salts, such as cetyltrimethylammonium bromide (CTAB). When a voltage is applied, CTAB is attracted by electrostatic forces to the capillary wall where it forms a dynamic, cationic layer. Anions are attracted to the layer and hence the charge of the electrical double layer is reversed, reversing the direction of the electroosmotic flow from the negative to the positive electrode, with the micelle phase attempting to migrate in the opposite direction (positive micelle charge – migration toward cathode). The order of analyte elution is therefore reversed.

Ion-pairing effects can further complicate the mechanism. For highly water-soluble compounds, the residence time predicted for an analyte in the micelle phase would be small. However, if the analyte is of opposite charge to the

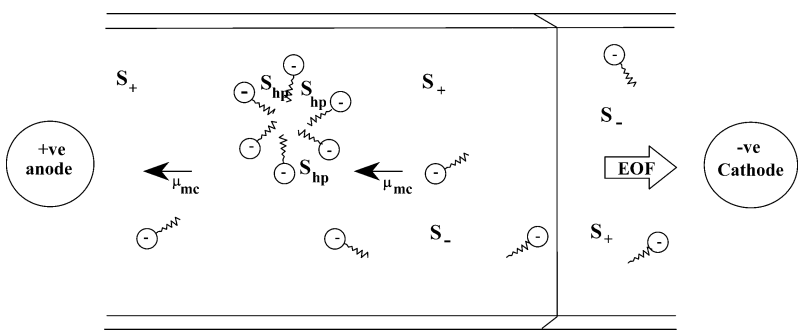


Figure 2.11 *Illustration of the MECC process, where S_+=positively-charged solutes; S_-=negatively-charged solutes; S_{hp}=hydrophobic solutes; and m_{mc}=micellar electrophoretic mobility*

micelle surface it will be attracted to that surface, increasing the probability of admission to the micelle. The behaviour of such mechanisms is difficult to predict due to their complexity, but they can yield powerful separations.

Where the separation could be aided by ion-pairing but, say, non-ionic surfactants are in use, the addition of metal salts to the buffer can facilitate interaction with the micelles by providing adsorbed metal ion charge sites in their surface.

MECC-MS. The general approach has been to carry out MECC in the first capillary then "heart cut" the band by voltage switching and buffer replacement, shunting the MECC band along the second capillary to the MS while the surrounding electrolyte with surface-active agents is sent to waste, thus avoiding the depression of the ion signal (Figure 2.12).

This valveless switching may also be used to cut fractions – albeit in micromolar quantities – for further study.

MECC compared with Microemulsion Electrokinetic Chromatography (MEECC) for Preservatives in Foods. The authors explain the difference between MECC and MEECC and describe the application of MEECC to the separation of preservatives in soft drinks, soy sauces and wines.[133]

Isoelectric focusing (IEF)

By filling a capillary tube with sections of electrolyte of graded pH (ampholytes), compounds with different isoelectric points (pIs) will, when an electroosmotic force (EOF) is applied to the electrolyte, migrate to the point in the tube where they have zero charge – their pI. Thus, after phoresing the sample for some time, the components of similar pI will be concentrated at various points along the column. The "column" of electrolyte can then be advanced say, hydrodynamically, to drive each component sequentially towards the next stage of separation, where each band acts as a discrete sample for further analysis.

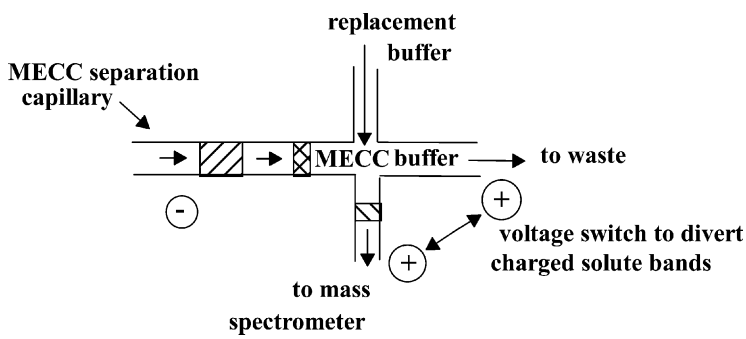


Figure 2.12 *Valveless switch for cutting fractions for further study*

Capillary Isotachophoresis (CITP)

Principles and Practice. Figure 2.13 illustrates the principle of CITP. A capillary is filled with a discontinuous buffer system, *i.e.* two solutions in which the analytes will have different mobilities. On the anodic side of the separation zone an electrolyte of high electrophoretic mobility is used as the *leading electrolyte* (LE), and at the cathodic end a low mobility buffer is employed as the *terminating electrolyte* (TE). The analytes migrate in consecutive sample bands, which yield a step-like profile called an *isotachopherogram* at the interface between the two separation media. The band length depends upon the concentration of the sample. The technique requires a capillary that has been treated to eliminate EOF and cannot be applied to determine both cations and anions simultaneously. CITP has the disadvantage of a lower resolution than CZE.

This low resolution was to limit the application of ITP as a separation technique. But when it is used as a pre-concentration method, placed in tandem with a high-resolution separation technique, it provides a starter separation and concentrates the components of the initial sample into fairly crude but discrete fractions. If "stacking" methods are used too (Figure 2.14), these fractions or

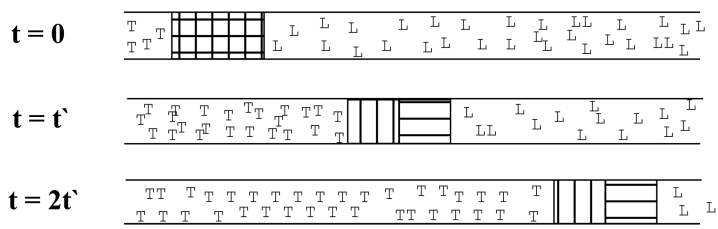


Figure 2.13 *After time* t = t′ *the components of the sample separate into two adjacent bands between the leading (L) and terminating (T) electrolytes, and are not further resolved after time* 2t′

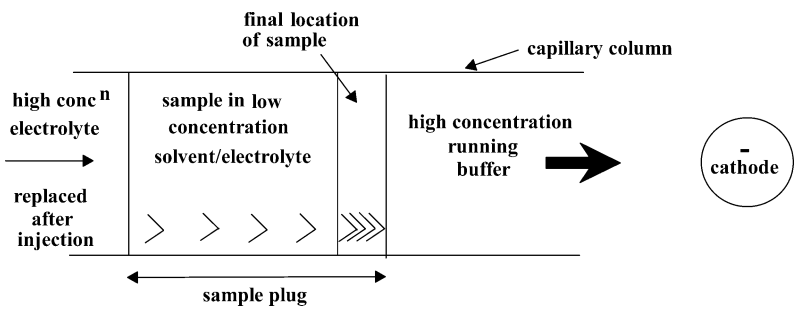


Figure 2.14 *The principle of sample stacking uses the fact that ions migrate faster in lower concentration electrolytes. The low concentration electrolyte used to load the sample and stack the analytes into the front section of the "plug" is replaced by the high concentration running buffer to effect the separation*

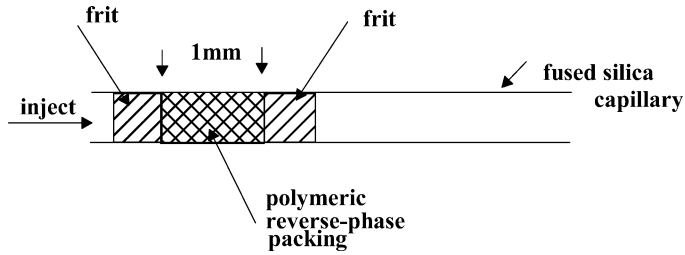


Figure 2.15 *On-column concentration trap*

extracts can be concentrated for direct injection into a coupled CZE or CEC stage for final separation.

Another method of concentrating sample for CE separation is the on-line adsorption trap (Figure 2.15). The sample is loaded by adsorption from the flowing matrix and desorbed by a small volume of a suitable solvent.

Application of Off-line CITP Prior to HPLC of Wine. Off-line ITP was used to pre-concentrate and fractionate wine samples before separation by HPLC. The paper describes the optimisation of the ITP conditions for hydroxybenzoic, hydroxycinnamic acids and flavanoids from various types of wine, exploiting the concentrating power of ITP.[134]

Capillary Isotachophoresis–Capillary Zone Electrophoresis (CITP-CZE)

The principle of isotachophoresis (ITP) enables ionised sample mixtures to be separated into adjacent bands wedged between a terminal electrolyte and a leading electrolyte. If a selected band containing the analyte is "stacked" (concentrated by selective ion acceleration techniques) and then eluted into a zone electrophoresis column, further high-resolution separation can be effected and the pure analyte peak detected as it emerges from the second capillary column. These two operations can be coupled on a chip so that the analyte extraction and concentration from the sample medium and its purification and detection are both miniaturised and on-line. The ITP stage (and/or a stacking operation) is necessary as a preliminary concentration step to provide a sample intense enough for CZE separation and detection, and, therefore, is considered to be a sample preparation for extraction method.

EDTA in Mayonnaise. ITP was coupled on-line to CZE for the analysis of EDTA in mayonnaise.[135]

Free Sulphite in Wine. In an excellent example of modern method development, CITP-CZE was used in a feasibility study of the measurement of free SO_2 in wine.[136] It was argued that the time-consuming distillation step in the MWD

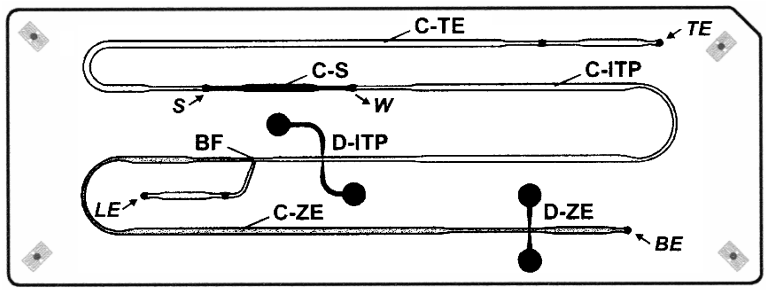


Figure 2.16 *Poly(methyl methacrylate) chip with (C-TE, terminating electrolyte channel), (C-S, sample injection channel),(C-ITP, separation), (D-ITP, detection), (C-ZE, separation), and (D-ZE, detection) channels. Inlet/outlet (S)/(W), (BF, branching point for introduction of LE, the leading electrolyte), TE, introduction of terminating electrolyte, and BE, introduction of background electrolyte*
(Reprinted from the *Journal of Chromatography A*, vol. 1026, M. Masár, M. Danková, E. Ölvecká, A. Stachurová, D. Kaniansky, and B. Stanislawski, "Determination of Free Sulfite in Wine by Zone Electrophoresis with Isotachophoresis Sample Pretreatment on a Column-coupling Chip", pp. 31– 39, © 2004, with permission from Elsevier)

method was a serious limitation in modern analyses. Although free sulphite will migrate in CZE, it was also decided that the formation of a stable sulphite-formaldehyde complex, hydroxymethanesulphonate (HMS), was a prudent precaution against losses during analysis. Sample preparation entailed simply adding formaldehyde to the wine sample and diluting before loading a sample onto the columns-on-a-chip system. Figure 2.16 shows the chip system.

ITP-CZE of Phenols in Red Wine. The increased sensitivity and resolution of CE in this form was reported for the analysis of 14 flavonoids and phenolic acids from red wine samples.[137] The LE was 10 mM HCl (pH 7.2), with Tris as counterion, and the TE was 50 mM boric acid (pH 8.2) adjusted with $Ba(OH)_2$. The CZE electrolyte was 25 mM MOPSO, 50 mM Tris, 15 mM boric acid, and 5 mM cyclodextrin (pH 8.5). All electrolytes were modified with 20% (v/v) MeOH. Picric acid colour marked the stacked zones extracted by ITP for separation by CZE.

The schematic for the phoresing voltage supplies, pumps and detector control units is shown in Figure 2.17.

The stepwise sequence of operations in the preparation, extraction and separation of the analyte are shown in Figure 2.18.

Capillary Electrochromatography (CEC)

Electrochromatography combines the high resolution of the CE family techniques with the selectivity and sample capacity of microbore HPLC, without creating a backpressure. This relatively new technique provides separation of

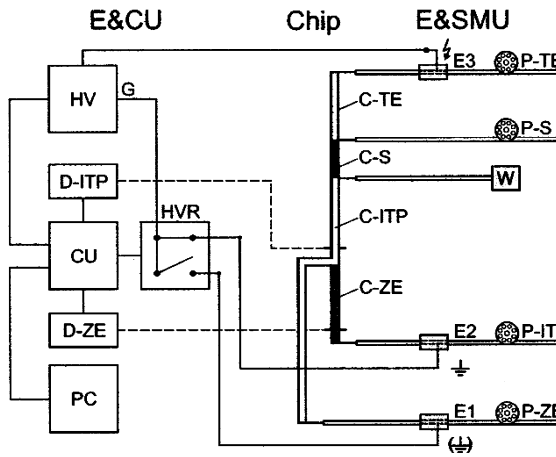


Figure 2.17 *Chip controls. E&CU, electronics and control unit containing; (HV) polarising electrode supplies, (CU) control unit, D-ITP and D-ZE conductivity detectors, (HVR) endosmotic flow (phoresing current polarity) switch, (G) ground potential. The chip, labelled as Figure 2.16, and the E&SMU, electrolyte and sample management unit containing P-ITP, P-ZE, P-S, and P-TE, peristaltic pumps, for ITP, CZE, sample, and terminal electrolyte, (W) waste, E1 and E2, driving electrodes for ITP and CZE stages, and E3, terminating electrolyte driving electrode*
(Reprinted from the *Journal of Chromatography A*, vol. 1026, M. Masár, M. Danková, E. Ölvecká, A. Stachurová, D. Kaniansky, and B. Stanislawski, "Determination of Free Sulfite in Wine by Zone Electrophoresis with Isotachophoresis Sample Pretreatment on a Column-coupling Chip", pp. 31–39, © 2004, with permission from Elsevier)

both charged and neutral analytes in a packed capillary column with the mobile phase driven by EOF, producing "plug flow" conditions rather than the laminar flow condition associated with chromatographic separations, *i.e.* with the potential for very high resolution. For a treatment of the migration of ions in CEC, the paper by J. Ståhlberg is recommended.[138]

Sample Preparation Techniques Coupled to CE

Subject Reviewed. Valcárcel *et al.* (2001) (Chapter 8, ref. 34) has reviewed the coupling of continuous separation techniques to CE; 457 references are quoted and the paper is recommended for the complete coverage of applications, many from the area of food analysis.

Continuous-flow System/CE. A continuous flow system was coupled to CE for the preparation of phenolic compounds extracted from citrus fruit.[139] The manual process was to prepare and centrifuge the pulp. The supernatant was the sample for the continuous flow system with a C_{18} minicolumn for SPE using MeOH as eluent.

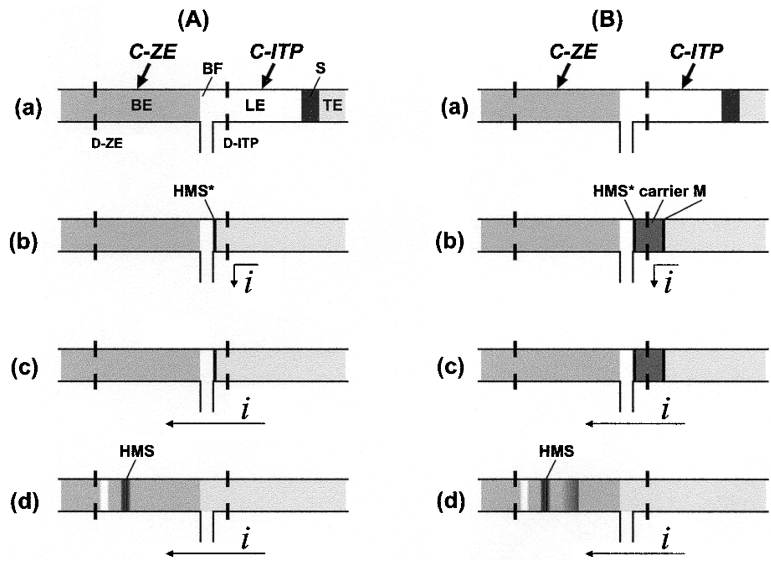


Figure 2.18 *The electrophoretic steps in the development of the resolution of the analyte.* (A) *Represents the conditions used initially.* (a)–(d) *the steps in the progression of the analysis. Symbols are the same as Figure 2.17.* (a) *Sample is loaded between the LE (chloride anion) and the TE (tartrate anion),* (b) *HMS band is allowed to migrate to the "T" junction with the LE venting to waste.* (c) *When the phoresing current direction is changed to the CZE column, the HMS band crosses the "T" and,* (d) *continues to separate. Unfortunately, with this arrangement, a sample matrix component, pyruvate, co-eluted with the HMS. Therefore, a change was made to the electrophoretic conditions shown in* (B). *A discrete spacer electroltye was introduced into the protocol.* (a) *Sample plus spacer (dichloroacetate anion) loaded as in* (A). (b) *The carrier/spacer separates the HMS from the pyruvate band labelled M (matrix component).* (c) *The driving current direction is changed to the CZE column, and* (d) *the HMS elutes free from pyruvate interference*
(Reprinted from the *Journal of Chromatography A*, vol. 1026, M. Masár, M. Danková, E. Ölvecká, A. Stachurová, D. Kaniansky, and B. Stanislawski, "Determination of Free Sulfite in Wine by Zone Electrophoresis with Isotachophoresis Sample Pretreatment on a Column-coupling Chip", pp. 31–39, © 2004, with permission from Elsevier)

"Extractionless Techniques"

Introduction

This monograph, collating extraction methods, comes at a time when the demise of the extraction method is in view! Remote spectroscopic sensing methods, in-line detection, *e.g.* by specific immobilised enzymes, augers the future of target compound recognition by an array of sensors monitoring the sample for analysis, or even "extracting" the analytical data from the bulk food as it is processed.

Direct Chromatography

There are already some assays where extraction is circumvented by careful choice of experimental conditions. Eight synthetic food colorants (Amaranth, Brilliant Blue, Indigo Carmine, New Red, Ponceau 4R, Sunst Yellow, Tartrazine, and Allura Red) were separated by HPIC using very low hydrophobicity anion-exchange (Dionex IonPac AS11) with 2.0 mol l^{-1} HCl–acetonitrile gradient elution such that the on-line column clean up allowed for continuous routine analyses of carbonated drinks, fruit juice, and fruit-flavoured instant drink powder.[140] The sample preparation was to dissolve the powder, degas the carbonated drinks, dilute and filter. The strong acid conditions suppressed ionisation.

Immobilised Enzymes

Aldehydes. The combination of the separation efficiency of HPLC, the sensitivity of electrochemical detection and the specificity of enzymes was put to work to determine aldehydes, indicators of deterioration, 5-hydroxmethyl-2-furaldehyde and 2-furaldehyde in particular. A cation micromembrane suppressor and enzyme reactors packed with VA-Epoxy on which aldehyde dehydrogenase from baker's yeast and NADH oxidase from *Bacillus licheniformis* were immobilised were utilised prior to HPLC analysis.[141] The efficiency of the method was demonstrated with honey, coffee, sherry, port, dry fruits and breakfast cereals.

L-Asparagine. The determination of L-asparagine in oranges, asparagus, orange and apple juices was effected using an asparginase biosensor and an asparginase reactor.[142] The principle being that asparagine is hydrolysed by the enzyme to aspartate and NH_3, and the NH_3 detected potentiometrically *via* a pH meter (biosensor) or spectrophotometrically. The biosensor was made by dissolving the enzyme in a polymer squeezed between two glass plates. Polymerisation took place at room temperature in 30 min. A disc of the membrane was cut for use with a pH electrode situated in a flowthrough cell in a FIA system. The reactor was made by adding VA epoxy to asparaginase and shaking for 50 h at room temperature. A buffer was added to the suspension, and after sedimentation the polymer carrier liquid was decanted and stored in buffer at 4 °C until used. The reactor was inserted into a FIA system upstream of the reaction coil, where mixing with the basic solution released NH_3, which diffused through the PTFE membrane in the gas diffusion cell into the acceptor solution flow line in the gas diffusion cell for transmission to the detector, where the pH change of the acceptor solution was measured. (Summarised from ref. 142 with permission from Elsevier)

Biogenic Amines. Putrescine, cadaverine, histamine, tyramine, spermidine, spermine and tryptamine in anchovies were detected electrochemically on a Pt

probe with diamine oxidase immobilised on its surface.[143] The immobilisation process and the pH were optimised, and the method compared to the use of IC-PAD.

Pesticides. OPPs were detected by the irreversible inhibition of cholinesterase activity using an electrochemical biosensor. Potentiometric detection uses cholinesterase immobilised on the surface of a pH sensing electrode, and amperometric sensors use choline oxidase in addition to esterases, indirectly monitoring choline by O_2 consumption or H_2O_2 production.[144]

Surface Plasmon Resonance (SPR)

Folic acid, vitamin B_{12} and pantothenic acid have been measured using SPR. Polarised light is reflected from a metal/liquid sensor surface forming the underside of a continuous flow channel. At a chosen angle and wavelength, SPR causes a reduction in the light intensity for analyte detection. Commercial equipment is manufactured by Biacore AB, Uppsala, Sweden.

μ-TAS

Introduction

Miniaturised total chemical analysis (μ-TAS) was created when the use of narrow bore fused silica tubing for CE demonstrated the high resolving power obtainable with <5 micron i.d. tubes. This could only be exploited in practice if equally low volume connections and injection and detection instrumentation was available. An early paper introduced the concept and looked at the theoretical performance of μ-TAS systems based on FIA, chromatography and CE.[145] Practical application of CE on a chip followed.[146] The modern μ-chip CE method was applied to the analysis of phenolic acids using amperometric detection.[147]

Microdialysis and μ-TAS

Microdialysis (Chapter 7) is another low volume extraction device that has been incorporated into the total chemical analysis armoury.[148] It was used for glucose and lactate measurement. Recently, a flow-through potentiometric sensor has been based on a microdialysis system using the μ-TAS and lab-on-a-chip approach for potassium monitoring.[149]

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CHAPTER 3

Partition

1 Introduction and Nomenclature

Partition is the core subject of any work on extraction methods. It is the process of dividing the sample into parts or the separation of one part from another – the extraction. To partition a mixture is to perfuse a part of it into a different compartment. In extraction processes, molecular partition at a boundary is dependent upon the affinity of the particle for the adjoining medium. Transfer across the boundary continues until the "pressure" (energy) on either side of the boundary is in equilibrium when the quantitative transference stops. At this point, the number of particles moving in one direction equals the number moving in the opposite direction.

Although there is no clear analytical division between partition and solvation (Chapter 4), it is possible to treat the fundamental concepts of partition in and between the various states of matter as an issue separate from the more practical business of solvent partition–extractions.

Because partition is fundamental to extraction science, a Case Study has been included in Chapter 6 to show some results of recent experiments.

Partition Equilibrium

One of the earliest methods of extraction in chemical analysis was to employ the partition equilibrium of a binary liquid/liquid phase system. A liquid (containing a mixture of solutes) was shaken with an immiscible solvent in a separating funnel. After a settling time, equilibrium was established between the two solvents such that the number of particles (ions or molecules) (P) of solute transferring across the interface in both directions was equal. If there is a change in any of the conditions governing the equilibrium then according to Le Chatelier's Principle, the equilibrium will move to compensate for the change (Equation 3.1).

$$P_A \rightleftharpoons P_B \tag{3.1}$$

For example, an increase in temperature will generate an endothermic change in the concentration relationship.

Partition Constant

In this steady state condition a fundamental relationship exists between a solute and the two partitioning solvents defined as the partition constant (K Equation 3.2),

$$K = \frac{[P_A]}{[P_B]} \tag{3.2}$$

where $[P_A]$ is the concentration of the solute in solvent A and $[P_B]$ is the concentration of the solute in solvent B. Square brackets, [ ], are the symbol used to signify concentration of solute in the solvent. Some writers use partition coefficient (K), others use distribution coefficient (K) or distribution constant (K).

In Figure 3.1, where $K_{A/B} > 1$, equilibrium is established with solvent A containing most of the solute. In analysis, an efficient extraction of the solute dissolved in solvent B could be made by partitioning it with solvent A, when the equilibrium would move to transfer molecules of the solute across the boundary into solvent A. This would be worth doing if other interfering solutes were less soluble in solvent A than in solvent B.

However, if $K_{A/B}$ had been = 1, the solute would have been equally distributed between the two solvents and there would have been no practical partition–extraction to concentrate the solute.

Partition–Extraction of Food Samples

Partition equilibrium may be established among various immiscible states of matter such that the concentration of molecules of a common solute will be different in the different states. Gas/gas, gas/liquid, gas/solid, liquid/liquid, and liquid/solid partitions form the bases of practical extractions as well as chromatographic separations. In precise physical terms, partition is not a separate extraction process, it is the result of molecular diffusion, permeation, solvation or adsorption of a common solute across a boundary permeable to the solute but largely impermeable to the solvents constituting the boundary. Thus the partition constant is the absolute means of quantifying the likely outcome of a proposed extraction.

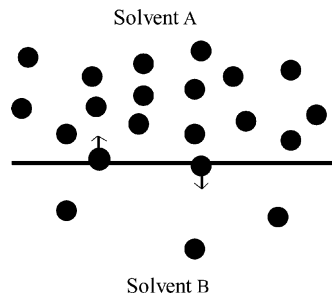

Figure 3.1 *Diagram of the state of dynamic equilibrium between solvents A and B, where ● represents a particle of solute whose partition coefficient $K_{A/B}$ is* >1

In the analysis of food, where all states of matter may be encountered, various partitions are involved in extracting components from the food matrix. For example, in flavour analysis the diffusion of volatile substances into the gaseous headspace involves gas/liquid and gas/solid partitioning from liquid and solid food matrixes respectively. If the headspace is a closed volume, as in analytical headspace sampling chambers, equilibrium will be reached and the headspace is said to be saturated in each component while the ambient (including the biological) conditions remain unchanged. It is helpful to define the types of partition processes that might be encountered in food analyses. However, unlike partition in analytical chemistry, the partitioning of a sample of food cannot be viewed as a precise science. Nevertheless, the following different categories may be recognised.

Liquid Food/Solvent Partitioning

A liquid food sample is partitioned with an immiscible solvent.

Solid Food/Solvent Partitioning

A disintegrated dried food sample (powder) is brought into intimate contact with a liquid solvent.

Suspended Solid Food/Solvent Partitioning

A simple method of extracting water-soluble food components is to grind dried foods to a powder, or freeze dry and comminute other foods, and then suspend the dried material in water to release the water-soluble constituents and partition them with an immiscible organic solvent. (Murphy *et al.*, 2002, Chapter 4, ref. 19)

Solid Food/Liquid/Liquid Partitioning

Organic compounds are extracted from foods using liquid/liquid partition extractions. Liquid 1 is used to extract the analytes from the solid food sample and liquid 2 is used to partition liquid 1. The partition process is used either to extract the target compounds from interferents or to remove interferents from the target compounds.

Solid Food/Liquid/Liquid Partitioning via *an Adsorbent Bed*

Often, the liquid extract from the food sample is applied to a bed of porous solid adsorbent and the adsorbed material partitioned from the bed by a second solvent such that the compounds of interest are eluted but unwanted material soluble in the first solvent is left behind. The modern term solid-phase extraction (SPE) applies, but the solvent extraction and partition stages are equally important parts of the process and the solid phase adsorption might equally well be viewed as a partition aid.

Simultaneous Solid/Liquid and Liquid/Liquid Partitioning

Comminuted foods of various moisture contents are partitioned with an immiscible non-aqueous solvent. This is the most ill-defined and, perversely, the most common area of food extraction.

Solvent and Solid-Phase Extraction

Solvent extraction is alternative nomenclature for the partition processes defined above. Many of the applications of partition–extraction fit better under the heading of solvation in the section on solvent extraction (Chapter 4). Many of the fundamental concepts of the partition constant and the use of a binary phase partition, set up between two pure liquids, belong here and will be expanded in later sections.

Often in the scientific literature a binary liquid extraction–partition of soluble analytes from food matrices is performed using a porous, powdered solid as an intermediate "holding" or adsorption phase, or partition aid. Method development will have established that the compounds of interest are soluble in both solvents and reversibly adsorbed onto the solid phase from solvent 1, and desorbed from it by solvent 2. The exchange of analytes from solvent 1 – the liquid/solid absorption – to, ideally, the more selective solvent 2 by desorption or partition will remove interferents in the following steps:

1. Solvent 1 is percolated through the adsorbent, when some solutes may not be adsorbed and run to waste.
2. Solutes adsorbed from solvent 1 are partitioned with solvent 2, when some adsorbed solutes may not be soluble to any practical extent in solvent 2, remaining on the solid phase.
3. The target analytes (and other co-eluted solutes) partition into solvent 2. The extraction–partition has concentrated the target analytes with respect to their starting concentration in the sample for analysis.

SPE is just such a process where the nomenclature is focused on the solid-phase adsorption of analytes from liquid 1 (crude extract) and their desorption (often in both purified and concentrated form) into the higher selectivity solvent, liquid 2. There may be many stages in the extraction–partition process involving liquid and solid phases in various combinations. The principles of extraction partition are exemplified here and the applications of it are dealt with in the next four chapters on extraction methods.

2 Gas/Liquid Partition (GLP)

Introduction

In flavour research, gas/liquid partition constants were put to use in the early days of the development of gas chromatography to build relationships between

instrumental and olfactory methods of the perception of volatile compounds being adsorbed by the stationary phase (model system) or by the olfactory epithelium.

Air/Water Partition Constants ($K_{A/W}$)

Flavour Analysis and Olfactory Sensing

One of the first examples of the use of partition constants in flavour analysis was reported by Buttery *et al.* in 1965 when they measured the air/water partition coefficients of some low-boiling aldehydes released from foods during the cooking process.[1] Conversely, headspace GC (HS-GC) was used to calculate the air/water partition constant, *e.g.* of ethyl acetate.[2]

The rate of release of aroma compounds from the food matrix into the headspace, for subsequent extraction by the sensory epithelium, is governed by the affinity of the compound for the matrix. A well-defined protein, β-lactoglobulin, used in food processing to simulate the sensation of fat in the mouth was used in various concentrations and at various pH in model studies of the changes occurring in the air/water partition constants of 20 aroma compounds.[3] Concentrations of the protein of 0%, 0.5%, 0.7%, 1.0%, and 2% generally depressed the partition constant. Effects of protein concentration, pH, functionality of the chemical compound, and its chain length, and the presence of other components on the air/water partition constants measured by GC were reported.

The perception of volatile compounds released prior to and during the eating of food involves two routes to the olfactory surfaces, the orthonasal (sniff) and the retronasal (in the mouth) routes. Models have been constructed that use the partition and mass transfer constants, and also take account of the mixing between phases and airflow rates. In a modern approach to the release of volatile substances from foods, Professor A. Taylor and his team at Sutton Bonington, UK, have built on physicochemical foundations to study the air/water partition constants ($K_{A/W}$) and mass transfer in both phases governing the release of volatile compounds, *e.g.* when food containers are opened to the atmosphere.[4] To do this, they considered how the headspace volatile profile changed as the equilibrium concentrations were disturbed when the headspace is diluted with air. In a study of the extraction of volatiles into the headspace of a solution, compared to the breath volatile content (exhaled from the nose and mouth) after consuming the same solution, Linforth *et al.*, (2002) found that the amounts of volatile in the breath were less than in the headspace.[5] Breath exhaled from the mouth was found to contain, on average, 8-fold more volatiles than breath exhaled from the nose. *Inter alia*, compounds with the lowest air/water partition constants were absorbed more at the nasal epithelia. It is difficult enough to measure partition constants in standard chemical experiments, but to measure them in biological environments is even more challenging. The subject is marginally appropriate for inclusion in this book, but the involvement with partition leads to the notion of the human subject extracting stimuli from the

environment and therefore the calculation of perception and odour intensity contain mass transfer and partition factors. It is not for the fainthearted to calculate the interactions of odour impact chemicals with the olfactory epithelia!

Application of Gas/Liquid Partition

Rapid Extraction of Volatile Organic Compounds (VOCs) from the Aqueous Phase. To speed up and automate the extraction of VOCs from aqueous samples for HS-GC, an on-line vaporiser (spray/gas stripping) was described that reduced the time required for stripping VOCs for GC analysis[6] compared to, say, SPME-GC. Cyclohexane, toluene, *o*-xylene, isopropylbenzene and *o*-dichlorobenzene were used for the trials. The vaporised substances were trapped downstream cryogenically and vaporised for GC analysis. Correlation with Henry's law constants (K_H), relative volatilities from modified matrices, and aqueous diffusion coefficients showed that analyte stripping from water was modelled best by relative volatilities from modified matrices. For isopropylbenzene, 0.1% MeOH in water reduced the extraction time by 35%, and 0.1% NaCl by 22%, but 0.01% SDS increased the extraction time by 13%.

Steam distillation/condensation stripping of VOCs was used early on in the development of the rapid analysis of cooked food aroma using cryogenic (focusing) enrichment GC.[7]

A spray and trap system was described recently which continues the development of rapid, automated extraction methods for VOCs in aqueous samples.[8] Benzene, toluene, ethylbenzene, *m*-xylene, and *o*-xylene were used in the study.

Measurement of Henry's Law Constants. A proton transfer reaction MS method of measuring K_H that defines the air/water partition was applied to breath analysis and to the emission of VOCs from fruit, coffee, and meat. The dependence of Henry's law constants on temperature and salt content of water was studied.[9]

Vinyl Chloride/Water Partitioning. The movement of gases across an air/water interface was studied in a vinyl chloride water model system of interest in food chemistry. The diffusion constant of vinyl chloride (VC) into water, the partition constant of VC at the gas/liquid N_2 carrier gas/water interface, mass transfer constants for VC in the N_2 and water phases, and the Henry's Law constants for the absorption of VC in water were determined using mathematical analysis and reversed-flow GC.[10]

Air/Tetraglyme Partitioning. The use of tetraethylene glycol dimethyl ether (tetraglyme), a water-soluble organic liquid, in the measurement of air/tetraglyme partition constants ($K_{A/T}$) of chlorinated alkanes, alkenes, and monocyclic aromatic hydrocarbons has been reported.[11] The effect of tetraglyme on the air/water partition of organic compounds was investigated.

Essential Oil/Air Partitioning. Adsorption of air pollutants by essential oil plants prompted the study of the $K_{EO/A}$ partition constant for monocyclic aromatic hydrocarbons (MAHs).[12] The five MAHs, benzene, toluene, ethylbenzene, *p*-xylene, and *o*-xylene, are also important volatiles in foods.[13] Nine species of aromatic plant leaves were extracted in a modified Likens–Nickerson apparatus using DCM in multiple extractions as required, and the total solvent evaporated in a rotary evaporator. Overnight equilibrium was established for equal volumes of the MAHs over the essential oil extract in 5.5 or 1.5 ml vials closed with a Miniert valve (Alltech) and an aliquot of headspace taken for GC analysis. Two orders of magnitude difference in $K_{EO/A}$ were recorded among the five compounds. The importance of the work for food science is the observed difference between $K_{EO/A}$ and $K_{O/A}$ for lipophilic systems, and therefore the need to measure partition constants directly.

Sources of Air/Water Partition Constant Data

Table 3.1 *References to partition constants*

Chemical class	*Chapter*	*Authors*	*Reference*
Gibberellins A_3 and A_8	3	R.C. Durley and R.P. Pharis	*Phytochemistry*, 1972, **11**, 317
Printing ink solvents	–	G.W. Halek and E. Hatzidimitriu	*J. Food Sci.*, 1988, **53**, 568
Packaging solvents	–	G.W. Halek and A. Chan	*J. Food Sci.*, 1994, **59**, 420
Five MAHs ($K_{EO/A}$) EO/A=essential oil–air	3	R. Keymeulen, B. Parewijck, A. Górna-Binkul and H. Van Langenhove.	*J. Chromatogr. A*, 1997, **765**, 247
Benzaldehyde	7	S.M. Mousavi, S. Desobry and J. Hardy.	*J. Food Eng.*, 1998, **36**, 453
Five aroma compounds	3	M. Marin, I. Baek and A.J. Taylor.	*J. Agric. Food Chem.*, 1999, **47**, 4750
DMDS, DMTS, MTB	7	F.X. Pierre, I. Souchon and M. Marin,	*J. Membr. Sci.*, 2001, **187**, 239
Food colour red No. 106 (acid red)	4	H. Oka, M. Suzuki, K-I. Harada, M. Iwaya, K. Fujii, T. Goto, Y. Ito, H. Matsumoto and Y. Ito	*J. Chromatogr. A*, 2002, **946**, 157
PCBs	7	M. Schellin and P. Popp	*J. Chromatogr. A*, 2003, **1020**, 153
20 Aroma compounds	3	S.M. Van Ruth and E. Villeneuve	*Food Chem.*, 2002, **79**, 157
Alkylresorcinols	8	A.B. Ross, P. Åman, R. Andersson and A. Kamal-Eldin,	*J. Chromatogr. A*, 2004, **1054**, 157

Water/Air/Water Partition in Microdiffusion Extraction

The Petri dish is a simple way of arranging for a liquid/air/liquid partition of a volatile substance, e.g. ammonia, from the liquid sample into the liquid extraction medium within the headspace environment under the airtight lid. By modern standards, it takes a long time for the equilibration of the three-phase system to transfer the volatile analyte from one liquid to the other (typically 20 h), but the microdiffusion/extraction is a low cost process capable of large batch processing and application in the field.

Ammonia Analysis

The amount of ammonia in canned meat products (pork in natural juice, beef with bacon, and luncheon meat) was measured using a microdiffusion method.[14]

Total Cyanogens as HCN in Cassava Flour

Cassava flour is an important component in the diet of 400 million people. The extraction of the toxic cyanogenic glucosides is normally achieved satisfactorily through the use of proper processing procedures. Analytical extraction methods have been developed for monitoring the level of glucosides such as linamarin and lotaustralin.[15] A modification was developed that would be easier to use in the field.[16] Flour (30 g) was homogenised with 170 ml extraction medium (0.1 M orthophosphoric acid containing 25% v/v ethanol) for 3×1 min, with an interval of 1 min, in a Waring blender. With a further 30 ml extraction medium, the homogenate was washed onto a glass fibre filter and the extract collected under vacuum. Then, 0.2–0.5 ml aliquots of the extract were mixed with 1 ml phosphate buffer 0.5 M, pH 6.0 and 0.1 ml linamarase solution (3 EU ml^{-1} in 0.1 M orthophosphate buffer pH 6) in a 2 ml tube. The tubes were placed in an incubation block closed by a picrate sheet and incubated overnight (<20 °C). Microdiffusion of HCN was measured using a microplate reader.

Fluoride Extraction

Fluoride is usually extracted from foods using a microdiffusion method.[17] HF is diffused for 20 h at 50 °C from fresh or freeze-dried samples (0.1 g dry wt) in a polystyrene Petri dish containing 2 ml 40% $HClO_4$ and 0.3 g Ag_2SO_4 and absorbed on the lid by 0.1 ml 0.5 M NaOH. In this case, method verification was by spiking infant foods with NBS SRMs. The method was the subject of an interlaboratory trial (12 laboratories) using 12 samples (1 replicate) of infant foods, milk, pears, and peas containing 0.2–5 ppm F. Mean CVs were 7.06% for 3 sets of blind duplicates and 21.6% for determination of 12 samples.[18] The MDE method was reliable and accurate (<8% error) in the extraction of 113 baby foods with fluoride concentrations ranging, *e.g.*, from 0.01 to 0.31 mg F per kg for baby milk products.[19] Steam distillation was compared to a modified MDE for the analysis of fluoride in shrimps.[20] The values were similar,

but some loss of fluoride might have occurred during the ashing process needed for the StD method.

3 Liquid/Liquid Partition (LLP)

Organic Solvent/Water Partition Constant

A sample of dried milk powder, contaminated with DDT, is dissolved in water (W) and the solution added to a separating funnel containing an approximately equal volume of organic solvent, *e.g.* octanol (O). If the contents are shaken and allowed to settle, there will be a distribution or partitioning of DDT between the water (milk), and the organic phase. DDT is very soluble in octanol and therefore the ratio of the concentration of DDT between the two phases will be >>1 (Equation 3.3),

$$K_{o/w} = \frac{[DDT]_o}{[DDT]_w} \tag{3.3}$$

where $K_{o/w}$ is the partition constant of the solute, DDT. The partition constant of DDT in the octanol/water binary phase system is given in Table 3.2, along with some other chemical compounds by way of comparison. Halogens are more soluble in carbon tetrachloride than in water $K_{o/w} > 1$, while acetic acid and chloroacetic acid are more soluble in water than in benzene $K_{o/w} < 1$.

The values in the table are calculated from a standard addition of the solute to the binary phase system. In food analysis, there may need to be a correction applied for the effect of, *e.g.*, the milk powder and its solubility (complete or not) in the phasic mixture.

Octanol/Water Partition Constant ($K_{o/w}$)

If you wish to extract DDT from the aqueous liquid phase, the octanol/water system (Table 3.2) is highly efficient at removing the pesticide from the aqueous matrix into a volatile organic solvent, which, after separation from the aqueous

Table 3.2 *Partition constants of some organic compounds in three different binary solvent systems*
(Table 6, reprinted from Chemical Ideas, Salters Advanced Chemistry, by the University of York, with permission from Harcourt Education)

Solute	*Solvents*	$K = \frac{[solute(organic)]}{[solute(aq)]}$
Cl_2	CCl_4–H_2O	10
I_2	CCl_4–H_2O	83
CH_3COOH	C_6H_6–H_2O	6.3×10^{-2}
$CH_2ClCOOH$	C_6H_6–H_2O	3.6×10^{-2}
DDT	Octanol–H_2O	9.5×10^{5}

phase, can be concentrated by careful evaporation. In general, the logarithm of the octanol/water partition constant (log P) is popular with food analysts because it relates directly to lipophilicity.[21] Model systems for the estimation of log P for 345 drug or related structures were described,[22] and extended in a new method for predicting log P, for over 12000 organic compounds from the PHYSPROP database of the Syracuse Research Corporation.[23] Other databases – CAST-3D and MAY – have been assessed for log P calculations.[24]

The octanol/water partition constant is used in food contamination studies, especially for polyaromatic compounds (PACs), *e.g.* polyaromatic hydrocarbons (PAHs), dioxins, polychlorinated biphenyls (PCBs), simply because the extraction into octanol is so efficient for these chemical classes.[25] Similarly, Guillén[26] considered the octanol/water partition constants of PACs in relation to water solubility.

The authors of a recent publication[27] collected data on 287 odorous compounds having a rigid molecular structure, in order to simplify their studies of interaction at the olfactory receptor proteins. From their database, *inter alia*, they estimated the log P, for comparison with values for odour quality, thresholds and safety of the molecules.

Effect of pH. Polar and ionic compounds dissolve readily in polar solvents. Non-polar and neutral compounds dissolve in organic non-polar solvents. Ionisation, or more precisely, partial ionisation, leads to the formation of a mixture of ions and neutral molecules in the aqueous layer, whose composition depends on the ionisation constant of the solute and the pH of the aqueous layer.[28] The partition constant (K_P) alone is no longer sufficient to explain the situation.

If a binary phase system of octanol (o) and water (w) contains solute A that ionises in aqueous solution to give B^- and H^+ with an ionisation constant K_w, then the partition ratio (P), the ratio of the sum of the species (ions and molecules) in each phase, is given by Equation 3.4.

$$P = \frac{[A]_o}{[A]_w + [B^-]_w} \tag{3.4}$$

By substitution, the relationship between K_P and P can be shown to be Equation 3.5.

$$P = \frac{K_p}{1 + \dfrac{K_w}{[H^+]_w}} \tag{3.5}$$

From this equation, when the hydrogen ion concentration $> K_A$, P approximates to K_P, and if K_P is large solute A will be mainly in the octanol layer. If the hydrogen ion concentration is $< K_A$, then P will be small and solute A will remain in the aqueous layer. Furthermore, the separation efficiency is normally independent of the concentration, making solvent partition extremely versatile in applications from trace to preparative scale extractions.

Hexane/Acetonitrile Partition Constant

Extraction of Pesticides from Fats. An early example of the use of liquid/liquid partition *via* an adsorbent bed used a partitioning column of Florisil to separate pesticides from fish, beef, and butter fat with an efficiency of 97–100%. The hexane extract was partitioned with acetonitrile, and nine pesticides having hexane/acetonitrile partition coefficients of ≤ 0.05 were separated from fat with good recoveries.[29]

Essential Oils. Isidorov *et al.* (1998) made an important contribution to the hexane/acetonitrile K_P database, working experimentally on 70 common essential oil components in relation to other physical properties such as the GC retention–structure parameters.[30] Their interest in GC retention indices (I) and retention–structure and solubility–structure relationships enabled them to calculate additional K_p. The sample, an essential oil or a standard terpenoid, was added to equal volumes (0.5 ml) of the two solvents in a 3 ml flask with 1 µl toluene (internal standard) and 5 µl *n*-alkanes as relative retention markers. The flask was shaken for 30 s and the settled phases separated and a 1 µl sample of each phase taken for GC analysis. I values were calculated from the relative retention indices (RRIs). Work on the hexane/acetonitrile partition was extended to over 250 alkyl aromatic hydrocarbons and esters. They concluded that for group identification it is better to use $j = kI - \log k_p$ to include the GC retention index.[31]

Oil/Water Partition Constants

In food systems the presence of oil in the structure and the importance of oil and oil-based components in flavour, makes $K_{oil/water}$ measurements particularly apposite for food studies. Pollien and Roberts[21] prefer a method for determining $K_{oil/water}$ that can measure the concentration of the solute in both phases, an important step in accelerating the rate of collection of reference data [see also gas/liquid partition analysis (GLPA), Chapter 6].

This is critical for many lipophilic solutes since the C_{Liq} term is often very small. The ppb level attainable with SPME (Chapter 6) gave it this capability. They were satisfied also with the reproducibility and accuracy of the method but, in common with most users of SPME, they found it to be relative, requiring the same conditions to be used for all comparative studies. They discuss the development of their SPME method and compare it with the HPLC method used for pharmaceutical analysis.[32,33]

The rationale for the use of predicted log P to standardise log K_w and log $K_{oil/water}$ values are discussed. The values for the 12 compounds chosen to span the lipophilicity range encountered in flavour research are recorded in Table 2 of Pollien and Roberts.[21]

In 1996, it was reported that a discrepancy existed between the instrumental and the sensory measurement of the oil/water partition constant.[34] The sensory intensity above water solutions was higher than expected. This was suspected

to be due to the presence of water vapour above the water solution. The discrepancy was investigated in 2002 by measuring the instrumental and the sensory oil/water partition constants of four test compounds, acetophenone, benzaldehyde, linalool, and 1-octen-3-ol.[35]

Partition Behaviour of Phenolic Compounds in Olive Oil

Extra virgin olive oil (EVOO) was studied in a model simulating the canned-in-oil food system. Chemical modification and partitioning of major phenols towards the brine (5% salt) phase was induced by thermal processing (sterilisation). Hydroxytyrosol, tyrosol, and their complexes decreased in the oily phase and moved towards the brine phase, contributing to the loss of phenolics when EVOO was used as a filling in canned foods.[36]

4 Solid/Liquid Partition (SLP)

Introduction

The use of an organic liquid to partition components of a comminuted, solid, dry food sample is an example of solid/liquid partition, but more common is the partitioning of a comminuted, solid food in an aqueous matrix when the process is both solid/liquid and liquid/liquid partitioning. In practice, the operation is better described as a simple solvent extraction of the food sample. For that reason, the practice of solid/liquid partitioning is dealt with in Chapter 4.

Solid/Liquid Partition Constants

Recoveries in the SPE, using 200 mg Lichrolut-EN (Merck), of trace volatiles from wine were verified using breakthrough volumes calculated from the solid/liquid partition constants.[37] A phase partition constant of 0.38 at the solid/liquid interface in the freezing of carrot juice was found to be virtually independent of the freezing temperature in the range –6.5 to –14.2 ºC.[38]

5 Applications of Partition–Extraction

Liquid/Liquid Partitioning of Liquid Foods

Penicillins

LLP-E was used in the extraction of five penicillins from milk. The acetonitrile extract was partitioned with DCM for LC-MS.[39]

Levamisole

In the estimation of the anthelminthic drug, levamisole, the sample of milk was made alkaline and extracted with ethyl acetate and a series of LLP steps were

used to effect LODs down to 0.5 ng g^{-1} by GC with nitrogen/phosphorus detection.[40]

Fat-soluble Vitamins and Provitamins

Milk was partitioned with hexane in the simultaneous estimation by RP-HPLC of vitamins A, D2, D3, E and K1, retinyl acetate, retinyl palmitate, tocopherol acetate, ergosterol, and 7-dehydrocholesterol.[41]

Lycopene from Tomato Products

A rapid method for the determination of lycopene in tomato and tomato products was developed using the simple preparation of making a puree. Fresh tomatoes were sliced, cubed and homogenised in a Waring blender until chunks were less than 4 mm^3. The samples were diluted 1:1 (w/v) with deionised water if necessary and pureed in a Brinkmann Polytron Homogeniser with a 20 mm blade (Brinkmann Instruments Inc., Westbury, NY), without heating or frothing. Low volume hexane extraction was carried out by adding 5 ml 0.05% (w/v) BHT in acetone, 5 ml 95% EtOH, and 10 ml hexane, and shaking at 180 rpm for 15 min on ice. Deionised water (3 ml) was then added and the vial shaken for a further 5 min on ice. Analytes partitioned into the upper hexane layer were taken after phase separation for spectroscopic analysis.[42] Preparation was under subdued lighting.

Solid/Liquid and Liquid/Liquid Partitioning of Liquid Foods

Organochlorine Pesticides

A hexane solution of organochlorine pesticides in oils and fats was dispersed onto Extrelut-3 solid phase, connected to the C_{18} solid-phase cartridge in series and partitioned through both phases continuously with AcCN. The eluent was partitioned with water–petroleum ether (40–60ºC) and the petrol phase separated, dried through Na_2SO_4 and evaporated to dryness. The residue was dissolved in hexane and chromatographed on Florisil and eluted first with *n*-hexane–benzene (fraction 1) and then with *n*-hexane–benzene–ethyl acetate (fraction 2).[43]

Multiple Liquid/liquid Partitioning of Liquid Foods

Benzo[a]*pyrene, in Olive Oil*

Olive oil was partitioned with cyclohexane in a separating funnel using the multiple extraction method (Chapter 4 Section 2). The cyclohexane was extracted with multiple aliquots of DMF–water (9 : 1, v/v).[44] The extract was diluted with 1% Na_2SO_4 solution and re-partitioned with cyclohexane (multiple extractions).

The cyclohexane extract was washed with distilled water (twice), dried over Na_2SO_4 and rotary evaporated to a small volume for final clean up on silica gel, partitioned with cyclohexane, dried under N_2 and dissolved in acetonitrile for HPLC analysis.

Liquid/Liquid Partitioning of Liquid and Solid Foods

Alternatively, the liquid extraction (stage 1) and liquid partition (stage 2) are completed and then the analytes are concentrated by solid/liquid extraction (SLE) and further purified by desorption partition in a selective solvent–SPE (stage 3).

Pyrethroid Pesticides

The determination of 12 pyrethroid pesticides in processed fruits and vegetables used a miniaturised extraction–partition procedure with small amounts of non-chlorinated solvents.[45] Tomato puree, peach nectar, orange juice, and canned pea samples were extracted with acetone and the acetone partitioned with ethyl acetate–cyclohexane (50:50, v/v). A final clean up with Florisil concentrated the pesticides in the partitioned material for GC-ECD and GC-MS analyses.

Liquid/Liquid Partition of Solid Food Samples

Pesticide Residues

A very simple extraction method was described for the estimation of six pesticides, azinphos methyl, Bromopropylate, chlorpyrifos, dimethoate, parathion methyl and phosalone, in apricots and peaches.[46] An acetone extraction of the homogenised sample [8000 rpm in Ultra-Turrax T25, (IKA, Germany)] was followed by LLP with a mixture of (1:1) DCM–petroleum (40–60°C), and centrifuged at 4000 rpm for 5 min and the filtered supernatant liquid further processed for GC-MS analysis. Recoveries were 97–120% for Bromopropylate and phosalone at 0.2–2.0 mg kg^{-1}, 96–145% for chlorpyrifos and parathion methyl at 0.02–0.2 mg kg^{-1}, 75–97% for azinphos methyl at 0.05–0.5 mg kg^{-1} and 73–93% for dimethoate at 0.1–1.0 mg kg^{-1}. The limits of quantitation (LOQ) were in the range 0.01–0.1 mg kg^{-1}.

Biogenic Amines

Perchloric acid extracts were partitioned using *n*-hexane, and the supernatant layer discarded. The cleaned up aqueous layer was sampled for IEC with integrated pulsed amperometric detection to separate and measure biogenic amines and some amino acid precursors from kiwis, pilchards, cheese and sausages (salami).[47] LODs were 1.25–2.50 ng at a s/n ratio of 3:1.

Liquid/Solid/Liquid Partitioning of Solid Foods

Organophosphate Pesticides

A fast, single-step partition between *n*-hexane and acetonitrile was effected on ready-to-use disposable mini-columns packed with Kieselghur-type material for the extraction of nine organophosphate pesticides from lipidic extracts of cereals, oil seeds and legumes.[48]

Pyrethroid Pesticide Residues

Again, a single-step LSLP was developed to combine several processes into one, and to avoid emulsions common with separating funnel methods. Aqueous acetone extracts of strawberry, apple and orange containing 14 pyrethroid pesticides were extracted in acetone, adsorbed onto disposable, ready-to-use cartridges filled with macroporous diatomaceous earth material and eluted with light petroleum–DCM (75 : 25, v/v). This one-step process replaced a separating funnel partition, drying with Na_2SO_4 and a partial adsorption clean-up.[49]

Sodium Sulphate Drying in Liquid/Liquid Partitioning

The Karl Fischer titration method of moisture determination was used to monitor the residual water in solvents after LLP.[50] n-Hexane and petroleum ether had low levels of residual water (0.1 mg ml^{-1}). Residues of 8–10 mg ml^{-1} were found in diethyl ether, while wet ethyl acetate had 20–25 mg ml^{-1}, and wet acetonitrile had 60 mg ml^{-1} of water remaining. Anhydrous Na_2SO_4 removed 20–25% water from ethyl acetate and diethyl ether, but was ineffective with acetonitrile.

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CHAPTER 4

Solvation

1 Introduction

In classical food analysis, the shaking of a comminuted sample of food with a solvent followed by filtration and concentration would have covered most of the activity in the area of solvation. However, there are several recent developments towards the more efficient use of solvents for extraction that now must be included. The following headings have been used to subdivide the chapter.

Solvent Extraction

The solvation of compounds of interest in the food sample by a liquid solvent is most often referred to as solvent extraction, but the term "leaching" is used and vividly brings to mind a process where the soluble substances are dissolved in a percolating liquid. Both terms appear in the literature pertaining to the use of solvents to extract analytes directly from samples of liquid food and indirectly from solid foods after disintegration.

In textbook terms, solvent extraction is the liquid/liquid partitioning of a solute between two immiscible pure solvents. The principles of the subject were dealt with in Chapter 3 under the title "Partition". In practice, it is important to make sure that the sample matrix is amenable to efficient extraction of the soluble material. With liquid food samples, emulsion or precipitate formation should be avoided, while with solid samples it is important to make sure that the structure is sufficiently dispersed to allow intimate contact between the powder or slurry – depending on the moisture content and texture of the sample – and the extracting solvent. Extraction aids such as anticoagulants for liquid samples and dispersants to maintain an open structure in the solid food matrix may be employed.

Matrix Solid-phase Dispersion

An adsorbing powdered solid is added to the sample to open up the structure to the permeating liquid solvent and the mixture packed into a column for solvent extraction. Analytes may be desorbed from the column as a complex mixture

and subjected to further purification or, by using a series of selective solvents, as fractions containing a narrower range of solutes, clean enough for chromatographic separation.

Subcritical Fluid Extraction

Pressurised Liquid Extraction (PLE)

Enhanced solvation is possible if the pressure of a liquid solvent is raised to near its critical point. Improvements in extraction efficiency are reported, for example, for pesticides in foods. Static immersion and dynamic flow extractions are used.

Sub-critical Water Extraction (SWE)

An efficient extraction of apolar solutes, using water made less polar by pressurising it near to its critical point, is processed on-line by merging the aqueous eluent from the extraction chamber with an organic solvent so that when the water is subsequently depressurised its polarity increases again, rendering its apolar solutes more readily partitioned with the organic solvent.

Pressurised Hot Water Extraction (PHWE)

In practice, both temperature and pressure are increased to approach the critical point and the process is called PHWE. The potential for automation, as with PLE, is attractive for modern analyses.

Supercritical Fluid Extraction

As the name suggests, the technique takes advantage of the merged gas and liquid properties exhibited by solvents such as carbon dioxide in the supercritical state. SFE is popular because non-toxic materials like CO_2 may be used, and it has the obvious advantage of returning the solvent to its vapour state after extraction for easy removal from the analytes that are automatically concentrated in the process.

2 Solvent Extraction

Liquid/Liquid Extraction

Introduction

LLE is a term reserved in this book for the interaction of an immiscible solvent with a liquid food. Normally, the liquid food matrix will be aqueous and therefore the extraction will be from the aqueous phase into an immiscible organic phase. An example would be the extraction of fat from milk into diethyl ether.

In Chapter 3 the notion of the partition constant was developed to quantify the distribution of a solute between two immiscible solvents. In practical terms, if the partition constant for a given solute is not equal to 1 there will be more of the solute in solvent 2, say, than in solvent 1, and that can be used to effect an extraction. Using the principle of multiple liquid/liquid extraction, after separation, re-extracting solvent 1 with solvent 2 the remaining solute will again be partitioned in favour of solvent 2. Combining the aliquots of solvent 2, the solute may be more effectively transferred to solvent 2. The further the value of the partition constant is from unity the fewer extractions will be required.

Applications

Contaminants in Bovine Milk. An obvious demonstration of the use of LLE in food analysis is to examine the extraction of a liquid food such as bovine milk. Several contaminants have been analysed directly from the solvent extract of the aqueous phase. Nitroxynil was extracted with acetone and acetonitrile.[1] Ivermectin was removed by LLE and then further cleaned using alumina B SPE.[2] Similarly, ochratoxin A was removed by LLE and cleaned up on a home-made silica gel column.[3] Acid hydrolysis was required before LLE and SLE in order to extract albendazole.[4]

C_{18} SPE showed better detection limits than LLE for the analysis of 15 PCB congeners.[5] SPE was also faster and more economical in solvent use than LLE for the extraction of methyl parathion and methyl paraoxon.[6] Furthermore, a semi-automatic method involved LLE for the analysis of PCDD and PCDF residues.[7]

Two widely used LLE methods were compared for the extraction of pesticide residues.[8] A 15 laboratory collaborative study was made of the LC method adopted by the AOAC International for the extraction of vitamin D that included LLE, saponification, and SPE.[9]

Carbohydrates from Fatty Matrices. Problems with the extraction of carbohydrates from matrices containing even small quantities of polar lipids, *e.g.* milk, have been researched. As the clean-up of chloroform–methanol extracts, such as water washing or silicic acid SPE, degraded glycolipids improvements were suggested.[10]

After saponification, 25 g samples of liquid and reconstituted powdered infant formula were washed and multiply extracted with *n*-hexane (five extractions, 2×50 ml and 3×25 ml aliquots, bulked, evaporated and filtered) for HPLC analysis.[11]

Plant Pigments. Five solvent systems, chosen from previous extractions in the literature, were compared for their efficiency in extracting 16 carotenoids from processed tomato juice:[12]

1. Ethanol–hexane (4:3, v/v)
2. Acetone–hexane (3:5 v/v)

3. Ethanol–acetone–hexane (2:1:3 v/v/v)
4. Ethyl acetate–hexane (1:1 v/v)
5. Ethyl acetate

Tomato juice (8 g) and $MgCO_3$ (0.2 g) were mixed in a 60 ml vial and then solvent added (40 ml). The vial was shaken for 30 min at 140 rpm. The upper layer was decanted and the lower layer re-extracted with the same solvent and then partitioned with hexane (twice) and the decanted layers pooled, homogenised and filtered. This solvent layer was partitioned with water (150 ml) and 10% NaCl (100 ml) and the supernatant collected. The lower phase was extracted with hexane and the supernatant combined, evaporated to dryness and the residue taken up in methylene chloride (1 ml) for HPLC. Ethanol–hexane was the best solvent for extracting lycopene, the major carotenoid component of tomato juice.

This extraction is an example of liquid food/solvent partitioning where the organic solvent is further partitioned with an aqueous phase solvent system to remove hydrophilic interferents.

Patulin in Apple Juice. The high acidity of apple juice required the ethyl acetate extract to be partitioned with Na_2CO_3 solution, which was then back-extracted with ethyl acetate and dried over anhydrous sodium sulphate for RP-LC.[13]

Simplification of the LLE Estimation of Cholesterol

A decreased sample size and operation in one tube improved the estimation of cholesterol. A food may be saponified with say, 0.4 M KOH in ethanol at 60 °C and the unsaponified material subjected to LLE, *e.g.* in hexane.[14] In this case the extract was clean enough for direct injection onto an efficient capillary column for GC analysis. Thus a simple extraction replaced a multiple extraction method with its intrinsic, cumulative losses at each stage. This example reinforces the principle of taking an overview of the total procedure to identify the minimum pre-treatment necessary when preceding a high-resolution separation/analysis step.

Solid/Liquid Extraction

Introduction

When a liquid solvent is put into intimate contact with a finely divided sample of a solid food the soluble components will partition into the solvent. After a time, equilibrium – different for each component – will be reached when no further changes in mass transfer from sample to solvent will occur. Using extraction aids (Chapter 2) can shorten the time. Separation of the two equilibrated phases, *e.g.* by filtration, constitutes a SLE. In the time available in any practical assay,

most of the more-soluble constituents have been extracted from most of the less-soluble constituents, but the ultimate total extraction is not realised for several reasons. It is unlikely that comminution will allow solvent access to all of the potentially soluble material and the composition of the solid/liquid food matrix might render a percentage of the solute immobile through binding to proteins, carbohydrates, *etc.* Solubility is difficult to define and the rate of solution will limit the extraction of certain components, and some material might be mobilised in the solvent in an unmanageable, colloidal or particulate state. Nevertheless, solid–liquid extractions, *e.g.* using acetone to remove high value solutes from oleoresins, are used commercially to yield economic recoveries.

Applications

Aflatoxins from Palm Kernels. Aflatoxins B_1, B_2, G_1, and G_2 were extracted from palm kernels with acetone–water (80:20, v/v) and the crude extract subjected to SPE (phenyl bonded phase) chromatographed with H_2O–MeOH (93:3, v/v) and the analytes eluted with 3 ml CH_3Cl for bi-directional HPTLC (Appendix 2).[15]

Fumonisins in Corn. Three solvent mixtures (CH_3CN–H_2O, 50:50, v/v; MeOH–H_2O, 75:25, v/v; and 100% H_2O) were compared for the extraction of fumonisins from corn. C_{18} SPE was used as a clean-up following solvent extraction. The acetonitrile solvent mixture gave the fastest and most efficient extraction.[16]

Hop Proanthocyanidins. The complicated job of analysing plant proanthocyanidins starts with the removal of pigments and lipids (and lipid solubles) by partitioning into DCM, and is followed by air drying and grinding the residue. Three water–acetone extractions with continuous stirring were vacuum rotary evaporated to remove the acetone and the aqueous extract washed with hexane, and then with DCM to remove remaining lipid soluble material, and rotary evaporated again to remove the organic solvents.[17] The extract was then ready for fractionation on Sephadex LH-20, partitioned with various water–MeOH mixes to remove, first, glycosides and then the catechin-epicatechin monomers and dimers. Much more detail is provided in the source paper.

Isoflavones–Extraction Protocols. Hendrich (2002) reported on the bioavailability of isoflavones.[18] They have therapeutic properties, which include reducing the risk of cardiovascular disease and lowering the rates of various cancers, and improving bone health.[19] In a detailed practical project, Murphy *et al.*[19] (2002) tested four organic solvents, AcCN, acetone, EtOH, and MeOH, in binary aqueous partition for the solvent extraction of 12 phytoestrogenic soy isoflavones from five soy-based food matrices (soy flour, tempeh, tofu, TVP and soy germ), with and without the addition of 0.1 N HCl, in quadruplicate. Special

precautions were needed to reduce thermal decomposition. They concluded that acetonitrile was better than acetone, ethanol or methanol for most foods.

Liggins *et al.* (1998) used 2.5 g soy flour, covered with 5 ml 80% aqueous methanol and sonicated for 10 min to degrade the cellular material and enhance the efficiency of the following extraction process. The matrix was left to soak in the solvent for a further 1 h, filtered, and the solid phase washed with, *e.g.*, 5 ml 80% aqueous methanol. The filtrate was evaporated and the enzyme cellulase added in 5 ml 0.1 M acetate buffer (pH 5). The samples were further sonicated and then incubated overnight in a shaking water bath at 37 °C. The hydrolysed aglycones (daidzein and genistein) were partitioned from the aqueous hydrolysis solution into ethyl acetate (three washes with ethyl acetate).[20] Recovery and quantification of the assay were tested using synthetic isoflavone aglycones. Hydrolysis of the glycosidic bond was studied, as was the effectiveness of the assay over a wide concentration range. This was effected by making up mixtures of soy flour of known isoflavone content and white wheat flour (which contains virtually no isoflavones). The method was accurate down to 100 ng g^{-1} (the lowest level tested). The defatting of extracts with hexane caused losses of isoflavones and has been omitted from this protocol. The authors warn that the enzyme hydrolysis was optimised for soy flour and recoveries for other foods would have to be determined. (Summarised from ref. 20 with permission from Elsevier)

Natural Food Colours. Cochineal is the aqueous alcohol extract of the dried female *Dactylopius coccus* cacti insect containing a glucoside of hydroxyanthraquinone, and Annatto Natural Orange (E160b) is the extract of the resinous seed coating of the *Bixa orellana L.* containing the oil-soluble carotenoid *cis*-bixin.[21] Various solvents were tried in the extraction of annatto components, α- and β-norbixin and α- and β-bixin, from cheese, butter, margarine, and hard candy.[22] The extract was transferred into aqueous acetic acid in MeOH for HPLC analysis. Spiked recoveries in the range 1–110 μg g^{-1} of, *e.g.*, norbixin averaged 92.6%.

Recently, solvent extraction was the method of choice for the removal of annatto from a wide range of food commodities permitted to contain it. The major and minor colour principals were studied.[23]

Monocyclic Aromatic Hydrocarbons. The pollutants benzene, toluene, ethylbenzene, and the xylenes were isolated by solvent extraction from fruits and vegetables for SIM-GC-MS analysis.[24] Absorption of MAHs was found to be species and morphological part dependent. Parsley leaves contained the highest concentrations of *m*- and *p*-xylene and orange peel the highest toluene, but the possibility of natural biosynthesis cannot be ruled out.[25]

Polycyclic Aromatic Hydrocarbons. For the analysis of samples of liquid smoke flavourings and smoked foods, alkali treatment was necessary to remove some of the smoke components before using proven solvents – cyclohexane

and DCM – to extract the PAHs.[26] Cyclohexane was preferred for its higher specificity – avoiding the extraction of other smoke flavourings.

In the development of a method for measuring PAHs in seafood, the FDA clean-up method to attain ppb levels of detection was evaluated.[27] Eighteen PAHs at 1–5 ppb were subjected to a LLE followed by silica, alumina, and C_{18} SPE stages in the purification for GC-MS analysis.

PCBs and Organochlorine Pesticides. Total diet samples and individual foodstuffs were solvent extracted and treated with concentrated H_2SO_4 for SIM-GC-MS isomer-specific analysis. Very low levels were found in Finnish market basket samples, hospital diets, milk, cheeses and egg samples.[28]

Vitamins. The fat-soluble vitamins D and E have been extracted from solid foods using light petroleum,[29] diethyl ether,[30] hexane,[31] and methylene chloride[32] solvents. Vitamin K has been extracted with a mixture of propan-2-ol and hexane,[33] and vitamin A with hexane[34] and trichloromethane–acetone.[35] Data are presented in Table 4.1. Luque-Garcia and Luque de Castro (2001) have reviewed the extraction of fat-soluble vitamins.[36]

Zearalenone in Rice Cultures. A solvent extraction with AcCN–H_2O was partitioned with hexane to remove the fat, diluted in MeOH, and evaporated to dryness. The residue was dissolved in AcCN and diluted in H_2O for LC analysis. Spiked recovery was 76–94%.[37]

Table 4.1 *Solvents used in the extraction of fat-soluble vitamins from solid foods. Solid–liquid extraction was normally followed by preconcentration for solid and semi-solid food samples (see also MSPD)*
(Constructed from data reprinted from the *Journal of Chromatography A*, vol, 935, J.L. Luque-Garcia and M.D. Luque de Castro, "Extraction of Fat-solute Vitamins", pp. 3–11, © 2001, with permission from Elsevier)

Solvent	*Vitamins*
$CHCl_3$	A
CH_2Cl_2	D, E
$CHCl_3$–acetone	A
Diethyl ether	D, E, A
Ethanol	E[a]
Ethyl acetate–butanol	A
Hexane	D, E,[a] A
Hexane–propan-2-ol	K
Light petroleum	D, E

[a] Extracted from feedstuffs using this solvent in the Soxhlet method.

Buffered Aqueous Extraction of Protein

To measure protein solubility, extruded pea and bean seeds were extracted at various pH and using different solvents. In the range pH 2–10, raw and extruded samples were more soluble in salt solution than in water, and when either 2-mercaptoethanol (2-ME) or SDS or both were added to buffered aqueous systems, protein extraction was increased to almost 100%.[38]

SLE Methods for Total Lipids

A good example of the use of SLE is in the estimation of total lipids by various established solvent extraction methods, the outlines for some of which are given as follows:

Bligh and Dyer (B & D) Method.[39] 1 aliquot of sample was mixed with 4 parts of MeOH and 2 of $CHCl_3$, and vortexed. A further 2 aliquots of $CHCl_3$ were added and shaken. Four aliquots of distilled water were added, vortexed, centrifuged and the lower layer re-extracted with the MeOH–$CHCl_3$ mixture and the process repeated. The two organic extracts were combined and evaporated to dryness.

Modified Bligh and Dyer Method. When the use of chlorinated solvents was strictly controlled, alternatives to the B & D method were sought for the analysis of marine organisms.[40] Several solvent mixes were tried and a propan-2-ol–cyclohexane mixture to replace the MeOH–$CHCl_3$ was recommended. The water–propanol–cyclohexane mixture was 11:8:10. Plaice, mussel, and herring samples were used in a comparative study of the Soxhlet, B & D, and the Smedes modified B & D methods (Table 4.2). Students of food extraction are recommended to read this paper which contains much valuable background information and a careful record of the optimisation of the modified B & D experimental method. de Boer (1988) discussed the low values for the Soxhlet method for plaice and mussel in relation to the work.[41]

Roese-Gottlieb Method (AOAC 1990). This method considered the state of the lipids and a hydrolysis step was added.[42] To a 1 ml aliquot of sample, add 6 of boiling water and vortex. Allow to cool and add 1 ml 25% ammonia and vortex again; add 7.5 ml of MeOH and vortex. Finally, add 17 ml diethyl ether, shake and add 17 ml petroleum ether, centrifuge and evaporate the upper layer to dryness.

Comparison of Total Lipids Methods. Solvent extraction methods for total lipids in fish tissues were evaluated and a method using $CHCl_3$–MeOH and an Eberbach jar was optimised for solvent ratio, solvent–sample ratio, and phase

Table 4.2 *Lipid content on a wet weight basis for three marine tissue types and three extraction procedures. Sets labelled superscript a, b and c were significantly different ($p < 0.01$)*
(Table 3, from *Analyst*, F. Smedes, vol. 124, 1999, pp. 1711–1718. Reproduced by permission of the Royal Society of Chemistry)

	Concentration (mg g^{-1})		
Sample	*Soxhlet method*	*Bligh and Dyer method*	*This work*
Plaice (muscle)	9.5[a]	13.9[b]	12.8[c]
	9.3	13.7	12.6
	9.7	13.7	12.5
Mussel	20.0[a]	25.6[b]	24.5[c]
	20.6	25.6	24.5
	20.3	25.5	24.6
Herring (muscle)	110.7[a]	No result[b]	109.8[c]
	109.9	104.0	109.9
	109.4	102.6	108.8

separation time.[43] The article by Manirakiza *et al.* (2001) compares the above solvent extraction methods with two forms of the Soxhlet distillation method (Table 1.1, Chapter 1, ref. 25). The authors' comment that the results should be viewed with caution seems apposite. Based on the manufacturers fat content, the values obtained for powdered chocolate and milk are reasonable by all five methods; however, the liquid milk and egg values suggest that only the Bligh and Dyer methods are acceptable, whereas the Soxhlet methods are to be preferred for the fat content of margarine.

Assisted SLE

Chloramphenicol. Egg powder samples were extracted with acetonitrile and assisted by ultrasound followed by centrifugation. The supernatant was diluted with water and defatted with *n*-hexane for adsorption onto graphitised carbon black.[44] The eluate was derivatised for GC analysis.

Organochlorine Pesticides. A one-step extraction of 11 organochlorine pesticides from milk with ethyl acetate–acetone–MeOH was assisted by ultrasound, and the supernatants re-extracted with C_{18} SPE for GC analysis.[45]

Automated SLE

LVI-LC-MS-MS. One objective in the development of SLE methods is to reduce the amount of solvent used, in keeping with modern environmental requirements. LVI-LC-MS for the determination of pesticides in carrots and potatoes uses only 3 ml per 2 g sample.[46] The vegetable sample was ground in

a blender for 15 min and a 2 g aliquot extracted with 3 ml solvent (MeOH, AcCN, or CH_3COCH_3) with UAE for 45 min, filtered and diluted with buffer for injection *via* an autosampler vial. The extraction was optimised as AcCN extraction followed by dilution with ammonium formate (85:15, v/v) for LVI-LC-ESI-MS-MS in the selected reaction monitoring mode. Selected reaction monitoring was used to improve the resolution of the detector. Quadrupole 1 was fixed at either $[M+H]^+$ or $[M-H]^-$ values for positive ion and negative ion modes, respectively, and Quadrupole 3 at the mass of an intense product ion. This is an example of optimisation of whole assay resolution. The very high selectivity of detection allows the preparation to be a simple, rapid solvent extraction.

SLE Coupled to CE. A hot water infusion of green tea using MAE was put on-line to CE with a flow injection (FI) system. The polyphenols analysed were caffeine, theophylline, epigallocatechin-3 gallate, epigallocatechin, epicatechin-3 gallate, (–)-epicatechin, (+)-epicatechin, gallic acid, quercetin, and caffeic acid. The LOD was 0.04 μg ml^{-1} for flavanols and 1.2 μg ml^{-1} for caffeine. [47]

Collaborative Study of SLE Methods

Fumonisins. Fumonisin B1 and B2 were spiked into maize flour, corn flakes, extruded maize, muffins, and infant formula for an interlaboratory collaborative trial among four laboratories.[48] The collaborating laboratories studied five factors – extraction solvent, extraction mode, volume of solvent, sample size, and the clean up. HPLC of the *o*-phthaldialdehyde derivatised extracts was the end determination method. AcCN–water (1:1, v/v) and immunoaffinity column SPE was preferred to MeOH–water (3:1, v/v) and strong anion exchange SPE, but inaccuracies due to phase separation occurred with AcCN–water.

Multiple Liquid/Liquid Extractions

Theory

The well-known rule of solvent extraction is, for a given volume of solvent: several small volume extractions are more efficient than one large volume extraction. Christian (Chapter 3, ref. 28) derives the following equations and sets examples.

Where V_o and V_a are the volumes of the organic and the aqueous phases, and $[S]_o$ and $[S]_a$ are the concentrations of the solute S in the organic and aqueous phases respectively, the percentage extracted into the organic phase from the aqueous phase is

$$\%E = \frac{[S]_o V_o}{[S]_o V_o + [S]_a V_a} \times 100\% \tag{4.1}$$

Where $D = [S]_o/[S]_a$, dividing by $[S]_a$ and then by V_o gives

$$\%E = \frac{100D}{D + \left(\frac{V_a}{V_o}\right)} \tag{4.2}$$

Divide by 100 and subtract from 1 gives

$$F = 1 - \frac{D}{D + \left(\frac{V_a}{V_o}\right)} \tag{4.3}$$

where F is the fraction left unextracted, or, rearranging,

$$F = \frac{V_a}{DV_o + V_a} \tag{4.4}$$

If W_a is the initial quantity of solute in the aqueous phase then the quantity (W) remaining in the phase after one extraction is

$$W = W_a\left(\frac{V_a}{DV_o + V_a}\right) \tag{4.5}$$

and after n extractions

$$W = W_a\left(\frac{V_a}{DV_a + V_a}\right)^n \tag{4.6}$$

These equations serve as an excellent introduction to multiple extractions in general and to countercurrent distribution and multiple solid-phase microextraction in particular. (From G.D. Christian, *Analytical Chemistry*, John Wiley and Sons, New York, © 1994, with permission from Wiley)

Application of the Manual Method

Non-starch Compounds. Multiple extraction with 1% sodium dodecyl sulphate and mercaptoethanol removed non-starch compounds from wheat, corn and potato starch granules.[49] Further details are provided about the effect of the extraction on the residual starch granules. Surface lipids were cold extracted with a *n*-propanol–water mixture (3:1, v/v) and internal lipids were hot extracted with the same solvent mix. After a single extraction, mainly low molecular weight (LMW) compounds were found from the three types of starch, while in the cereal starches some high molecular weight material was also found. Lysophosphatidylcholine was the main non-carbohydrate LMW product extracted from wheat starch.

S-*adenosyl-L-methionine*. Multiple extractions with an acidic phosphate buffer were used in the removal of *S*-adenosyl-L-methionine from dietary supplement tablets. The extract was separated by ion pairing on a reversed-phase C_8 column.[50]

Countercurrent Distribution

Introduction

If the distribution ratios of two solutes between two solvents are similar, a single extraction is unlikely to produce an efficient separation and a multiple extraction technique should be employed.

Countercurrent distribution is a continuous multiple extraction method and the Craig countercurrent distribution equipment is well known for this purpose. The apparatus consists of a framework to support large numbers of interconnecting glass tubes like the one shown in Figure 4.1. The number of tubes can be increased to provide additional resolution, as required.

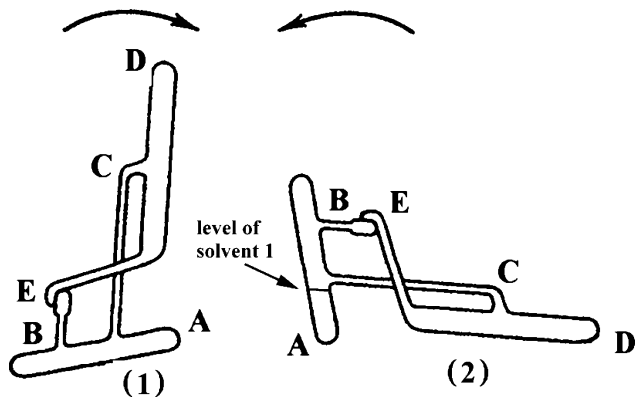

Figure 4.1 *Craig countercurrent distribution tube. The tube position shown in* (1) *can be rotated through 90° to position* (2)*, and also rocked continuously backward and forward in position 1 through a few degrees when solvent mixing is required. Extraction starts with the insertion through inlet E, into chamber A of all the tubes, an amount of the more dense solvent 1 so that none of it passes into chamber D through tube C when the tube is rotated to position* (2) *(*i.e. *up to the level shown). The sample for extraction is introduced in solvent 1 in the first distribution tube only. The less dense extracting solvent 2 is introduced to tube 1 through inlet B. The tube is rocked for a while to allow mixing and then held steady while the phases separate. Then, by turning the tube through 90°, the extracting solvent 2 passes through tube C into chamber D, and by rotating it back again the extract flows via tube E into the next distribution tube. The process is repeated after filling tube 1 with a second aliquot of solvent 2*
(Figure 16.5 from G.D. Christian, *Analytical Chemistry*, John Wiley and Sons, New York, © 1994, with permission from Wiley)

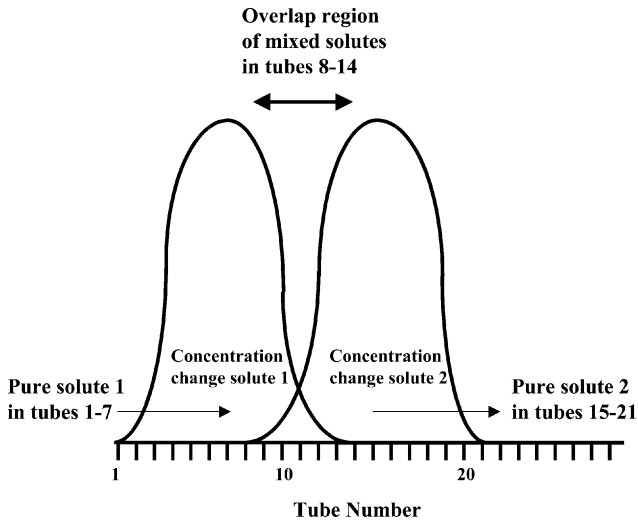

Figure 4.2 *The concentration distribution of solutes among the tubes in a countercurrent distribution extraction. In this case, at the end of the run, Solvent 1 recovered from the first 6 tubes may be bulked to collect pure solute 1, the next 6 tubes may be collected as a mixture of the two solutes (for a repeat extraction if required) and the final 7 tubes may be bulked as pure solute 2*

If there are 2 solutes with different partition ratios (in this case $K_2 > K_1$) in solvent 1 in the first distribution tube then the more tubes in the line the greater the potential separation. After a number of partitions, up to the number of distribution tubes in the line, there will be two Gaussian distributions of solute concentration among the distribution tubes with solute 2 leading solute 1 (Figure 4.2). The resolving power of the system will depend upon the number of partition–extractions and the difference between the partition constants of the solutes.

Applications

A convenient place to start is with the review by Casinovi (1963), which covered the earlier separations of organic substances.[51] The designer L.C. Craig co-authored a number of papers demonstrating the use of countercurrent distribution for the extraction of biochemicals from biological matrices. Amino acids were prevalent as components to isolate. An early paper compared the results obtained by gel filtration and countercurrent distribution on the amino acid sequence of bovine parathyroid hormone.[52] Peptides and particularly antibiotics were needed in pure state and the rather lengthy extractions were not considered to be a problem in the 1960s[53–56] and 1970s.[57–59] During this period, Craig[60] attempted to explain the attainment of high resolution in countercurrent extraction, and the technique was used to extract gibberellins.[61] The application of partition constants (Chapter 3) to the countercurrent distribution of gibberellins was also considered.

Countercurrent Chromatography (CCC)

Marston and Hostettmann reviewed the technique in 1994.[62] A prototype pH-zone-refining countercurrent chromatography instrument was used by Oka *et al.*, 2002, to purify 300 mg of the Food Colour Red No. 106 (acid red) (San-ei Chemical Industry, Osaka, Japan).[63] The two-phase solvent system, *n*-butanol–water had 40 mM sulphuric acid (retainer) in the organic stationary phase and 30 mM ammonia (eluter) in the aqueous mobile phase. The authors give a detailed record of the choice of the two-phase solvent system, and the use of partition constants to adjust the distribution between the phases. The column of the multi-layer coil planet centrifuge (Shimadzu, Kyoto, Japan) was filled with upper organic stationary phase and the sample loaded. The column was rotated at 800 rpm, and the lower aqueous phase pumped into the head of the column at a flow rate of 1 ml min^{-1}. 1 ml fractions of eluate were collected. The stationary phase retained fractions were removed from the column by pressurised nitrogen gas in the tail-to-head elution mode. Some 262 mg of pure (99.9%) Food Colour Red No. 106 was recovered. Milligram quantities of the other components were not pure.

CCC was used to extract polyphenols from red wine and a mutagen was recognised by enzymes and further purified by RP-HPLC. The active compound was identified as rutin.[64] Theaflavins and epitheaflavic acids from black tea[65] and the catechins, flavonol glycosides, proanthocyanins and strictinin from green and black tea[66] were extracted using high speed CCC (HSCCC) on a preparative scale. Gram quantities can be extracted from tea by this method.

Countercurrent Extraction and Isoelectric Precipitation

The countercurrent extraction method of Galanos and Kapoulas[67] was compared with dialysis in petroleum ether for the fractionation of polar and non-polar lipids.[68] Combined countercurrent distribution and isoelectric was used in the isolation of proteins from rapeseed.[69]

3 Matrix Solid-phase Dispersion

Introduction

Sand, with its sharp cutting surfaces, was often mixed with wet food samples in a mortar in the grinding process to form a slurry or paste of the tissue. The paste was then solvent extracted to release the soluble components. Sand has also been used to increase the surface area of food samples prior to oven drying for total solids analysis. In these ways the sand acts to disrupt or disperse the sample material to assist the extraction. The idea of dispersing/disrupting the solid and semi-solid food samples to gain access to more of the extractable material has been adapted in MSPD not only to do that but also to provide an adsorbing powder as the dispersant.[70] If the mixture of adsorbent and dispersed solid sample is then packed into a glass column and an organic solvent percolated

through, components may be eluted. The bulk sample may be extracted using a general-purpose solvent and the eluent taken through a further stage of separation, or a solvent with a narrower solubility band used to elute target compounds sequentially. Therefore, in some respects only, MSPD[71] is a modern version of SLE. The reader is referred to the review by Ahmed (2001) and references therein (Chapter 8, ref. 76) for further study. It also solves some of the problems associated with SPE. The method was reviewed in 1993.[72]

The processes involved in the application of MSPD in food analysis are illustrated in Figure 4.3. The literature originating from the developers' laboratory is recorded and many applications (analytes and commodities) are tabulated in the review by Barker (2000, Chapter 8, ref. 9).

The advantages of MSPD are the combination of extraction and clean-up stages, good recoveries, and reduced solvent use (compared to other SLE methods). The technique has been applied to pesticide residue analysis in particular.

Development

Furazolidone in Pork

In an early development by the originator, furazolidone was extracted from pork muscle tissue.[73] Blank or furazolidone-fortified pork muscle tissue samples were blended with C_{18} (18% load, endcapped, 2 g) derivatised silica as the sorbent. A column packed with C_{18}/pork matrix was first washed with hexane (8 ml), followed by elution of furazolidone with ethyl acetate. The ethyl acetate extract was then passed through an activated alumina column. At this early stage the method was linear over a useful range and had an average percentage recovery of $89.5 \pm 8.1\%$ for the concentration range (7.8–250 ng g^{-1}) examined, and resulted in an LOD of 390 pg on column. MSPD was also applied to tetracycline antibiotics.[74]

Heterocyclic Aromatic Amines

Ten aromatic HAs were used in the development of a model system to study their formation from precursors, creatine, carbohydrates, and free amino acids during high temperature food preparation.[75] The extraction from beef, pork chops, chicken and turkey breasts was effected by dissolution in 12 ml 1 M NaOH and homogenisation for 1 h at 500 rpm. The solution was mixed with 13 g diatomaceous earth and extracted with ethyl acetate and the eluate re-extracted through an Oasis MCW cartridge eluted with 2 ml MeOH–conc. NH_3 (19:1, v/v).

Pesticide Analyses

Oysters. The development that generated many followers was the study of 14 chlorinated pesticides (alpha-BHC, beta-BHC, lindane, heptachlor, aldrin, heptachlor epoxide, p,p′-DDE, dieldrin, endrin, 4,4′-DDD, endrin aldehyde,

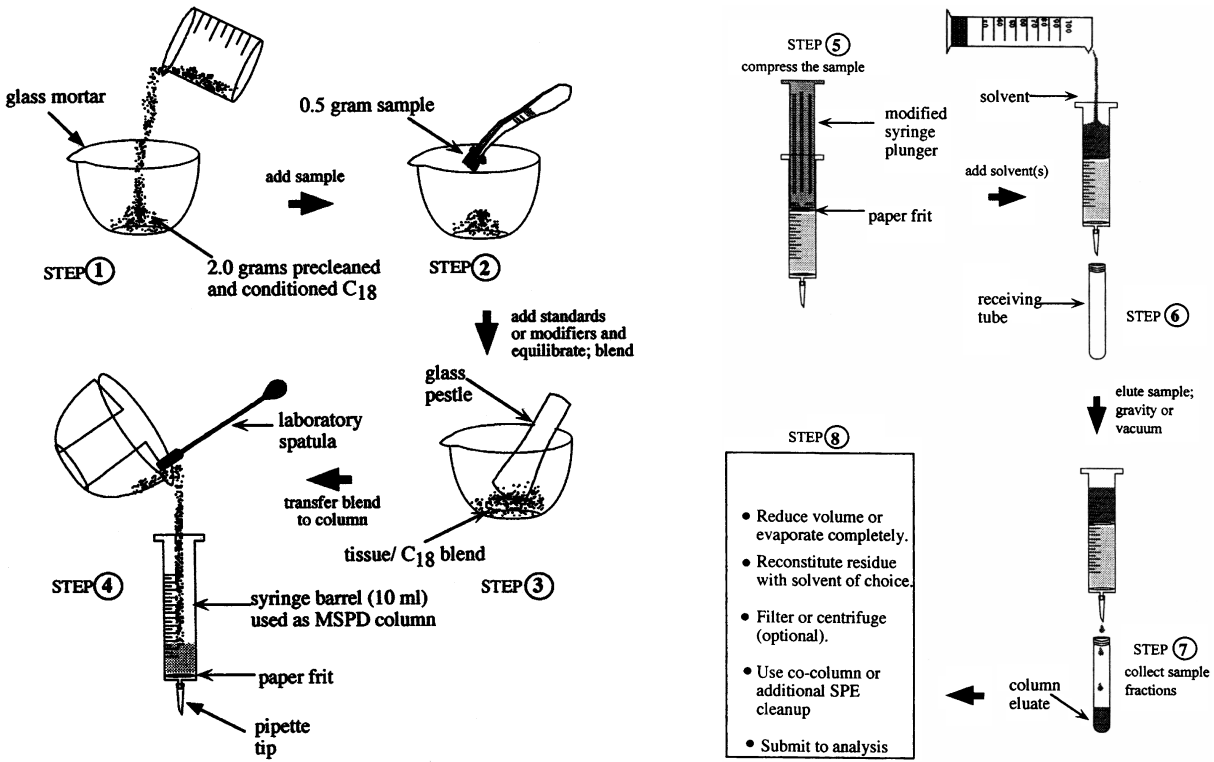

Figure 4.3 *Stages in the MSPD process*
(Reprinted from the *Journal of Chromatography A*, vol. 885, S.A. Barker, "Matrix Solid-phase Dispersion", pp. 115–127, © 2000, with permission from Elsevier)

p,p′-DDT, endosulfan sulfate, and methoxychlor) from oysters.[76] Oyster puree was fortified with the pesticides and δ-BHC, as an internal standard, blended with 2 g C_{18}-derivatised silica, and the pesticides eluted with 8 ml acetonitrile–methanol (90:10) from an extraction column composed of C_{18}/oyster matrix blend and 2 g activated Florisil. Average relative percent recoveries over the range of concentrations examined ($66 \pm 12.7\%$ to $84 \pm 25.3\%$, $n = 25$ for each pesticide) showed the general success of the method.

Fruits and Vegetables. In a development exercise testing alumina, silica and Florisil as dispersants for pesticides in vegetables, the recoveries with dichloromethane were similar, but again Florisil produced the cleanest extract.[77]

Thirteen carbamate pesticides were extracted from orange, grape, onion and tomatoes by MSPD.[78] Different solid phases (C_{18}, C_8, cyano, amine and phenyl) for the sorbent were tested. C_8 was found to produce the cleanest extract. Mean recoveries using C_8 varied from 64–106% and RSDs from 5–15% in the concentration range of 0.01–10 mg kg^{-1}. LODs were typically in the 0.001–0.01 mg kg^{-1} range, which were between 10 and 100 times lower than the maximum residue levels (MRLs) established by the EU.

LLE has been the method of choice for extracting chlorinated pesticides from fruit and vegetable samples, but MSPD with the above advantages promised to be environmentally friendly and furnish a faster analysis. The extraction of endosulfan isomers and endosulfan sulphate from tomato juice was optimised by choosing pure ethyl acetate rather than mixtures of it with *n*-hexane as the solvent, and Florisil over alumina as the adsorbent for the cleaner extracts produced.[79] UAE improved the yield of extractant with both solvents and for all extractants, so MSPD was performed in an ultrasonic bath at room temperature for 15 min. Recoveries were 81–100% with the RSD $\leqslant$ 10%.

A MRM based on MSPD microextraction was developed for the carbamate, benfuracarb, and urea insecticides, diflubenzuron, flufenoxuron hexaflumuron and hexythiazox, used in control of citrus pests. Optimisation of different parameters led to 0.5 g of orange sample, added to C_8 bonded silica as sorbent and DCM as solvent. Spiked sample recoveries, at concentrations below the maximum residue levels established by the Spanish Government, were between 74 and 84% with RSDs ranging from 2 to 4%.[80]

The Codex Alimentarius MRL for ethylene bisdithiocarbamate (0.1 mg kg^{-1}) and the optimisation of the MSPD (washed sand as support and NaOH as defatting agent) and SPE clean-up procedure (C_{18} or alumina and acetonitrile) produced LOQs of 0.05 and 0.07 mg kg^{-1} for ethylenethiourea and ethylenebis (isothiocyanate) sulphide, respectively, in samples of almonds.[81]

Animal Fat Tissue. A salutary report[82] warns that a non-standard dispersant material, a locally bought Florisil, can introduce impurities that pass as pesticide components in an analysis of chlorinated pesticides in animal fat tissue, even when high resolution CC-GC is used.

Development of Automated Assays. A miniaturised and automated MSPD extraction of organophosphorus pesticides and a pyrethroid from oranges, later tested on apples, pears, and grapes, used only 25 mg samples and 100 µl of solvent.[83] The LOD was 4–90 µg kg^{-1} and the recoveries were 83–118% (in oranges) with a RSD of 10–13%.

Applications

Pesticide Residues

Organochlorine Pesticides. Milk (5.0 ml) was blended with 2.0 g of C_{18} octadecylsilyl-derivatised silica and 1.5 ml AcCN in a syringe barrel. After the aqueous phase had been removed by vacuum aspiration, the pesticide residues were eluted from the C_{18}/milk matrix with AcCN. The AcCN extract was then re-chromatographed through a Florisil SPE column, and the final eluent evaporated under nitrogen and the residue dissolved in petroleum ether. The MSPD and the AOAC International multiresidue method for pesticides in milk produced comparable results for milk samples containing incurred organochlorine pesticide residues and also delivered a 90% reduction in organic solvent consumption and a 95% reduction in the hazardous waste generated when compared with the AOAC method.[84]

The MSPD method was found suitable for the extraction of an organochlorine pesticide, hexachlorobenzene, from perirenal and dorsal fatty tissue.[85] The perirenal fatty tissue contained significantly higher levels of HCB than the dorsal fatty tissue ($P < 0.01$). All levels were below the criteria for MRLs established by Croatia and the EU.

Chloramphenicol Residues. A column filled with a mixture of C_{18} bonded silica adsorbent blended with animal muscle tissue was washed with *n*-hexane and AcCN–water (5:95, v/v) and the chloramphenicol residues eluted in AcCN–water (50:50, v/v). The eluate was partitioned into ethyl acetate.[86] Fortified samples at 5, 10, and 15 µg kg^{-1} had mean recoveries of 93, 96, and 98%.

Abamectin Residues. A 96% recovery of abamectin residues from oranges using C_{18} bonded silica as dispersant and DCM as eluent was reported by Valenzuela *et al.*, (2000).[87,88]

Organophosphate Residues. Twenty four organophosphate pesticides were dispersed on diatomaceous material and extracted from milk.[89] MSPD was used to effect a simultaneous extraction of 12 organophosphorus pesticide residues: carbophenothion, chlorpyriphos, chlorfenvinphos, diazinon, ethion, fenitrothion, malathion, methidation, methylparathion, phosmet, quinalphos, and tetradifon from citrus fruits for SIM-GC-MS.[90] Over 32% of samples contained pesticide residues and almost 7% exceeded EU MRLs.

Fungicide Residues. Eight fungicides were quantified from 0.5 g samples of spiked orange, apple, tomato, artichoke, carrot and courgette dispersed with C_{18} bonded silica (the clean-up sorbent was silica) and eluted with ethyl acetate.[91]

Five post-harvest fungicides (dichloran, flutriafol, *o*-phenylphenol, prochloraz, and tolclofos-methyl) were extracted from fruits (bananas, lemons, and oranges) and vegetables (chards, onions, and peppers) using MSPD.[92] More recently, the same team[93] has studied dithiocarbamates and their metabolites, applying MSPD to the analysis of dazomet, disulfiram, and thiram, from EU recommended food groups by composition: cereals and dry crops; rice, and oats, high water content; lettuce, cherries, peaches, and tomatoes, high fat content; avocados and nuts, and fruits with high acid content; lemons and oranges. Sampling was also according to EU directives. The optimised method was dispersion with carbon (chosen from alumina, Extrelut, Florisil, silica, carbon, C_8, and C_{18}) and elution with DCM–MeOH for LC–MS.

Estimation of Daily Intake. Some time later, methods to measure the estimated daily intake of five newly developed pesticides (diflubenzuron, flufenuxuron, hexythiazox, benzfuracarb, and hexaflumuron) from oranges also used MSPD.[94] The LOD was between 0.002 and 0.05 mg kg^{-1}.

Aflatoxins

Aflatoxins B1, B2, G1 and G2 were extracted from peanuts. Optimisation of parameters produced the following protocol: 2 g peanut sample was mixed with 2 g C_{18} bonded silica as the MSPD sorbent and AcCN was used as the eluting solvent. Recoveries of each aflatoxin spiked to peanut samples at 2.5 ng g^{-1} (5 ng g^{-1} for aflatoxin G2) level were between 78 and 86% with RSDs ranging from 4 to 7%.[95] The investigation was expanded to examine cereals, dried fruits, herbs and spices, pulses, snacks, nuts and nut products that had been analysed previously by ELISA.[96]

Anabolic Drugs

Three C_{18} MSPD sorbents were compared for the determination of clenbuterol in bovine liver fortified at 5 ng g^{-1}. MSPD grade C_{18} sorbents were more efficient for the blending and packing of the material and for subsequent washing and elution compared with non-MSPD grade sorbent. Liver extracts were enzymatically deconjugated. The mean recovery of clenbuterol was similar for all sorbents, and in the range 86–96% in two intra-assay studies ($n = 3$). MSPD grade C_{18} (end-capped) was preferred.[97]

Polychlorinated Biphenyls and FAMEs

UAE was employed to extract fat from the food sample and then MSPD fractionation provided PCBs for GC analysis.[98] PCBs in fish (grass carp) were

determined with a mixture of C_{18} derivatised silica and acidic silica gel sorbent, allowing smaller samples, shorter analysis times, and lower costs.[99] Fatty acids in the UAE extract were converted into their methyl esters using sodium methylate and also subjected to GC analysis, using suitable columns.

Phenolic Acids

MSPD was used for the extraction of lemon balm. Different MSPD sorbents and elution solvents were tested to optimise the extraction conditions to obtain extraction recoveries greater than 90% for all analytes.[100]

Pyrethroids

Six synthetic pyrethroids (fenpropathrin, cyhalothrin, permethrin, cypermethrin, fenvalerate and deltamethrin) in 5 g samples of West Indian gherkin, eggplant, pak-choi, cabbage and garden peas were extracted by MSPD.[101] A Florisil-based sorbent in an MPSD column and *n*-hexane–acetone (9:1) extractant provided recoveries for 0.1 and 0.5 μg g^{-1} fortification levels of between 92 and 113%. LODs were between 5.1 and 91.5 ng g^{-1}.

Sulphonamide Antibacterial Drugs

An early application of MSPD was to the extraction of sulphonamide from milk.[102] A reviewer at that time welcomed the replacement of SE with SPE or MSPD techniques as a step forward in the determination of sulfonamides in foods of animal origin (meat, milk, and eggs), noting that significant improvements in sensitivity had been achieved.[103] Very recently, a MSPD method using hot water as the extraction solvent for 0.8 g samples deposited on sand (crystobalite) was used to determine the residues of 12 sulphonamide antibacterial drugs in cattle and trout muscle tissues,[104] and MSPD with neutral alumina and with 70% (v/v) aqueous alcohol solvent was used for the analysis of six sulphonamides in chicken.[105]

Vitamin K_1

A LOD of 6.6 pg, a LOQ of 22 pg, a linear response range of 45 to 908 pg, and a recovery of 97.9% ($n = 25$) on a spiked zero reference material (ZRM) was reported for the extraction of vitamin K_1 from medical foods,[106] and an LOD of 12 pg, a LOQ of 38 pg, a linear response between 0.55 and 22.1 ng ml^{-1}, and recovery of 91.7% ($n = 25$) for milk-based infant formula.[107]

β-Carotene in Medical Food

Isocratic normal-phase chromatography was used to quantify β-carotene extracted from medical food by MSPD.[108] No saponification was necessary to

release the analyte. ZRM was spiked with β-carotene and recoveries averaged 91.2% ($n = 25$) with RSDs from 0.5 to 3.1%.

MSPD in Combination with Other Extraction Methods

Vitamin K_1. For the automated extraction of vitamin K_1 from medical foods, the sample was extracted by MSPD and then the eluate processed by ASE.[109] The LOD was 6.6 pg and the LOQ 22 pg. Recoveries averaged 97.6% ($n = 25$) for a spiked ZRM.

Penicillin derivatives. A MRM was developed for the analysis of five penicillin derivatives in animal tissue. MSPD preceded C_{18} reversed-phase SPE with phosphate buffer–acetonitrile as mobile phase.[110] The LOD was 20 ng g^{-1} and recoveries were in the range 40–90% for samples fortified at 40 and 200 ng g^{-1}.

Comparison with Other Methods

MSPD was compared with SBSE (using solvent extraction with ethyl acetate as the classical standard) for the analysis of 10 pesticides (bitertanol, carbendazim, fenthion, flusilazole, hexythiazox, imidacloprid, methidathion, methiocarb, pyriproxyfen and trichlorfon) from oranges. MSPD was superior in recovery and RSD values, while SBSE was more sensitive but not so widely applicable.[111]

4 Sub-critical Fluid Extractions

Several new experimental approaches to the efficient use of solvents for extraction have exploited the favourable conditions at elevated pressure and temperature as the critical point is approached. Sub-critical solvent extraction methodology has been variously called pressurised liquid extraction (PLE), pressurised fluid extraction (PFE), pressurised solvent extraction (PSE), accelerated solvent extraction (ASE), pressurised hot water extraction (PHWE) and enhanced solvent extraction (ESE).

Pressurised Liquid Extraction (PLE)

The origins of PLE go back to the mid 1990s, *e.g.* Richter *et al.* (1995).[112] The technique uses solvents pressurised to near their critical pressure to take advantage of the enhanced extraction properties in this region[113] A scheme of the basic equipment is shown in Figure 4.4.

The commercial equipment has a temperature range to 200 °C and a maximum pressure of 21 MPa with extraction cell volumes 1, 5, 11, 22, and 33 ml, and collection vials of 40 or 60 ml volume. Commercial SFE equipment can also be used for PLE, when pressures up to 70 MPa can be reached. Carousels are available for multiple extract operation.

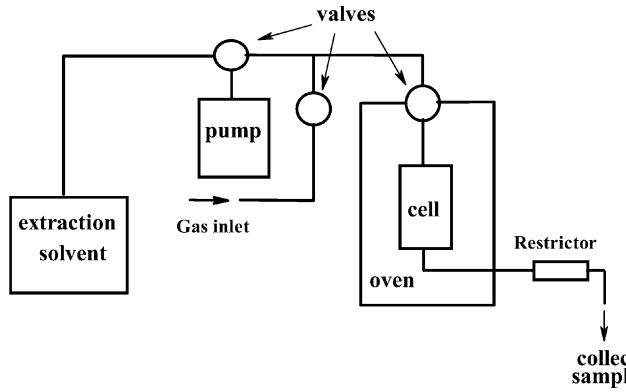

Figure 4.4 *Schematic of the basic requirements for PLE. The sample in the cell is extracted by organic solvent at pressures up to 20 MPa and at temperatures up to 200 °C. Extracted material is flushed from the cell and collected after the flow has been depressurised*
(Figure 1, redrawn from the *Journal of Chromatography A*, vol. 975, L. Ramos, E.M. Kristenson and U.A. Th. Brinkman, "Current Use of Pressurised Liquid Extraction and Subcritical Water Extraction in Environmental Analysis", pp. 3–29, © 2002, with permission from Elsevier)

The sample is bathed in organic solvent (static method) at elevated temperature, whence its viscosity and surface tension fall, while the diffusion rate into the sample and sample solubility increase. The increased pressure lowers the boiling point and increases the depth of penetration into the structure of the matrix. After a time the flow is re-established so that, after depressurisation, the solvent and its extracted solutes can be collected. The apparatus is flushed with a small amount of solvent and then purged with nitrogen, in readiness for the next sample.

The dynamic PLE method – also called dynamic high-pressure solvent extraction (DHPSE) – has utilised SFE equipment[114,115] and laboratory-built equipment.[116] Contrary to theoretical predictions based on Fick's law, DHPSE has not, so far, been shown to have superior efficiency over the static method. As a general guide to the choice of solvent for PLE, several papers, the most recent of which is ref. 117, suggest that the solvents used for Soxhlet extractions are suitable. Mixtures of polar and apolar solvents are often more efficient than single solvents, *e.g.* the use of a AcCN–DCM mixture in place of iso-hexane. Also, more polar solvents, *e.g.* toluene, are preferred to apolar solvents, *e.g.* *n*-hexane, when high adsorption matrices are encountered (Popp *et al.*, 1997, ref. 145).

Method Development

A preliminary study of pesticides in baby foods compared two solvation extraction methods with an adsorption method (SFE, ESE, and SPE) for detection by ELISA or GC-MS.[118]

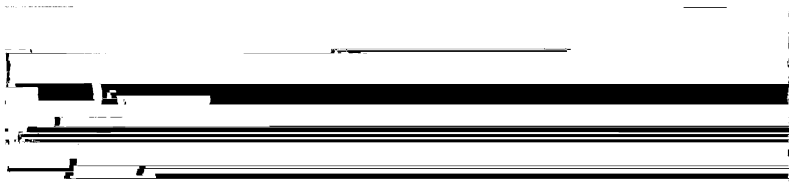

Figure 4.5 *Routes investigated in the optimisation of the method. The ASE extraction was optimised from two solvents (AcCN and ethyl acetate) and three temperatures (80, 100, and 120 °C) as AcCN, 80 °C and 2000 psi pressure. The subsequent SPE was optimised from C_{18}, basic alumina, and ENVI-Carb (Supelco, graphitised non-porous carbon) phases. C_{18} and basic alumina were inferior when fatty foods were being extracted. Seven different eluting solvent combinations were tested and the preferred sequence was 30 ml AcCN (fraction 1), 60 ml 20% DCM in AcCN (fraction 2), and 30 ml 20% DCM in AcCN (fraction 3). Nevertheless, interfering substances were still present and the compromise was based on the separation of remaining interferences so the pesticide target compounds were resolved enough by taking two fractions for GC-MS separation and detection*
(Drawn from data in *Analytica Chimica Acta*, vol. 444, J.C. Chuang, K. Hart, J.S. Chang, L.E. Boman, J.M. Van Emon and A.W. Reed, pp. 87–95, © 2001, with permission from Elsevier)

In a second major comparison of methods of pesticide analysis for large numbers of baby foods, the National Exposure Research Laboratory, USEPA, tested and rejected off-line SFE (using either 100% CO_2 or 15% AcCN) followed by GC-MS, because in their hands it gave only poor recoveries (<50%). They went on to evaluate ASE-ELISA and ASE-GC-MS (Figure 4.5).[119] The main problem addressed was the analysis of pesticides in baby foods containing fat, normally requiring extensive preparation and extraction for chromatographic separation. The paper contains a full account of the experimental protocols, and the optimisation of the preferred ASE summarised here.

Applications

Acaricide Residues in Honey. ASE was optimised in terms of solvent composition, temperature, static extraction time, and solvent flush volume for synthetic acaricides (amitraz, bromopropylate, cymiazole, coumaphos, T-fluvalinate, and flumethrin) and their residues in honey, in preparation for HPLC separation.[120] The acaricides were extracted by hexane–propanol (1:3, v/v) at 95 °C and 2000 psi for 8 min. The LOD was 0.01 to 0.2 μg^{-1}.

Acrylamide Analysis using PLE. The genotoxic compound acrylamide has been found in high concentrations in heated carbohydrate-containing foods.[121] Fast methods of analysis are needed to survey large numbers of samples of carbohydrate foods that have been subjected to high temperatures during processing, and PLE has been used.[122] 5 g samples, in a 34 ml extraction cell,

were extracted in 20 min using water or 10 mM formic acid in water in the ASE 100 or ASE 200 system (Dionex, Sunnyvale, California, USA). Food samples prone to swelling were put in Soxhlet thimbles and then inserted into the extraction cell. Co-extractables were removed by ion-exchange chromatography. The method was used on potato chips, potato crisps, tortilla crisps, wheat snacks, and crisp bread. The precursors of acrylamide are thought to be asparagine and related amino acids. The presence of these amino acids in potato extracts has been measured using the EZ:fast® procedure. Lyophilised potato sample was extracted in 25% acetonitrile in water for 1 h at 40 °C (0.05 g to 1 ml liquid extractant).[123]

Catechins. Catechin and epicatechin were extracted from tea leaves and grape seeds by PLE for HPLC analysis.[124] The stability of catechins to PLE was tested and recoveries began to fall off to <95% at 130 °C and above. Optimum extraction was obtained with MeOH (from MeOH, EtOH, ethyl acetate, and water) using PLE (from magnet stirring, UAE, and PLE) in 10 min with RSDs of 3.21 and 2.96% for catechin and epicatechin, respectively (Appendix 1).

PAHs. Fish tissues and ground pork samples were used to check recoveries for ASE of PAHs in biological samples.[125] Recoveries were better than or equal to the Soxhlet method. The method was then used for smoked meat samples found to contain from 3 to 52 ng g^{-1} wet weight.

Pesticides. Some 28 compounds from 8 pesticide classes were extracted from fresh pear, cantaloupe, white potato, and cabbage with better than 70% recovery and LODs varying according to compound and matrix in the range 0.0019–0.14 μg g^{-1}.[126] *N*-Methylcarbamates in foods were extracted by AcCN at 100 °C and 2000 psi pressure in less than 20 min. A second clean up used a carboxylic acid mini-column eluted with either 10% or 30% acetone in hexane. Seventeen *N*-methylcarbamates spiked at 0.2 ppm gave recoveries of 70–100%.[127] Organophosphorus residues were extracted with ASE at 100 °C and 1500 psi followed by GPC and GC. Four foods were spiked with 19 compounds at 0.1 ppm, giving 80–90% recoveries.[128]

PLE and SPE of Polyphenols from Hops and Hop Pellets. Initial PLE with pentane removed hydrophobic resins and oils for on-line SPE-LC/MS analysis in a convenient and efficient assay[129] (Appendix 2). The use of different solvents sequentially to remove interferents before eluting the analytes was recommended.

Total Lipids and Hydrolysed Fatty Acids. Total lipids in poultry meat by PLE required minimal preparation. The homogenised sample was mixed in a mortar with Hydromatrix (Varian SpA, Torino, Italy), and 2:1 v/v $CHCl_3$–MeOH [the same solvent mixture used in the Folch method (Chapter 1)], at 20 MPa, using 2

static cycles of 10 min each – reducing the solvent consumption to less than half that of the Folch method. The extract was dried over anhydrous Na_2SO_4, filtered and evaporated. The ASE was optimised for time, temperature, and pressure of extraction. In comparison with the Modified Folch and AcHyd methods, PLE for 10 min at 120 °C and 20 MPa gave similar results for total lipids and $C_{12:0}$ to $C_{22:6}$ fatty acids.[130]

Zearalenone from Wheat and Corn. PLE produced a solution ready for injection and analysis by LC/ESI-MS. PLE parameters were optimised for fortified cereals at 80 °C (40, 80 and 120 °C), 5 min (5 or 10 min), and 2 cycles of MeOH–H_2O from AcCN–water (9:1, v/v), MeOH–water (8:2,v/v), and MeOH–AcCN (1:1, v/v).[131] Using IPT samples, recovery was 118% (RSD 5.2%, $n = 3$) for wheat and 107% (RSD 2.2%, $n = 3$) for corn.

Sub-critical Water Extraction (SWE)

The apparatus, shown in Figure 4.6, consists of two pumps, one to pump deoxygenated water through the extraction cell *via* a pre-heating coil, and the other to present the organic solvent for mixing with the water after the extraction occurred. Once the required pressure and temperature have been reached the extraction is started and the eluting mixed solvent stream passes through a cooling coil where rapid cooling causes the sub-critical water to return to being a polar solvent, whereby the dissolved substances partition readily with the organic solvent. Concentration of the separated organic layer produces the

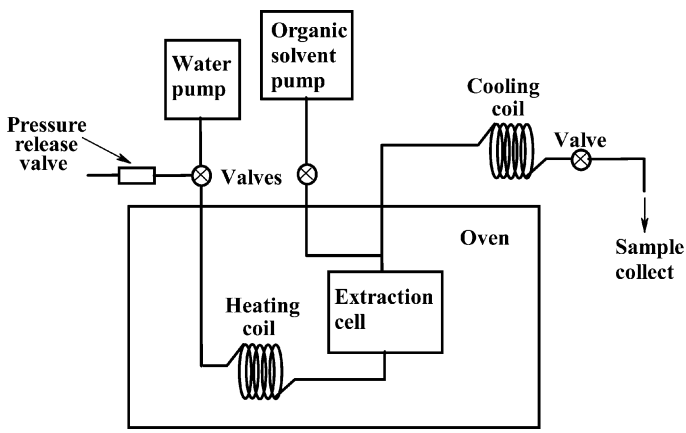

Figure 4.6 *Schematic diagram of the basic SWE equipment*
(Figure 2, redrawn from the *Journal of Chromatography A*, vol. 975, L. Ramos, E.M. Kristenson and U.A. Th. Brinkman, "Current Use of Pressurised Liquid Extraction and Subcritical Water Extraction in Environmental Analysis", pp. 3–29, © 2002, with permission from Elsevier)

sample for further processing (if required). SPE has been used to minimise the dilution caused by the high volume solvent mixing stage[132] (see also Lou *et al.*[134]).

The use of water as extraction solvent, up to the critical point at 374 °C and 22 MPa, is known in the literature by several names (see SWE in Glossary). At a chosen temperature and a pressure sufficient to maintain the liquid state below the critical point this provides a very useful solvent system.

The effect of changing the temperature of water at elevated pressure is to lower its dielectric constant, which is related to polarity. An increase in temperature significantly improves the solubility of low polarity compounds. Also, these changes lower the surface tension and viscosity of the solvent, further increasing the solubility of apolar solutes. Thus, a temperature program will help in the selection of optimum conditions to solubilise different chemical classes. Steam can be used to further reduce the dielectric constant, but steam is corrosive and, therefore, only useful where stable solutes are being extracted. Yang *et al.* (1995) extracted PCBs from soil using steam, and reported a reduction to 30% of the extraction time needed for SWE.[133] While polar phenols can be extracted below 100 °C, it is necessary to reach 200 °C for some pesticides[134,135] and low mass PAHs[136] and 250–300 °C for PCBs and high mass PAHs[137] to be extracted from soils and sediments. This information will be of general interest to food analysts who will need to translate it in terms of the diverse matrices encountered. Also, the development of sub- and supercritical water extraction methods for PCBs may be applicable to foods.[138]

MSPD Clean-up for Combined SWE and SPME

Aqueous SWE extracts were effectively sampled by SPME probes.[139,140] MSPD with XAD-7 HP as dispersant was used to clean up a sample of beef kidney for an ethanol-modified SWE of atrazine, which was subsequently sampled with a carbowax/DVB SPME fibre for GC injection.[141] The SWE utilised a PSE unit at 100 °C and 50 atm and a number of parameters were optimised; 30% ethanol in water (v/v) extracted all the atrazine. SIM ion trap GC-MS allowed a spiked LOD of 20 ng g^{-1} and % recoveries of 104 and 111 from 2 and 0.2 μg g^{-1} spikes were recorded respectively.

Pressurised Hot Water Extraction

Development of Automated Systems

In a program to develop an integrated extraction and clean-up procedure for samples for analysis by LC and GC, pressurised hot water extraction was considered for the extraction of PAHs, and polychlorinated and brominated compounds. Special extraction vessels are needed and commercial equipment is not yet available. Figure 4.7 shows the laboratory-scale set-up. The pressurised water is preheated to the required temperature before passing through the extraction column containing the sample. The pressurised water and solvent-extracted

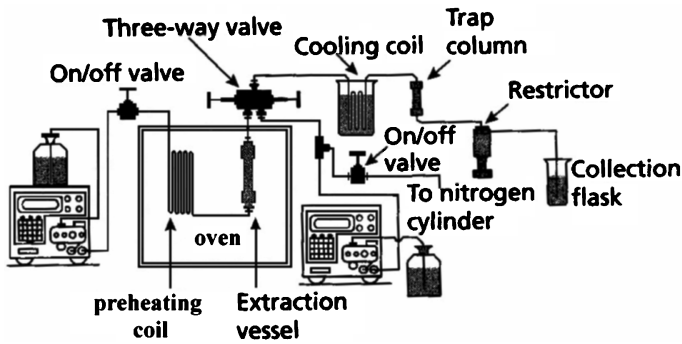

Figure 4.7 *Schematic of the pressurised hot water extraction described by Hyötyläinen and Riekkola*
(Reproduced from, *LC•GC Europe*, T. Hyötyläinen and M-J Riekkola vol. 15, p. 298, © 2002, with permission from Advanstar Communications (UK) Ltd)

material passes through a cooling coil and then the solutes are either adsorbed on a solid phase trapping column or partitioned in an organic solvent at ambient pressure. The trap column is dried and the extracted material dissolved in a small quantity of organic solvent ready for injection into GC or LC for separation. Liquid partition collection is preferred for impure samples.

Although supercritical water is too corrosive for most applications, water at < 350 °C and 20–250 bar has been used to provide greatly increased solubilities, *e.g.* that of benzo[*a*]pyrene was increased from negligible to 1 mg g^{-1} for an increase in temperature from ambient to 250 °C.

Obviously, under these harsh conditions only stable solutes are suitable for extraction. However, the solutes spend only a short time at the elevated temperatures in this dynamic extraction procedure.

Combination with Microporous Membrane Liquid–Liquid Extraction

PHWE was combined with microporous membrane LLE (MMLLE) (Figure 4.8). The principles of MMLLE are explained in Chapter 7. Briefly, it is a continuous membrane extraction that in this configuration replaces a solid-phase trap. Both techniques can be run dynamically and coupled to GC or LC for direct separation of the extracted compounds. This approach is ideal for computer-control of the whole analytical process and developments in this area should lead to shorter total analysis times.

Comparison of PLE and SWE with the Soxhlet Method

In their excellent review,[142] Ramos *et al.* compare the performance of PLE and SWE with the classical Soxhlet method. Their main area of interest was the

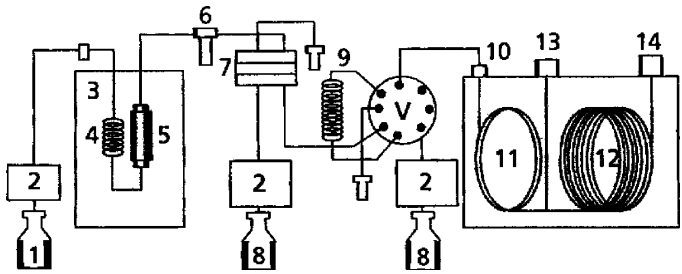

Figure 4.8 *Combined PHWE-MMLLE. 1 = water, 2 = pump, 3 = oven, 4 = preheating coil, 5 = extraction vessel, 6 = restrictor, 7 = membrane unit, 8 = toluene, 9 = sample loop, 10 = on-column interface, 11 = precolumns, 12 = analytical column, 13 = solvent vapour exit, 14 = detector, V = transfer valve*
(Reproduced from, *LC•GC Europe*, T. Hyötyläinen and M-J. Riekkola vol. 15, p. 298, © 2002, with permission from Advanstar Communications (UK) Ltd)

extraction and sampling of environmental material, especially contaminated soil samples, but several food materials were included in the survey. Furthermore, the comparison with the Soxhlet method makes the evaluation of these relatively new procedures so much easier. In common with many extraction method developers, they recognise the need to reduce the time taken to extract and clean up a sample for analysis; additionally they emphasise the need to reduce the consumption of solvents and sorbents, and also recognise the future need for on-line total assays. On-line sample preparation methods have been reported for aqueous samples,[143,144] but when solid samples have to be extracted, because the variety of matrices is extensive, a universally acceptable procedure is hard to design. Thus, the tried and tested Soxhlet method continues to be been used.[145] Ramos *et al.* presented clearly the details, and their review is essential reading for anyone starting out in this area. To summarise, these authors point out that the performance of PLE in relation to desorption of polychlorinated and polyaromatic compounds from environmental sample matrices rivals classical methods such as Soxhlet extraction. Shorter analysis times and smaller amounts of solvents are used. PLE combines "good recoveries and adequate precision with rapid and rather selective extraction."[142]

5 Supercritical Fluid Extraction

SFE can be considered as a dense-gas extraction process. When a fluid is above its critical temperature and pressure it is in the supercritical phase (Figure 4.9).

In food analysis, the most common gas is purified CO_2 because of its widespread acceptance in the food industry. The use of CO_2-SFE for the removal of caffeine from coffee has been in use for some time, and other large-scale applications have emerged. The combination of gaseous and liquid properties of a solvent in its supercritical state is ideal for optimising extraction of stable,

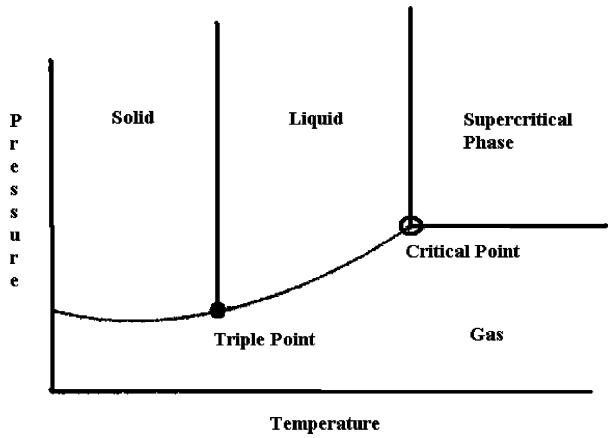

Figure 4.9 *Phase diagram. The interface among the solid, liquid and gaseous phases is the triple point, at which changes in temperature and/or pressure cause a change of state. The critical point is the combination of temperature and pressure above which the liquid or gas enters the supercritical region and their properties change*

soluble components in continuous processes. Supercritical carbon dioxide, for example, is non-toxic and non-flammable.

The food sample is placed in an extraction vessel and pressurised with CO_2. Solutes dissolve and the solution is transferred to the collection vessel where it is depressurised to cause the solute to precipitate. By manipulating the temperature and pressure, analytes can be selectively dissolved and extracted from the matrix. For quantitative analytical extraction, the integrity of the analyte to the harsh supercritical conditions has to be known.

There are now several commercial systems available with various combinations of extraction vessels and solvent recovery systems.

Developments

Ion-pair SFE for Clenbuterol

As an alternative to SE, simultaneous ion-pair/SFE for clenbuterol extraction as its 10-camphorsulphonate was studied in lyophilised milk and liver. CO_2 SFE at 383 bar and 40 °C for 30 min was used with the ion-pairing reagent added to the chamber before extraction. Recoveries were in the range 12–87%.[146]

Markers for Food Irradiation

2-Alkylcyclobutanones are used as markers for γ-irradiation in lipid-containing foods, *e.g.* freshwater and sea fish. CO_2-SFE was developed as an alternative to Soxhlet–Florisil chromatography for their isolation.[147] The SFE method was considerably faster.

Countercurrent SFE (CC-SFE)

Antioxidants. The effect of the solvent/feed ratio on the efficiency of extraction was studied while the countercurrent flow CO_2-SFE method was used to isolate flavanoids from orange juice in a pilot-scale operation.[148] RP-LC/MS was used in the identification of the components of the different CC-SFE fractions.

Fish Oils. Vitamin A palmitate was extracted from model cod liver oil/vitamin mixtures using pure CO_2.[149] CO_2–EtOH mixtures were used to obtain solubilities in fish oils and squalene. Supercritical CO_2 was also used to obtain phase equilibria of fish oil ethyl esters.[150]

Vitamins and Carotenoids

Commercial SFE Instrumentation. Work on the evaluation of two commercial SFE instruments for the analysis of vitamins A and E in meat, liver paste, milk, and milk powder reported that SFE and saponification provide a good alternative to conventional LLE, while having the distinct advantages of reduced running costs and reduced solvent use.[151]

The HP 7680T (Wilmington, DE, USA) with solid-phase trapping and the Isco SFX 3560 (Isco, Lincoln, NE, USA) with solvent collection SFE systems were compared with SE for the four samples. The food matrix was loaded with layers of hydroflow SFE wet support matrix, 0.5 g ascorbic acid, and 1 ml MeOH into the extraction thimble. All-*trans*-Retinol, retinol esters, α-tocopherol and tocopherol esters were extracted in a one-step process taking 80 min. The solvent was evaporated in a stream of N_2 at 40 °C. The extract was saponified (4 ml EtOH, 10 mg ascorbic acid, and 1 ml 50% KOH at 40 °C) to hydrolyse the glyceride and convert the vitamin esters into the vitamin. The reaction mixture was shaken vigorously every 10 min, and then re-extracted into water–petroleum ether with further vigorous shaking. The organic phase was water washed, evaporated to dryness under argon and reconstituted in 0.5 ml EtOH for HPLC. The saponification step was optimised as lipids will also be hydrolysed and may consume an amount of alkali, leaving the reduction of vitamin esters incomplete. With an excess of KOH, 30 min saponification was recommended to give 100% release of vitamin A, and the addition of 2 ml EtOH to the extraction cell was necessary to optimise the release of vitamin E. Too long a saponification time will reduce vitamin A recovery, and therefore the balance is important. (Summarised from ref. 151 with permission from Elsevier)

Based on this thorough development, the Lund University team extended the methodology in an exemplary exercise in analytical chemistry, providing complete details of their new methods based on SFE and described for the measurement of fat-soluble vitamins in processed foods.[152] Vitamins A, E and β-carotene were determined from UHT milk, milk powder, minced meat, liver paste, infant formula, canned baby food and margarine, doubling the sample-processing rate. Although accuracy and precision have not been determined, the detection limit was below 0.1 $\mu g\ g^{-1}$, and recoveries of almost 100% were obtained. The

validation trial involved five laboratories and the intercomparison test was undertaken by ten laboratories. The within laboratory RSD was 11% or less and the between laboratory RSD was 23% for the validation trial and 40% for the intercomparison.

Ruggedness tests showed that different types and models of equipment did not have as much effect on the RSD as the level of experience of the participants. MeOH or EtOH was added to aid the extraction from the food matrix, and an antioxidant was added to protect the vitamins during the assay.

Vitamin K_1 was extracted from commercial soy protein- and milk-based powdered infant formulas with CO_2-SFE at 8000 psi and 60 °C in 15 min, and avoids the problems with lipophilic materials experienced with SE.[153]

SFE and Micellar Electrokinetic Capillary Chromatography (MECC). CO_2-SFE (Applied Separations, Allentown, PA, USA) at 62 MPa and 50 °C was used to extract fat and lipid soluble substances from freeze-dried orange juice in preparation for the analysis of 14 water-soluble vitamins and vitamin cofactors by MECC (Hewlett-Packard, Waldbronn, Germany).[154] MECC on test vitamins gave excellent electropherograms, but on defatted orange juice gave a great many peaks, some of which interfered with the known vitamins. Nevertheless, the predictability of the elution order, based on the affinity of some species for the cholate micelles, turns micellar chromatography into a valuable extraction method if put on-line in flow switching format followed by further separation. In this case, the positively charged surface of the micelle attracted negatively charged species and the EOF was in the direction of the cathode. The order of elution was cations followed by neutrals, followed by anions of increasing charge. While the cations had very little interaction with the micelles, the neutrals to some extent and the anions increasingly as the charge increased were "extracted" by the micelles from the moving buffer and thus delayed for later elution.

Multi-sample Extractor for Lipid Extraction

Total diet studies required large numbers of large samples to be extracted. A multivessel CO_2-SFE was constructed and used for total lipid extractions from a range of foods.[155] SFE at 10000 psi, 80 °C, expanded gaseous flow rates of 4–5 L min^{-1} (at 35 °C), and 1–3 h extraction times gave reproducible recoveries from pork sausages, corn chops, cheddar cheese, and peanut butter.

Pesticides in Meat and Fatty Tissues

A method based on GC was developed for the analysis of OPPs in fatty tissues.[156] Parameters of density, temperature, flow rate, and extraction time were studied and temperature and density were identified as the most important in the recovery of OPPs. The analysis of chlorpyrifos, chlorpyrifos-methyl, malathion, pirimifos-methyl and prothiofos demonstrated the polarity range covered by

SFE. LODs of 0.01–0.03 mg kg^{-1} were recorded. The advantages of the new method were said to be speed, less labour intensive, reduced solvent use, and capable of automation.

SFE Compared to Soxhlet and UAE for Isoflavones

Another substantial contribution to the development of SFE was made using SE of isoflavones from soybean flour (Table 4.3).[157] The development of the SFE method was compared with the classical Soxhlet and UAE methods. To use SE methods, the samples were thoroughly ground in a disc grinding mill to start the dehulling and continued in a knife grinding mill, before being defatted in a Soxhlet extractor using 10 ml g^{-1} hexane. The standard Soxhlet extraction was carried out in a 500 ml glass thimble with 80% (v/v) MeOH–water at 70–80 °C for 9 h. For the ultrasound method, 1 g sample was mixed with 20 ml of 80:20 (v/v) MeOH–water for 1 h at ambient temperature.

The SFE development used the Hewlett-Packard 7680A module with 1 g of sample in a 5.46 ml (effective volume) thimble. MeOH–water (70:30, v/v) was the modifier under pressures of 200–360 bar at 40–70 °C. A 10 min static extraction was followed by a 20 min dynamic extraction and the CO_2 flow rate was 1 ml min^{-1}. The variation in the extraction efficiency among the three methods for the three isoflavones genistin, genistein, and daidzein is worthy of note. The maximum amount of total isoflavones was 312 μg g^{-1} by ultra-sonification, 213 μg g^{-1} Soxhlet and 86 μg g^{-1} CO_2-SFE.

On-line SFE–Piezoelectric Detection

An automated SFE–piezoelectric detection system was developed for the quantitative analysis of total fats in foods using skimmed milk and cocoa as examples.[158] Diatomaceous earth was used in the extraction thimble at 100 °C

Table 4.3 *Variation in the integer amount of isoflavone extracted from soybean by extraction method*
(Modified from Table 2, *Food Chemistry*, vol. 8, M.A. Rostagno, J.M.A. Araújo and D. Sandi, "Supercritical Fluid Extraction of Isoflavones from Soybean Flour", pp. 11–17, © 2002, with permission from Elsevier)

	Extraction method			
Isoflavone (μg g^{-1})	*Soxhlet*	*Ultrasound*	*CO_2-SFE 50/360*[a]	*CO_2-SFE 70/200*[b]
Genistin	205	300	54	52
Genistein	4	8	2	2
Daidzein	3	3	31	17
Total	213	312	86	71

[a] 50 °C and 360 bar. [b] 70 °C and 200 bar.

and a CO_2 fluid density of 0.60 mg ml^{-1} was applied. The new method was compared to Soxhlet extraction.

Applications

Adsorption of Food Aromas by Packaging Polymers

The comparative adsorption of low-molecular weight aroma chemicals into food packaging polymer films was monitored by GC analysis. Films were stored in an aqueous solution of 10 apple aroma compounds and then extracted by SFE.[159] The chemical classes adsorbed and the differences among commonly-used packaging films was studied.

Cholesterol

CO_2-SFE was used to extract cholesterol, and capillary column SFC was used with supercritical CO_2 as the mobile phase to determine cholesterol in certain foods.[160]

Nitrosamines in Cured Meats

The development of the SFE method for nitrosamines was to replace distillation (MOD and LTVD) and SPE methods, which were time and solvent consuming. The paper describes the SFE-CO_2 analysis of NPYR and NDMA found in fried bacon. Eighteen samples were analysed by SFE, SPE, LTVD and MOD, all followed by TEA[161] (Appendix 1).

Pesticides in Meat and Fatty Tissues

An automated SFE method for organochlorine and organophosphate pesticide residues in fats, such as butter fat and corn oil, was described in which supercritical CO_2 was modified with 3% acetonitrile at 27.58 MPa and 60 °C.[162] A C_1 bonded phase preparative column at 95 °C was used to effect the separation of 86 out of 117 non-polar to moderately polar pesticides from fat. Ten of the thirty-one pesticides not recovered could not be recovered with the classical Florisil absorbent. The method used a 5 g sample of fatty food containing less than 18% fat and 70% moisture. The new procedure simplified the extraction and reduced the amount of solvent and hazardous waste created.

Pigments

Carotenoids and chlorophyll were extracted from a freeze-dried powder of microalgal food additives by SFE to avoid forbidden solvents. It was necessary to work at 400 bar and 60 °C with the Isco model SFX 220 (Nebraska, USA) to obtain a significant yield.[163] Temperatures above 60 °C were not advisable, to

avoid thermal decomposition, and while the carotenoids, being of low polarity, were conducive to CO_2 extraction, the more polar pigments such as chlorophyll a were less suitable.

Vitamins

SFE has been reported for the extraction of vitamin A from liver[164] and cereal products.[165] It was also used as one of the extraction processes when vitamins A and E were extracted from milk powder.[166]

Volatiles

Extraction of cheese volatiles by SFE was optimised in relation to preparative dehydration and sonication, addition of alumina adsorbent, reconstitution solvent (2:1 *n*-hexane–acetone), and an ODS trap held at −5 °C.[167]

PCBs

PCB congeners were extracted from lyophilised fish tissue using CO_2-SFE. Compared with Soxhlet extraction, SFE gave quantitative recoveries with an LOD of 0.5–2 ng g^{-1} and SDs of <5%.[168]

Supercritical Fluid Extraction Method Compared to Others

Herbs

When compared to SDE, SFE produced better RSDs and avoided thermal degradation or solvent contamination for the extraction of oregano, basil, and mint (Diaz-Maroto *et al.* (2002) (Chapter 5, ref. 135, and Appendix 1).

Pesticides

CO_2-SFE was compared to solvent extraction with acetonitrile, and Soxhlet extraction as standard, for recovery of 22 organochlorine pesticides spiked into eggs.[169] C_{18} and Florisil SPE were used to clean up the extracts. With 0.1 ppm spike, recoveries were 52–100% by SFE and 53–93% by solvent extraction.

Essential Oils

The yield of ground fennel seed extract by CO_2-SFE (10%) was compared to that of StD (3.0%), SE (hexane) (10.6%), SE (alcohol) (15.4%). Although the SE methods gave similar yields, the sensory evaluation showed that quality was down on that achieved by SFE.[170]

Supercritical Fluid Chromatography

Introduction

Analytical scale supercritical fluid chromatography (SFC) is not an extraction technique *per se* but preparative SFC has been used to isolate target compounds for further study (Buskov *et al.*[171]). One or two examples of SFC are given to highlight its potential for use in the capillary column mode with miniature workstations, and in the preparative mode as an extraction technique for on-line coupling to GC-MS.

Applications

Trichothecenes. Capillary column SFC and SFC/MS are reviewed for the analysis of trichothecenes from cereals and other foodstuffs.[172]

Glucosinolates and Ascorbigens. The enzymatic hydrolysis products, *e.g.* the isothiocyanate and the nitrile, of sinalbin (4-hydroxybenzylglucosinolate) and the aqueous reaction product, 4-hydroxybenzyl alcohol, and the ascorbic acid reaction product, 4-hydroxyascoribigen were studied by SFC using a bare silica column.[171] A preparative SFC method was developed and used to extract target compounds for further study. Degradation products of glucobrassicin (indol-3-ylmethylglucosinolate and the ascorbigen and products) were also analysed by SFC, and preparative SFC used for the extraction and purification of indol-3-ylmethyl oligomers.[173]

A further study of ascorbigen, neoascorbigen and 5-methoxyascorbigen from brassica species was made using normal phase SFC. Again a preparative scale separation was developed to extract these analytes.[174]

Fatty Acids. The separation of FAMEs or FFAs by packed and open-tubular packed capillary SFC has been reviewed.[175] A main topic discussed was the avoidance of the use of modifiers by adjusting the polarity of the stationary phase to increase the range of polar compounds that could be analysed.

Sulphonamides. An early review (1992) of the determination of sulphonamides in meat covered, in 89 references, the use of TLC, HPLC, GC, GC-MS, LC-MS, and SFC/MS, and immunological methods.[176]

Mycotoxins. Analytical methods for aflatoxins and trichothecene mycotoxins were reviewed by Gilbert (1993).[177] Emphasis was placed on possible automation using immunoaffinity columns, while SFC-MS, LC-MS and MS-MS were showing potential.

Cloudberry Oil Volatiles. SFC-GC was used to extract and separate volatiles from cloudberry oil using CO_2 as mobile phase. Capillary SFC made the original

separation, and the volatile fractions were directed towards the GC for further separation. Sixty nine components were recognised using CI- and EI-MS.[178]

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CHAPTER 5

Distillation

1 Introduction

Latent heat is expended when a pure liquid evaporates (volatilises or vaporises) into the gaseous state. When sufficient heat is supplied to the liquid it will reach its boiling point, that is the temperature at which the saturated vapour pressure of the liquid equals the external pressure and the energy supplied by the boiling liquid fuels a change of state. The vaporised liquid will continue in the gaseous state until its temperature falls below the boiling point again when it condenses back to the liquid state. This evaporation/condensation cycle is called distillation. The condensed material can either return to the boiler (reflux) or be diverted (retort) for collection in an external receiver flask. The reflux and retort modes of distillation are used regularly in food extractions when the liquid is often water, some of which may be endogenous, but most of it is added in processing or sample preparation.

Organic liquids are also employed to remove by distillation the volatile components of food. If the water present in moist foods is soluble in the organic liquid the solution has interesting vapour pressure values defined by Raoult's law, depending on the nature of the organic–water molecular structure.

Vapour Pressure of Binary Solutions and Raoult's Law

The addition of salt to the water in which potatoes are boiled may add a "salty" flavour to the cooked potatoes, but it also raises the boiling point (lowers the vapour pressure) of the water so that more latent energy is contained in the boiling liquid system to effect increased thermally-induced chemical changes to the texture and flavour of the cooking product. Changes in vapour pressure also occur when the solute added is another liquid. The relationship between the two liquids in equilibrium is expressed in Raoult's law, which says

The vapour pressure of a solvent in a solution is equal to the vapour pressure of the pure solvent multiplied by its mole fraction in the solution.

For a binary system (A + B) at equilibrium:

$$P_{\text{Total}} = P_{\text{A}} M_{\text{A}} + P_{\text{B}} M_{\text{B}} \tag{5.1}$$

where P_{A} and P_{B} are the vapour pressures and M_{A} and M_{B} are their molar fractions. The law assumes that the bonding between the liquids is equal to the bonding within the liquids. Therefore, only mixtures of ideal liquids (ideal solutions) obey Raoult's law. For a mixture of two ideal liquids – *i.e.*, in practice, non-hydrogen bonding liquids that approach the ideal state – the vapour pressure–composition lines are shown in Figure 5.1.

Very dilute solutions approximate to the law, but at higher concentrations there are not many liquids that are ideal. Therefore most practical solutions deviate from the law.

Figure 5.2 shows four categories of deviant binary solutions. Deviations are due to increased or decreased interactions between the molecules of the two different liquids when they are mixed, compared to the intermolecular forces present in the pure liquid. When the inter–molecular bonding is reduced on mixing, the total vapour pressure will be increased giving a higher than ideally predicted value – a positive deviation, and when it is increased, *e.g.* by hydrogen bonding, then the vapour pressure will be reduced – a negative deviation. Other mixtures exhibit positive maxima; *e.g.* EtOH–water, and some have negative minima, *e.g.* DCM–acetone. The maxima and minima are points on

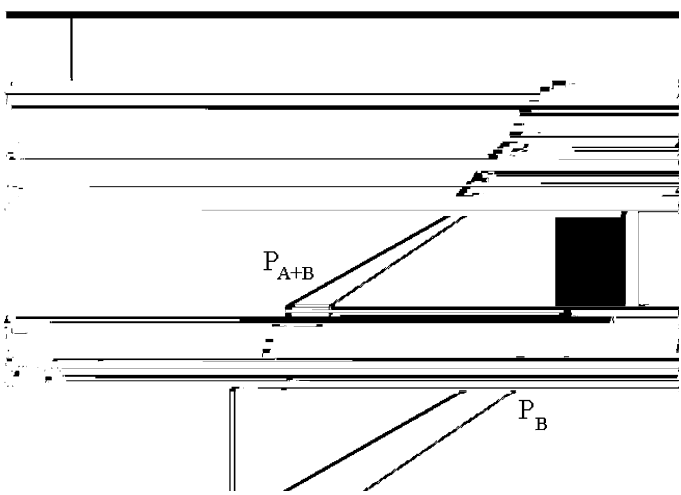

Figure 5.1 *Vapour pressure–composition curve for an ideal mixture of two liquids, A and B, in equilibrium in terms of their mole fractions. Liquid A with the lower vapour pressure will have the higher boiling point. The straight lines* P_A *and* P_B *are the vapour pressures of pure A and pure B from 0 to 1 mole fractions in a mixture of the two liquids. The line representing the vapour pressure of the mixture* (P_{A+B}) *is the sum of the individual vapour pressures of A and B,* i.e., *obeying Raoult's law*

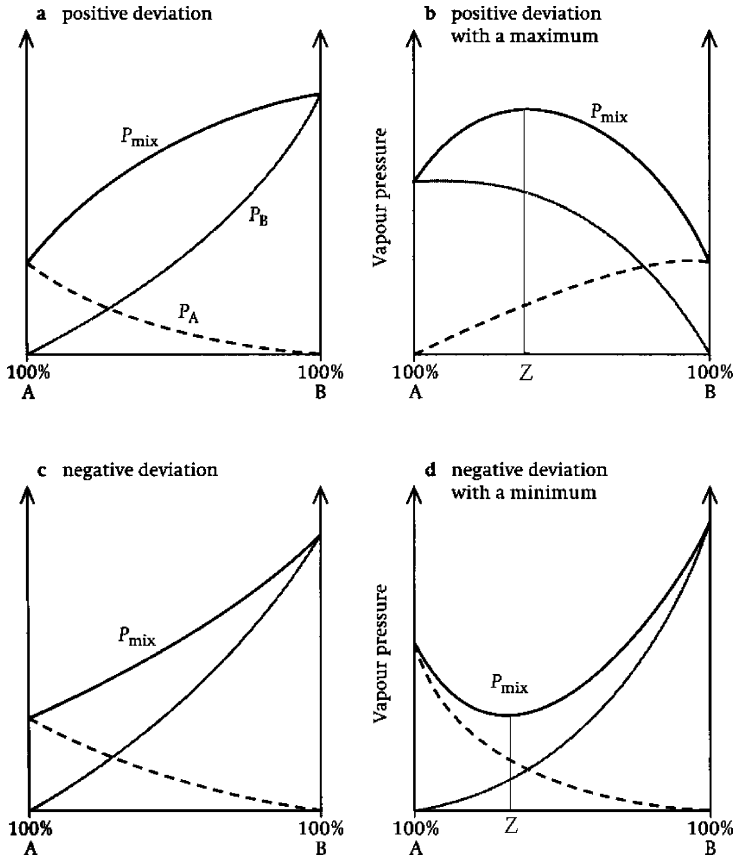

Figure 5.2 *The four types of deviation from Raoult's law.* (a) *A mixture of liquids A and B in molar fractions from 0 to 1,* i.e., *100% A to 100% B, exhibiting a positive deviation, where the total vapour pressure of the mixture is greater than the sum of the constituent vapour pressures, but does not exceed the maximum value of either constituent vapour pressure.* (b) *A mixture of two liquids showing a positive deviation with a maximum value greater than either individual value.* (c) *Two liquids when mixed producing a negative deviation.* (d) *Two liquids having a negative deviation with a minimum value less than either individual value. The maximum and minimum points (Z) are compositions that give rise to azeotropic or constant boiling mixtures*
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the curve at which the properties of the liquid and vapour are identical, *e.g.* point Z (Figure 5.2b and d), and at these azeotropic or constant boiling compositions separation by distillation is not possible. At all other points, the vapour has a different composition to the liquid and can be separated by fractional distillation.

Examples of Deviations from Raoult's Law

Mixtures of pentane and tricosane form negative deviations at 288 and 323 K,[1] while the three binary mixtures of Freon 113, halothane and isohalothane, and various mixtures of propane and cyclopropane form small positive deviations,[2,3] and binary mixtures of ammonia and pentafluoroethane form nearly ideal Raoult's law solutions.[4] Aqueous solutions of *N*-methyldiethanolamine showed a deviation of only 5.3 kPa.[5]

The various types of deviant solutions are encountered in food analysis, for example in the analysis of wines, in the Soxhlet extraction of moist foods, in the partitioning of pigments, vitamins, *etc.*, and in the use of SDE for the extraction flavour volatiles.

Vapour–Liquid Equilibria of Fatty Systems

Extraction of vegetable oils from plant sources involves extraction processes such as fatty acid and fatty alcohol distillations, refining and deodorisation and the attendant solvent recovery processes. A method of estimating the vapour pressures of fatty components, including, acids, alcohols, esters, acylglycerols, has been described.[6] Work is reported on the VLE of oil/solvent miscellas and the paper is a valuable database of vapour pressures from literature sources. It also presents VLE data on unsaturated compounds, fatty esters and acylglycerols, not found elsewhere. Further VLE data on octane–1,1-dimethylpropyl methyl ether and 2,2-dimethylbutane–1,1-dimethylpropyl methyl ether binary systems has been published by del Rio *et al.* in 2002[7] and 2004[8] (and references therein).

Vapour Pressures and GC Retention Data

Vapour pressures of 27 PCBs were plotted against retention indices on two GC phases, *i.e.* polar Dexsil-410 on a capillary column and non-polar OV-101 on a packed column. From these data and published RIs, the vapour pressures of 134 PCBs were estimated.[9]

Cooking, Distillation and Recovery of Volatiles

Volatile compounds may be present in the sample for analysis as a result of earlier enzymic activity or as a result of cell disruption during sample preparation, or they may be formed during heating from their involatile precursors. During distillation, the more volatile the components (*e.g.* low-boiling flavour compounds) are less likely to be retained efficiently at the temperature of the condenser, passing into the air (headspace) above the receiver flask unless, if these components are of interest, they are "trapped" in various ways for further study. In flavour research, it is necessary to know the difference between endogenous and exogenous volatile production.

Reflux Mode Distillation

There are important extraction methods in food analysis that use different types of reflux distillation. An overview is given here and more details are provided later.

Continuous Distillation

By placing a comminuted food sample in a porous container, above the boiler, in the path of the refluxing/condensing liquid solvent, soluble constituents are dissolved (leached) by the condensed, percolating solvent on its way back to the boiling flask. The food is being continuously extracted with freshly refluxed, pure solvent, *e.g.* the Wiley extractor.

Intermittent Extraction

If the food sample is not only placed in a porous container in the path of the refluxing solvent, but the container is kept immersed in freshly condensed liquid solvent (*i.e.* in a cistern with an overflow), then the soluble components are intermittently extracted and returned to the boiler after a period of immersion in the solvent. An example of this is the Soxhlet extractor.

Fractional Distillation

In the reflux mode, a vertical cooling condenser can serve also as a fractionating column, especially if the cooling surface area is increased. There are many patent methods of increasing the surface area of a fractionating column by filling it with glass beads, glass spirals, Fenske helices, or Raschig rings, or by increasing the internal surface area of the column itself, as in the Vigreux column. There are also many more variations on the shape of the distilling head best suited to the particular extraction. Fractionating columns in popular use are designed to provide a large number of theoretical plates in the path of the volatile mixture ascending the tube. The "height of a theoretical plate (TP)" is therefore a measure of the efficiency of the column. The more TPs per cm, the higher will be the separation efficiency of the column,[10] or, alternatively, the easier it will be to separate binary mixtures of similar boiling points. Fractionation creates an equilibrated volatility gradient (high-boiling point components lower down and low-boiling point components higher up the column) such that by intercepting the column at a chosen height a fraction with a required boiling point can be "tapped off".

Coffey Still. The Coffey Still for the commercial distillation of alcohol uses this principle. The rising steam extracts the alcohol from the descending wash (5% alcohol) and is drawn off at the top of the column as a "spirit" of around

85% alcohol. Vapours can be "extracted" at various heights up the column and condensed externally, forming fractions (extracts) of different boiling ranges.

Fractional Reflux/Retort Distillation

Completely miscible liquids, except those forming constant boiling point mixtures (azeotropic mixtures), may be separated by fractional distillation. The mixture boils when the sum of the constituent vapour pressures equals the external pressure or, conversely, the boiling point is the temperature at which the sum of the pressures of the mixed vapours is 1 atm.

The extraction of absolute alcohol (boiling point 78.3 ºC) from an aqueous fermentation uses fractional distillation. The vapour of the lower boiling point ethyl alcohol reaches the top of the large surface area reflux or fractionating column first and is retorted by a water-cooled condenser to an external collector. The aqueous vapour lower down the column condenses and returns to the boiler. Figure 5.3 shows the classical apparatus. By noting the temperature of the vapour at the top of the column, fractions can be collected over different temperature ranges. In the first distillation, raw ethyl alcohol from the fermentation of a sugar is collected in the fraction distilling below 95 ºC, and then on re-distillation the fraction between 78 and 83 ºC is collected. A point is reached where the water

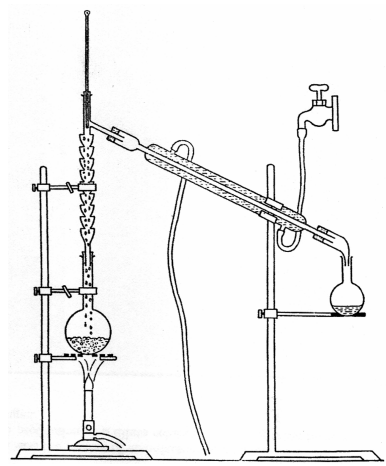

Figure 5.3 *The classical fractional distillation apparatus clearly illustrates the process. Volatile components with different boiling points can be separated into "boiling fractions" in the reflux condenser. For this process, the thermometer in the head of the still is used to monitor the temperature of the vapour that has reached the top of the fractionating column. In the extraction of absolute alcohol from a crude fermentation liquor, the fraction boiling at less than 95 ºC is collected and re-distilled when the fraction between 78–83 ºC is collected in the receiver flask* (The classical distillation apparatus reproduced from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd., London, (First Published 1933), (see Acknowledgements))

content has been reduced to around 4%, when a constant boiling mixture is formed that will not separate by distillation. Further purification may be obtained by chemical dehydration and re-distillation until pure absolute ethyl alcohol with a boiling point of 78.3 °C is obtained.

Retort Mode Distillation

Standard Distillation

This is the most common distillation process whereby the vaporised solvent and volatile material from the food sample co-distil into the headspace above the boiler and, once the apparatus reaches a temperature slightly above the condensation point of the solvent, the distilling vapour passes into the cooled area of the condenser, situated at an angle of greater than 90° from the vertical, allowing the condensate to be collected in an external receiver flask (Figure 5.4).

Vacuum Distillation

If the external pressure is reduced by distilling under vacuum, the boiling points of the components being distilled will be reduced. In other words, the energy efficiency of the extraction will increase. This process is especially useful to reduce the possible decomposition of thermally labile analytes.

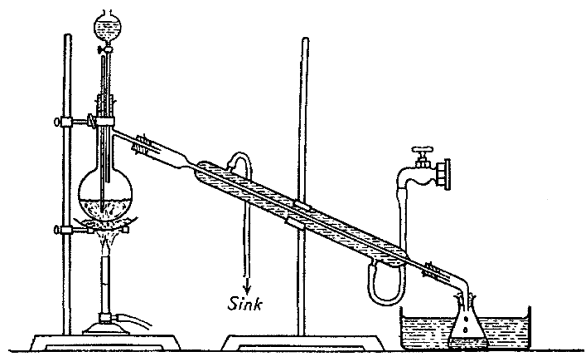

Figure 5.4 *Illustration of the classical distillation. In this apparatus, used for chemical preparation distillation, the thermometer records the temperature of the distilling mixture and the thistle funnel allows more reagent to be added. The water-cooled Liebig condenser transfers the distillate to the receiver kept at a constant temperature in the liquid bath. This principle applies to the collection of condensable analytes. Depending on the temperature of the condenser and the receiver, the non-condensable fraction will be lost to the atmosphere*
(The classical distillation apparatus reproduced from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd., London, (First Published 1933), (see Acknowledgements))

Cooked Cured Ham Flavour. Low temperature distillation was favoured in the flavour extraction process where thermal decomposition introduced products that adversely affected the olfactory quality of the extract. Vacuum distillation was a practical way of effecting the removal of volatiles at low temperatures. Glass traps cooled in liquid nitrogen condensed the vapours being evacuated. Four different vacuum distillation methods were compared using the example of the delicate aroma of cooked cured ham.[11] A direct distillation of a sample of ham for 5 h at 10 kPa and 30 ºC was method 1, a two-step distillation in which the dry residue left from method 1 was distilled for a further 4 h at 10 Pa and 30 ºC was method 2. For method 3 the ground ham was mixed with milliQ® ultrapure water (1:3, w/v), and for method 4 water was added to the ham and the supernatant subjected to two steps of centrifugation at 3500 *g* and filtered. The condensed volatile extracts were acidified to pH 2 with 2N HCl and extracted with DCM, the extract dried over anhydrous Na_2SO_4 and concentrated to 500 µl in a Snyder column of a Kuderna-Danish evaporator-concentrator for GC-MS. Method 3 was judged by an olfactory panel to be the most representative of the original ham aroma. (Summarised from ref. 11 with permission from Elsevier)

Wild Mango Seed Aroma. Thirty two odour-active compounds were released from wild mango seeds by SE and high vacuum distillation for aroma extract dilution analysis (AEDA).[12]

Solvents in Oleoresins. Vacuum distillation or reduced pressure distillation has been used for the extraction of residual solvents in oleoresins.[13] Seventeen different spice oleoresins were examined for DCM, ethylene dichloride, and TCE residues (TCE not found), which were distilled using toluene as the carrier solvent. Analysis was by GC on Porapak Q.

Volatile Nitrosamines. Low temperature vacuum distillation was used to extract nitrosamines from foods.[14] Several other accounts of the use of vacuum distillation in the extraction of N-nitroso compounds appear in the literature (*e.g.* Malanoski *et al.*, 1988, Appendix 1).

Aroma of Rennet Casein. An ether extract was high vacuum distilled and examined by GCO, AEDA, and GC/MS.[15] Reference is made to earlier work on the odour of skimmed milk.

Distillation and Adsorption Vapour Trapping

When the non-condensable fraction is the target, the distillate is isolated from atmosphere by a trap to adsorb the analyte(s) (Figure 5.5).

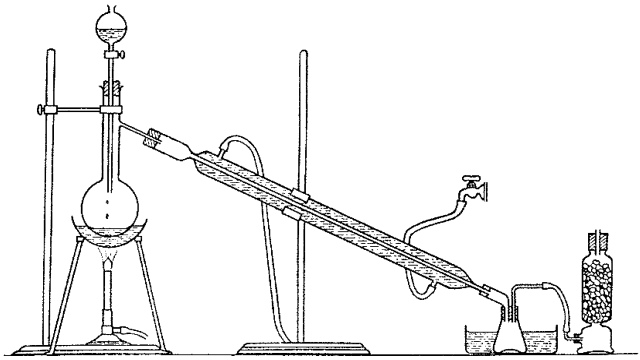

Figure 5.5 *Again, the classical apparatus is shown to illustrate the principle of the adsorption trap fitted to the standard still to retain the volatile compounds of interest. SPE is applied in flavour analysis where the activated carbon trap is popular for its universal adsorption of a wide range of chemical classes that can then be desorbed thermally or with the aid of a solvent. With micro-scale distillations it is possible to sample the headspace volatile substances directly using SPME*
(The classical distillation apparatus reproduced with permission from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd., London, (First Published 1933), (see Acknowledgements))

Distillation and Cryogenic Vapour Trapping

In flavour analysis, the retention of the full range of volatiles requires low temperature trapping – dry ice–acetone or liquid nitrogen are useful coolants. Volatile compounds associated with the aroma released from foods during cooking can be fractionated in a series arrangement of cryogenic traps (Figure 5.6) such that the ambient temperature condensable semi-volatiles, the dry ice condensables and the liquid N condensed compounds provide relatively crude fractions for further extraction.

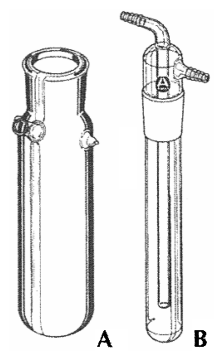

Figure 5.6 *The Dewar flask* (A) *ensures the efficient use of the coolant and the cold finger* (B) *provides a simple accessible condensation surface*
(Reproduced with permission from the Sigma Aldrich Corporation)

Distillation and Vacuum Degassing and Trapping

Devices similar to the cold trap in Figure 5.6 can be used as vacuum traps, although specially designed vacuum traps take into account the compromise between maximum internal diameter and cooling efficiency. High vacuum degassing is the method of choice for the isolation of volatiles from fatty foods.

Distillation and Chemical Reaction Trapping

The determination of daminozide in high protein foods (*e.g.* peanut butter) by GC/MS required the COI to be hydrolysed to unsymmetrical dimethylhydrazine (UDMH), by NaOH digestion, and the digested food matrix to be distilled.[16] Condensing UDMH was reacted with salicylaldehyde in the condensation trap and adjusted in the range pH 5 to 6 with glacial acetic acid. After incubation it was extracted into DCM for SIM-GC/MS of salicylaldehyde dimethylhydrazone using a modified Conditt and Baumgardner method.[17] The LOD was 0.01 ppm in a 50 g sample.

Processed food product samples were hydrolysed with NaOH to convert reaction product ethylene chlorohydrin into ethylene oxide (EO). The hydrolysate was then distilled into dilute H_2SO_4 and NaI, to convert EO into ethylene iodohydrin for GC analysis.[18]

Modern Application

An alcohol distillation is required in the detection of added beet or cane sugar in maple syrup by the site-specific natural isotope fractionation–nuclear magnetic resonance (SNIF-NMR) method. The syrup is diluted with pure water, fermented, and the alcohol distilled off for the detection of the proportion of ethanol molecules monodeuterated at the methyl site, which is decreased by the addition of beet sugar and increased by the addition of cane sugar.[19]

Distillation Processes

Aqueous or Steam Distillation (Section 2)

The special case of steam distillation as practised in SDE, for example, is valuable in food analysis for its ability to lower the boiling points of two immiscible liquids, thus helping to protect thermally labile solutes during the extraction. A mixture of octane (bp 126 ºC) and water boils at 89 ºC. Distillation of essential oils takes advantage of this phenomenon and the only drawback is the presence of small amounts of water in the final distillate that cannot be removed by chemical means if the oil is intended for use as a food additive. Otherwise, dehydration with Na_2SO_4, *etc.* produces a pure oil.

For analytical purposes it is convenient to distinguish between aqueous and non-aqueous solvents. Because water forms a major part of much of the food supply, steam distillation is inadvertently involved in food preparation. Food

aromas are steam distilled during the cooking process. Thus steam distillation is used as an extraction medium for cooked food flavour analysis. Steam distillation–extraction, as a special case of solvent distillation–extraction, is discussed in Section 2.

Some classical distillation methods survive the years with modifications that take advantage of modern materials and instrumentation, but still employing the original chemical concepts. The eponymous Monier-Williams acidified steam distillation–extraction and peroxide adsorption of SO_2 is a good example, employed in the determination of sulphite in preserved foods (Section 2).

Organic Solvent Distillation–Extraction (Section 3)

Solvent distillation–extraction has been employed widely in food analysis to measure some of the major components dealt with in the proximate composition section in Chapter 1, *e.g.* the fat content of foods.

There are other famous names in food extraction methodology, such as the Dean and Stark solvent distillation–extraction for the determination of moisture content. In this case, the water-insoluble organic solvent toluene, with a boiling point of 110.6 °C and a density of 0.865, vaporises moisture from the food sample and transfers it *via* a side-arm to a vertical reflux condenser from which the condensate falls into the external, graduated receiver for measurement below the toluene meniscus. This device allows refluxed, condensed vapour to be collected and quantified directly.

Mineral Oil Distillation (MOD) Method. The extraction of nitrosamines from food samples has used mineral oil distillation, which is considered to be a special case of solvent distillation extraction. The mineral oil acts as a "carrier" vapour, driving more volatile substances towards the condenser.

Simultaneous Steam and Organic Solvent Distillations – Likens–Nickerson Method (Section 4)

Another well-known simultaneous distillation–extraction (SDE), the Likens–Nickerson method, is covered in Section 4. This ingenious method of extracting those volatile substances that are soluble in a co-distilling organic solvent from the steam distilled food sample has found enormous popularity with food analysts and flavour chemists.

Sweep co-distillation (Section 5)

A stream of heated nitrogen gas is used to purge volatiles from a food sample mixed with a solid dispersant in a long glass tube. The volatiles swept out of the tube are condensed for separation and detection. The method uses only small amounts of solvent.

Commonly used distillation processes in food analysis are related in Figure 5.7.

Summary of Commonly Used Distillation Processes

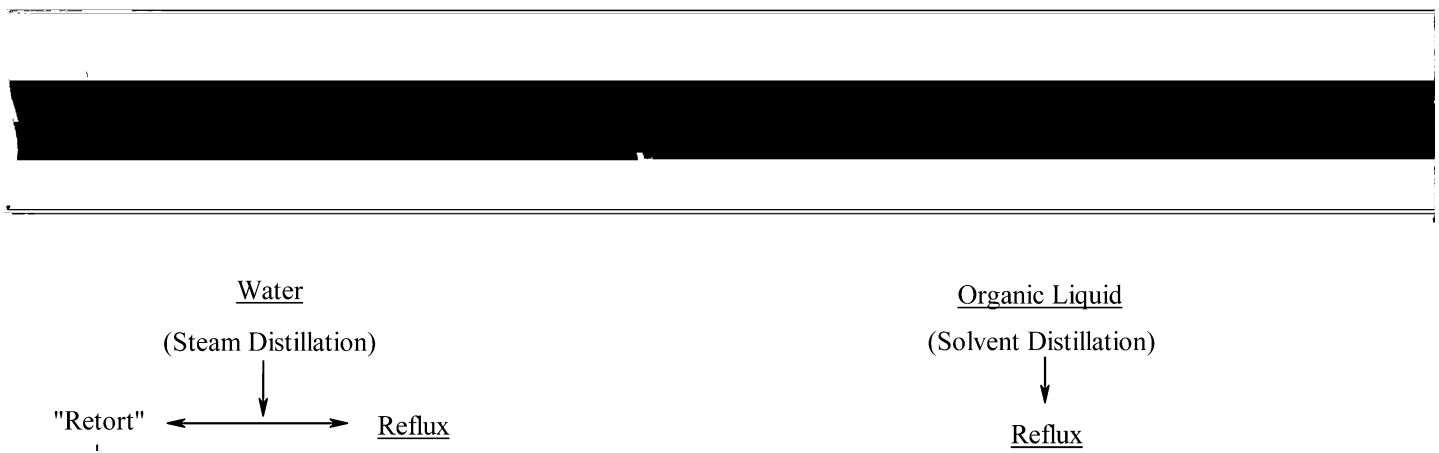

Figure 5.7 *Distillation in food extraction techniques. Nomenclature for distillation–extraction of food constituents. There are several terms used in the literature for various distillation techniques that can be confusing. The full title of simultaneous steam distillation/solvent distillation–extraction for the Likens–Nickerson extraction method is abbreviated to simultaneous distillation–extraction in most papers and the acronym SDE is in common use. The Karl Fischer water determination uses MeOH solvent to release residual water under reflux from powdered (*e.g.*, dehydrated) food samples (Osborne and Voogt, 1978). The Soxhlet method removes soluble constituents from food, returning them to the boiler, and Dean and Stark moisture determination uses an organic solvent,* e.g. *toluene, which is lighter than water and with a higher boiling point*

2 Steam Distillation

Introduction

According to Dalton's Law of partial pressures, a mixture boils when the sum of the partial pressures equals the external pressure. Nitrobenzene has a partial pressure of 27 mm Hg at 99 ºC and water at the same temperature has a value of around 733 mm Hg. Therefore a steam distillation of a saturated mixture of nitrobenzene and water, with individual boiling points of 210 and 100 ºC respectively, will boil at 99 ºC. Steam distillations are used for substances with boiling points above 100 ºC and up to around 250 ºC. They are particularly effective where analytes decompose below their boiling points.

If steam is heated in a separate boiler and passed into the food sample in the distillation flask, itself kept warm enough to prevent the steam condensing, then analytes will be extracted in the vapour and can be collected in a receiver flask after condensation in a water-cooled condenser (Figure 5.8).

Essential Oils

The extraction of volatilisable natural components of foods by distillation has been in use since earliest times, *e.g.* to extract essential oils for culinary purposes.[20,21]

The aromatic volatile essential oil constituents of saffron were steam distilled for GC/MS analysis in a study of the effect of irradiation on quality.[22] In a classical use of steam distillation, eight essential oils were extracted from the scented leaves of pelargonium species and cultivars and added in various concentrations to a quiche filling as a model food system in the study of their antimicrobial

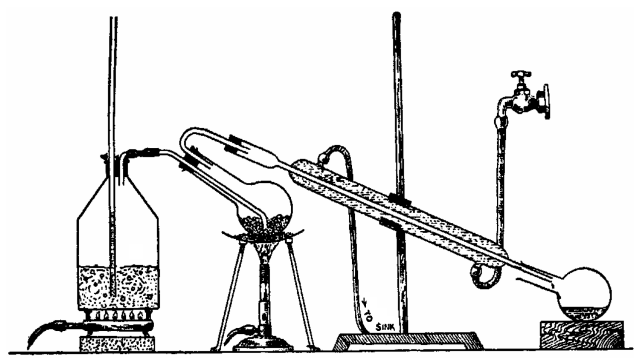

Figure 5.8 *Classical steam distillation experiment. Water is heated in a separate boiler and the steam generated is fed into the material to be steam distilled. Extracted volatile substances are carried in the water vapour and co-condensed with the steam for collection in the receiver flask*
(The classical distillation apparatus reproduced from *Organic Chemistry* by F. Sherwood Taylor, William Heinemann Ltd., London, (First Published 1933), (see Acknowledgements))

properties.[23] In a previous experiment, steam distillation was compared to petroleum spirit and methanol solvent extractions (Appendix 1).[24] A modern alternative approach would be to use SFE.[25]

It is reported that StD cannot extract the pungent principal of galangal rhizomes – galangal acetate – used in beverages,[26] whereas this compound was one of the major volatiles in headspace analysis by GC.

Taints and Off-flavours

Diesel oil contamination of canned fish products was recovered by steam distillation into a condensate with little carry over of fish oils, from which it was extracted by *n*-hexane.[27] Salmon muscle was hydrolysed with NaOH and $CaCl_2$ added before StD. The distillate was extracted with DCM for GC analysis of water-soluble hydrocarbons from seawater-soluble crude oil fractions.[28]

The migration of styrene from thermoset polyester cookware into olive oil was studied using a StD method for its extraction and concentration.[29] An automated steam distillation was applied to the screening of carbaryl as 1-naphthol in fruits and vegetables.[30]

StD was used to extract (*E*) 2-nonenal, a volatile beer ageing marker compound, with a "cardboard" aroma above its threshold of 0.1 μg l^{-1}.[31] A 100 ml beer sample was distilled until 15 ml distillate was collected. This was diluted to 100 ml with water and passed through the SPE column (Chapter 6 and Appendix 2). 2,4,6-tri-tert-butylphenol and related compounds were steam distilled from 101 food samples.[32]

A hydrolysis product of profenofos, 4-bromo-2-chlorophenol, was steam distilled from melons and associated with a taint.[33]

Volatiles – Flavour and Aroma Compounds

Distillation in Cooked Food Flavour Formation/Extraction

When foods are boiled in water at 100 °C (*e.g.* for the cooking process), volatilisable compounds are released or created and evaporated (steam distilled) into the headspace where they are either refluxed (condensed, *e.g.* on the saucepan lid!) back into the boiler or escape (evaporate) into the atmosphere (*e.g.* the steam and smell of cooking in the kitchen).

An early "scientific" investigation of the domestic boiling process used a reflux condenser to simulate the saucepan environment for the production of the aroma of potatoes being boiled. Headspace low-boiling volatiles were extracted and condensed for identification by GC[34,35] (Figure 5.9).

Fumigants

A modified Garman steam distillation apparatus was used in conjunction with solvent partitioning with hexane (for ethylene dibromide) or pentane (for

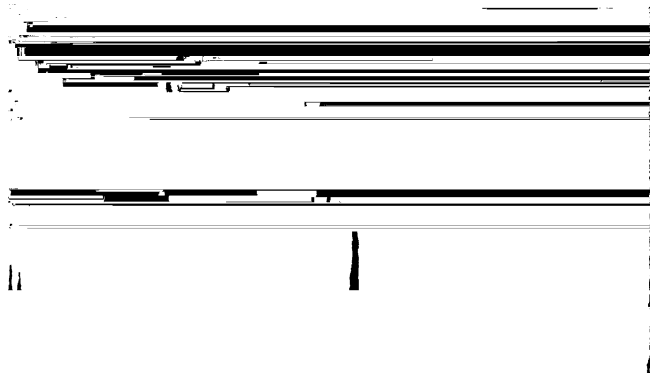

Figure 5.9 *Reflux distillation carried out in B19 Quickfit and Quartz glassware, (A) and (B). Stainless steel hypodermic needle tubing was fitted through the B19 stopper. A similar piece of tubing was bent into a "U" shape (D) and connected with a short piece of silicone tubing (making a butt joint to minimise adsorption on to the silicone tubing) to the stopper and to a motor-driven 1 ml hypodermic syringe (E) as shown. The "U" tube was immersed in liquid oxygen and 1 g potato in 0.8 ml boiled out distilled water was placed in tube A, which was immersed in an oil bath at 110 °C. After 30 min reflux boiling, 1 ml of headspace was extracted into the cryogenic trap. The trap was removed and fitted in the reverse flow direction into the carrier gas line of the GC and the extract vaporised for injection by plunging the trap into boiling water*
(Figure 1 of *Potato Research*, vol 7, p. 228, 1964, by T. Swain and R. Self, reproduced with permission from The European Association for Potato Research)

ethylene dichloride) and carbon tetrachloride in flour, flour-based mixes, baked cakes, breakfast cereals and citrus fruits.[36]

Sulphites

Monier-Williams Distillation (MWD) for Sulphite Estimation

Introduction. The classical MWD method[37] for sulphite determination in foods has been accepted as the standard against which others are judged. In this section, the development, modification, and application of the MWD are presented. Many thousands of analyses have been performed on a range of foods where sulphite was indigenous or incurred and general satisfaction has been expressed for the MWD method. However, as demand continues for increased sensitivity in the area of food intake, and as the need for smaller sample sizes for automation, and shorter analysis times for higher throughput also continues, other methods have been sought.

The faster iodometric method was developed with the needs of industry in mind, and has prevailed for many years. The sample is acidified and the sulphurous acid titrated with iodate solution using starch indicator. However, for red wines, the end point was indistinct and flow injection analysis (FIA) was developed.

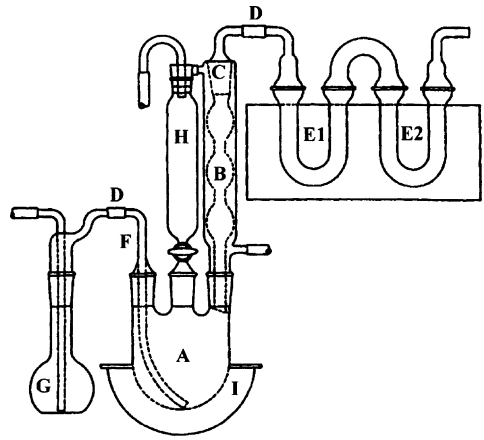

Figure 5.10 *Apparatus for the modified Monier-Williams method for sulphur dioxide. (A) 1 l distillation flask, (B) 30 cm Allihn reflux condenser, (C) right-angled hose connector, (D) silicone tubing, (E1) and (E2) ball-jointed "U" tubes with glass beads and rods, (F) curved glass inlet tube, (G) gas washing bottle, (H) 125 ml separator, (I) heating mantle*
(Modified diagram presented from *AOAC Official Methods of Analysis (2000)*: *Food Additives Direct*, Chapter 47, p. 26, figure 962.16A, with permission)

Experimental. The basis of the MWD method is to reflux a 6% HCl solution, added to the distillation flask (A) (Figure 5.10) from the separator (H), containing a quantity of blended food estimated to yield >45 mg SO_2, diluted to 400 ml in water, such that any SO_2 produced passes into the headspace of the water-cooled Allihn condenser (B), expanding through the double "U" tube traps (E1 and E2) containing hydrogen peroxide. The amount of SO_2 trapped in the H_2O_2 is titrated against NaOH.

Already by the early 1950s modifications to the Monier-Williams method were in use in Government laboratories in the UK and it continues to be used around the world, with further minor modifications, to this day. For a volatile additive like sulphite, the use of a distillation method to extract it from the food matrix was quick and simple.

Development and Application. In 1986, Warner *et al.* made a systematic evaluation of the method for sulphite in foods other than fruit products and wine, and found that for table grapes, hominy, dried mangoes, and lemon juice the recovery was better than 90%,[38] while for broccoli, soda crackers, cheese–peanut butter crackers, mushrooms, and potato chips it was less than 85%. The authors suggest that these data may in fact be correct and that a percentage of the sulphite had reacted irreversibly with food components. The levels ranged from 1400 ppm in dried apple slices to 25 ppm in cream sherry. A recent study to measure the natural abundance of the S isotope ratios in foods treated with

sulphite preservatives measured the isotope ratios of the sulphate obtained from the Monier-Williams distillation for the extraction of SO_2 from 33 retail foods.[39]

In 1989, an interlaboratory collaborative trial among 21 laboratories sponsored by the NFPA and FDA evaluated the FDA-optimised Monier-Williams method for determining sulphites in foods.[40] After familiarisation, three food matrices [hominy, fruit juice, and protein (seafood)] at three sulphite levels and blank as blind duplicates gave recoveries with reproducibilities with CVs of 15.5–26.6% for sulphite as SO_2 by weight at the 10 ppm level. At this stage, the FDA-optimised method was approved interim official first action, replacing the modified Monier-Williams method.

A survey was made in 1992 of sulphite levels in a range of foods (0 ppm in orange juice to 3722 ppm in dried fruit) using the optimised Monier-Williams method.[41]

The optimised Monier-Williams method was used to examine commercial butter flavouring materials for sulphite levels.[42] Levels of 1810 ppm in butter oil, 5760 ppm in butter flavour, and 14.5 ppm in butter (derivative) were found, although no sulphites were added. The presence of volatile fatty acids, compounds found to interfere with the optimised MWD, was thought to be responsible, and the authors recommended that alternatives to the optimised-MWD are used for foods high in volatile fatty acids. The sulphite residues in litchi fruit after SO_2 treatment, used to reduce skin browning and to provide some disease control, were extracted by the Monier-Williams method.[43]

MWD as Reference Standard for Sulphite Measurement

MWD Method and the Iodometric Committee Method. The MWD method was compared to the Iodometric Committee method for the determination of bisulphite in potato products.[44] Problems were experienced with both methods below 60 ppm. The method gave a sensitivity of 25 ppm, but accuracies were 47.4 and 78.2%, respectively, for the Monier-Williams and the Iodometric Committee methods, rendering them semi-quantitative at best.

Rapid Distillation and Iodine Titration Method. The AOAC debate continued with a method describing a rapid distillation followed by iodine titration. DeVries *et al.* (1986)[45] applied the method to various foods and food products, and only garlic and leek matrices presented any significant interference. Excellent correlation with the Monier-Williams method was reported. Compared to the 10 ppm detection limit reported here, a modified Monier-Williams distillation (M-MWD) followed by a polarographic method, measuring the reduction of sulphur dioxide at a dropping mercury electrode, attained screening levels of 1 ppm with cereals and up to thousands of parts per million in dried fruit.[46]

Differential Pulse Polarographic Method. An interlaboratory collaborative trial among eight laboratories compared the differential pulse polarographic (DPP) method with the official MWD method[47] for sulphite in shrimps, orange juice, peas, dried apricots, and dehydrated potatoes (Appendix 3). Later a nitrogen purge was added and the results compared to the optimised Monier-Williams method.[48]

Sulfitest Method. The Sulfitest sulphite qualitative test strip was evaluated against the modified MWD method for 90 food samples. Satisfactory results were obtained for lettuce, raw and cooked potatoes, but false positives were recorded for fin fish, red meats and poultry and false negatives for dried fruit and wine.[49]

Liquid Chromatography. A headspace LC amperometric detection method was compared to the FDA-modified MWD method for various foods.[50] A LOD of about 1 $\mu g\ g^{-1}$ was obtained for a 15 g sample.

An FDA-optimised Monier-Williams distillation (Op-MWD) extraction method was used for comparison with three liquid chromatographic separation methods for the determination of total sulphite, with similar results for lemon juice, white wine and golden raisins and differences among the methods for onion flakes and instant mashed potato.[51] An alkali sample extraction used for two of the methods yielded significantly higher values of sulphite in instant potatoes, and a large interfering peak observed in two of the LC methods prevented quantification of sulphite in onion flakes. As low as 1 $\mu g\ g^{-1}$ detection was obtained for most foods tested (Appendix 1).

Ion Chromatography (IC). IC offered an alternative to the MWD method in various of food matrices.[52] The 10 min chromatographic analysis time was preceded by a 10 min flash distillation, which provided not only a high speed technique, but also one that was sensitive, cost effective, and versatile, since other ions could be measured in the same assay. The IC technique was considered to be superior to the MWD method because the non-oxidising trapping medium was thought to be free from substances responsible for false positives in the Monier-Williams method. However, the distillation extraction was still needed.

The determination of total sulphite continued to attract attention with a paper by Kim[53] in which an alkali extraction/ion exclusion chromatography method was compared with the MWD method for enzymatic and non-enzymatic browning systems (fresh potato and dehydrated sulphited apple, respectively), in vegetables *e.g.* cabbage, containing naturally occurring sulphite, and a carbohydrate-type food additive – erythorbic acid (Appendix 1). Interesting differences reported between the alkali extraction and acid distillation method were thought to be a fraction of sulphite, binding to the browning reaction products, that was released by acid distillation but not by alkali extraction.

Nine laboratories took part in a collaborative study of the IEC-EC method using blind duplicates of starch, diluted lemon juice, wine cooler, dehydrated seafood, and instant mashed potato, with and without 2 levels of sulphite spike.[54] The initial sulphite levels varied from 0 to 384 ppm of SO_2 and the levels added varied from 10 to 400 ppm. Good agreement was obtained between the IEC-EC method and the MWD method for initial sulphite levels, but spiked levels were higher by the IEC-EC method. The method was adopted official first action. AE/ion exclusion chromatography and acid distillation/ion exclusion chromatography methods were applied to sulphite in grapes and compared to the MWD method.[55]

Flow Injection Analysis. An FIA method based on the decolorisation of malachite green by SO_2 gave a LOD in food extracts of 0.1 ppm. The method was tested on various sulphite-treated and untreated food products and the results compared favourably with the MWD, colorimetric (pararosaniline), and enzymatic sulphite oxidase (ESO) methods. The average differences for the MWD, CPR, and ESO results from the FIA results were 19, 11, and 12%, respectively, for those samples ($n = 12$) above 50 ppm SO_2. At lower levels the results were somewhat more erratic due to inaccuracies of the various methods at low concentrations.[56]

A collaborative study of an FIA method was made for the determination of total sulphite in shrimp, potato, dried pineapple, and white wine.[57] The sample solution was reacted with NaOH to liberate aldehyde-bound sulphite. The sample stream was acidified to produce SO_2 gas, which diffused across a Teflon membrane in the gas diffusion cell (Chapter 7) into a flowing stream of malachite green. The degree of discoloration of the malachite green was proportional to the amount of sulphite in the sample solution. Overall average reproducibility was 14%. Recoveries of sulphite added to samples averaged 80%. Comparison of FIA with the MWD method indicated comparable results by the two methods. The FIA method has been adopted official first action for determination of greater than or equal to 5 ppm total sulphite in shrimp, potatoes, dried pineapple, and white wine.

High-performance Liquid Chromatography. A HPLC method was combined with a modified Monier-Williams procedure and its efficiency compared with the conventional MWD method.[58] Residual levels of free and total sulphite in fresh and cooked burgers were determined by an HPLC method previously applied to fresh sausages, and the results compared with the optimised MWD method.[59] The peroxide-trapped SO_2 was separated by HPLC, which provided superior resolution (avoiding interferents).

Capillary Electrophoresis (CE). CE can provide very high efficiencies as a separation method and was combined with the Monier-Williams extraction to obtain a better resolution.[60]

Ion Exclusion Chromatography. The AOAC adopted method (Kim and Kim) using ion exclusion chromatography and direct-current amperometric detection is reported by Wygant *et al.* to have problems with fouling of the electrode.[61] To avoid (reduce) the problem, this paper describes the use of pulsed amperometric detection.

Coulometric Titration. A method for the determination of both free and bound S(IV) in white wine by coulometric titration and electrogenerated iodine was compared with the Monier-Williams method.[62] The method was validated by an average recovery of 97% for S(IV) spiked samples in the range 0.4–1.2 mM. With 150 mg kg^{-1} as the limit for sulphite in edible prawns and shrimps, the MWD method was used to check levels particularly because asthmatics are susceptible to sulphite.[63]

Pre- and Post-purification for MWD. The interference of allicin in the determination of sulphite in garlic was recognised and circumvented by an initial extraction with HCl. A further extraction by C_{18} SPE was required before RP-ion pairing LC.[64] The suitability of the MWD method for determining sulphite in garlic was discussed.

Membrane Diffusion Extraction. Sulphur dioxide determination in wines by gas-diffusion FIA utilising modified electrodes with electrostatically assembled films of tetraruthenated porphyrin was described as applicable to red, white and rosé wines and as being faster than the iodometric method.[65] The apparatus is shown schematically in Section 1, Chapter 7. The method was preferred to the MWD or iodometric methods.

Radiometric Chromatography. For highly sensitive detection of free sulphite in foods, Beck *et al.* (2000) devised a radiometric chromatography method of measuring [^{57}Co]sulphitocobalamin.[66] Foodborne sulphite residues freely extracted into pH 5.2, 0.05 M acetate buffer were resolved using SP-Sephadex C-25 gel chromatography. The sulphite detection range was 6.0 nM–0.3 pM with RSDs of 4.4–29.4%. Foodborne sulphite intolerances provoked by L-cysteine or sulphite additive use in bakery products required greater sensitivity than the conventional MWD, IExC, ECD methods could provide.

Miscellaneous

An interesting use of steam distillation was to extract cholesterol from animal fats that were then incorporated into diets for comparison with the unaltered fat.[67]

Finally, a GC method for the determination of prochloraz and its metabolites in vegetables, fruits, seeds, grains, and roots was simplified by omitting the

steam distillation stage.[68] The method still has several stages, including preliminary extraction, hydrolysis, extraction of the 2,4,6-trichlorophenol, and derivatisation; however, the minimisation of steps in the analysis is beneficial in analysis time and often in improved recoveries.

In a classical analytical experiment, coffee aroma constituents were steam distilled at normal pressure,[69] while in an unusual "steam distillation" by the water contained in potatoes being deep-fat fried was thought to be the cause of the loss of the antioxidant BHT (bp 265 °C) from the lard.[70] Although this is not the standard excess of steam used to lower the bp of the second solvent, to distil over analytes at a reduced temperature, the bp of BHT would have been lowered by the binary mixture formed by the oil and water, causing disproportionate losses of the relatively high-boiling preservative.

Combination of Steam Distillation and SPME

Nitrosamines

Eight volatile nitrosamines were extracted from six cured meat products using a pH adjusted steam distillation plus a concentration step (Groenen *et al.*, 1976, ref. 101). *N*-Nitrosodibutylamine and *N*-nitrosodibenzylamine were extracted from smoked hams by StD and the headspace above the distillate sampled by a polyacrylate coated SPME probe for GC-TEA (Appendix 2).[71] The method is solventless, environmentally friendly and rapid enough for monitoring purposes.

Essential Oils

The first report of combined StD and SPME for the extraction of essential oil components is a steam distillation – a continuous hydrodistillation – with continuous SPME taking place in the ascending vapour headspace in the reflux mode distillation.[72] The StD-SPME assembly is shown in Figure 5.11.

Comparison of Steam Distillation to Solvent Extraction

In an early experiment, sorbic acid was extracted by SE or StD in preparation for an acidified sodium chlorite reaction.[73] More recently, sorbic and benzoic acid preservatives, added to packaged vegetable products, were measured using three extraction methods:

1. Extraction with 60% MeOH and isocratic RP-HPLC
2. StD and isocratic RP-HPLC
3. StD and spectrophotometry (Appendix 1).

For the high concentrations of green olives (>100 ppm), the StD/spectrophotometry method was excellent; however, the HPLC methods were more efficient

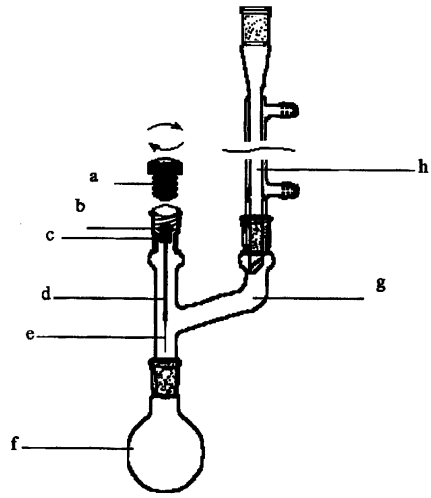

Figure 5.11 *StD-SPME assembly. a. Compression nut or cap, b. O-ring, c. Threaded nut of SPME fibre holder, d. Stainless steel sheath, e. Fibre, f. round-bottomed flask, g. Claisen distillation head, h. Water cooled condenser. The SPME holder fits into the thermometer O-ring. Volatile substances are adsorbed from the headspace onto the 100 μm polydimethylsiloxane surface on the fibre. Condensed vapour returns to the boiler*
(Reprinted from the *Journal of Chromatography A*, vol. 1025, M.R. Tellez, I.K. Khan, B.T. Schaneberg, S.L. Crockett, A.M. Rimando and M. Kobaisy, "Steam Distillation Solid-phase Microextraction for the Detection of *Ephedra sinica* in Herbal Preparations", pp. 51–58, © 2004, with permission from Elsevier)

over the whole range of concentrations (5–500 ppm), and had LODs of 1 ppm.[74] With tomatoes, cucumbers, caperberries, silver-skinned onions, and hot peppers, all three methods were excellent at high concentrations (500 ppm), but at 20 ppm methods 1 and 3 were unacceptable. A steam distillation method and a SPE method were compared for the extraction of preservatives, sorbic acid, benzoic acid, and *p*-hydroxybenzoic acid and its methyl, ethyl, iso-propyl, *n*-propyl, iso-butyl and *n*-butyl esters and saccharin, from foodstuffs in preparation for HPLC separation.[75] Better than 93% recoveries, RSD = 0.85–2.15%, were obtained for a coffee drink using the SPE method.

3 Organic Solvent Distillation–Extraction

Introduction

Two important applications of solvent distillation–extraction for food analysis come under this heading and are the estimation of fats and the determination of moisture content. Along with other analytical disciplines there are many general applications that employ a distillation step.

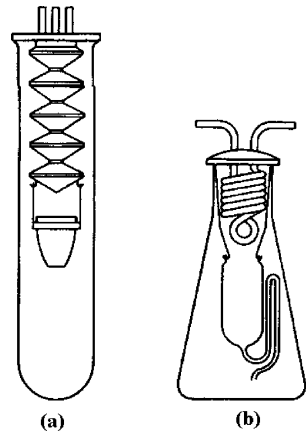

Figure 5.12 *Early examples of continuous extraction devices.* (a) *Wiley and* (b) *Underwriters Laboratories extractors. Samples were placed in a porous container and hung below the condenser (Wiley type), or placed in a porous container in the syphon tank inside the Erlenmeyer flask (Underwriters type). Solvent was introduced to the flask and boiled by a suitable heat source, depending upon the bp*
(Reproduced from *Methods in Food Analysis*, Second Edition, Academic Press, New York, ed. M.A. Joslyn, 1970, with the permission of the publishers, © 1970 Elsevier)

Continuous Extraction

Historically, the Wiley and the Underwriters Laboratories continuous extractors (Figure 5.12) arranged for the sample in a porous container to be bathed in the reflux-condensed vapour before it percolated back into the boiling flask, carrying soluble substances with it.[76] Therefore, these methods suited the extraction of stable solutes only, and other methods were sought to avoid labile analytes spending long periods of time in the boiling solvent in the flask.

An example of continuous extraction was the total reflux method used to extract lipids and surfactants from food products.[77] The extract from a 6.67 ml solvent per g sample after a 15 h reflux with 70 : 30 $CHCl_3$–MeOH, was filtered and evaporated and the lipids dissolved in petroleum ether. The method was recommended for products liable to form emulsions and for some troublesome Soxhlet extraction samples such as feta cheese. The solvent system was optimised.

Intermittent (Soxhlet) Extraction

The intermittent Soxhlet extractor was designed to extract solutes with the solvent at the condenser cooling water temperature rather than at its boiling point. Figure 5.13 shows early examples of the apparatus.

The boiling flask is of the Erlenmeyer type and the reflux condenser is either the Allihn (a), an efficient large surface area design, or a cold finger Hopkins

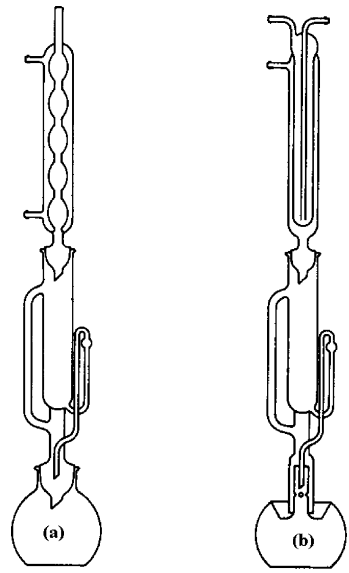

Figure 5.13 *Early examples of the Soxhlet extraction apparatus.* (a) *Glass sealed system with the Allihn condenser, and* (b) *mercury sealed system with the Hopkins condenser*
(Reproduced from, *Methods in Food Analysis*, Second Edition, Academic Press, New York, ed. M.A. Joslyn, 1970. with the permission of the publishers, © 1970 Elsevier)

type (b). The collector tube inserted between the boiler and the condenser houses a filter paper thimble in which is placed the sample to be extracted. The boiling vapour rises *via* the sidearm in the collector and after condensation fills the collector, bathing the sample in solvent, until the level of the solvent activates the syphon and the total solvent returns to the boiler *via* the overflow/syphon tube, taking dissolved material with it. The fact that the sample is bathed in cooled solvent and intermittently siphoned off and replenished improves the efficiency of this method, but the extract is still maintained over several hours, often, at the boiling point of the solvent, and while thermally stable solutes will survive some solute decomposition may occur and so use of the lowest boiling efficient solvent is advised.

Soxflo Technique

The Soxflo instrument was evaluated by Brown and Muellar-Harvey (1999)[78] for the determination of crude fat in foods. Samples packed in small columns are extracted with petroleum ether at room temperature. The method was tested with CRM and compared to the Soxhlet extraction when, in a lipid content range of 0.4 to 73.2%, recoveries were 99.7% and 100.7% respectively. An RSD of 1.81% was obtained compared to the Soxhlet method of 3.68%. Economies of

time (85%), energy (95%), water (100%) and solvents (50%) were significantly environmentally friendly.

Applications

Phenolic Compounds. Soxhlet extraction was chosen to determine a "broad spectrum" of organic compounds in finely ground and homogenised tissues of various fish species. Extracted phenolic compounds were acetylated and the extract (acetates plus neutral semi-volatiles) was further cleaned up with silica gel and SEC.[79]

Acrylamide. The complete extraction of acrylamide formed from carbohydrate foods that have been subjected to high temperature processing is currently occupying food analysts since its toxicity, even at low levels, has caused concern. A defatted sample of potato chips was Soxhlet extracted continuously with MeOH for 10 days. No further increase in the concentration of acrylamide occurred after 7 days.[80] Substantially more was extracted than by a static method in a previously published account.

Chlorophenols. Soxhlet extraction of pentachlorophenol and tetrachlorophenol from potatoes and carrots with acidified acetone (for 44 h) was the most efficient solvent extraction method of those tested. Diazoethane reaction of the extracts was followed by Florisil clean-up of the ethyl ethers of PCP and TCP.[81]

Lipid (Fat) Content. Chapter 1 dealt with total lipid estimation, under the heading of proximate analysis, and standard methods are to be found there. Here, other aspects of total lipid measurement by solvent distillation–extraction, such as method development, are discussed.

A filter paper thimble containing a weighed sample of fatty food is placed under a reflux condenser and a solvent such as petroleum ether used to extract fatty material into the boiling flask. The Soxhlet apparatus is ideal for this extraction. After a period of time, the solvent is evaporated and the fat deposit dried and weighed. The extraction is repeated until a constant weight is recorded.

Various rapid methods of fat content measurement have been devised whereby the food sample is digested and then cold-extracted with a fat solvent. The fat content of 340 samples of ready-to-eat foods was determined from extracts using mixed ethers.[82]

A concern for analysts using stable carbon isotope techniques such as IRMS was expressed in a paper by Schlechtriem *et al.* (2003).[83] A hot Soxhlet method using petroleum-ether as solvent was compared with the Bligh and Dyer and the Smedes methods for lipid extraction from fish and other foods. The Bligh and Dyer method used with chloroform solvent caused errors in the measurement of $\delta^{13}C$ when applied to diatoms.

The sensitivity of neuron-specific enolase in the Western blot for bovine and porcine brain, present in sausages, was increased by reducing the fat content by Soxhlet extraction.[84]

Ethoxylated Mono- and Di-glycerides. A gravimetric method for the quantification of ethoxylated mono- and di-glycerides in bread employed an azeotropic mixture of *n*-propanol–water in a 22 h Soxhlet extraction of air-dried pulverised sample.[85] The extract was evaporated to dryness and transesterified and the FAMEs extracted into petroleum ether. The ethoxylated mono- and di-glycerides were precipitated from aqueous solution with phosphomolybdic acid in the presence of barium ions. Quantification was achieved by taking standard compounds through the procedure.

Pesticide Residues. Organochlorine pesticides in fish samples were used to develop a single step sulphuric acid oxidation for lipids. Recoveries were corrected against tetrabromochlorine as internal standard, and grinding with sodium sulphate was more efficient than freeze drying; 18 h Soxhlet extractions removed most of the analyte.[86] Soxhlet extraction combined with solvent partitioning and gel permeation chromatography was chosen for development of a sensitive method (<1 ng g^{-1}) for the measurement of base/neutral and carbamate residues in dietary samples.[87] Recovery was >70%, RSD <25%, and LOD <1 ng g^{-1}.

Nitrated Polycyclic Aromatic Hydrocarbons. The Soxhlet method was combined with UASE, and UASE, GPC and SPE were also used in a development exercise to determine ultra-trace levels of nitro-PAHs in various matrices, including foodstuffs (complete human diet, mate tea, pumpkin seed oil, parsley, and sausages).[88]

Developments

Ultrasound-assisted Soxhlet Extraction (UASE). The developers of UASE decided that the classical Soxhlet method for the extraction of total fat was functionally better than many modern replacements, but its operational (long analysis time) and environmental (high consumption of organic solvents) performances were unacceptable in modern analyses. Their solution was to design the apparatus shown in Figure 5.14.[89]

Comparison of Methods for Total Lipid Estimation. The Soxhlet distillation method was compared to three LLE methods (Manirakiza *et al.*, 2001, Chapter 1, ref. 25, Table 1.1)

Examples of Soxhlet Extractions from the Literature. A selection of papers using Soxhlet extraction has been collated in Appendix 5.

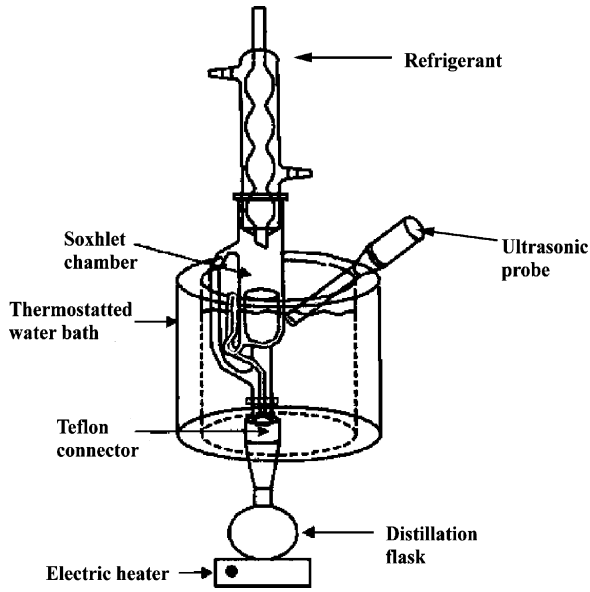

Figure 5.14 *Proposed UAE device. A Soxhlet extractor was placed in a thermostatted water bath with the ultrasonic probe focused on the sample chamber* (Reprinted from the *Journal of Chromatography A*, vol. 1034, J.L. Luque-Garcia and M.D. Luque de Castro, "Ultrasound-assisted Soxhlet Extraction: An Expeditive Approach for Solid Sample Treatment. Application to the Extraction of Total Fat from Oleaginous Seeds", pp. 237–242, © 2004, with permission from Elsevier)

Dean and Stark Extractor

Moisture Determination

The Dean and Stark solvent distillation method for the quantitative extraction of water from food has been in use for many years. It uses a reflux condenser with the vapour supplied from a boiler mounted on a side arm such that the mixed aqueous and immiscible organic vapour, after co-condensation, can fall vertically into a calibrated 10 ml collector tube (Figure 5.15).[90]

Toluene, with a boiling point of 110 °C, is a common choice of solvent, since it readily vaporises water, transferring it to the reflux condenser and then into the 10 ml graduated collector tube, and because water is denser it accumulates below the lighter toluene which returns to the boiler. After a period of time, all the water has distilled over (when two consecutive readings are the same) and the amount is read directly off the scale.

Applications

Dean and Stark moisture estimators are commercially available. They were used to prepare steam distillates containing petroleum hydrocarbons from water

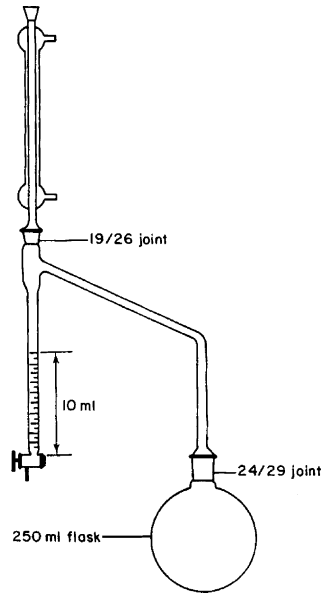

Figure 5.15 *Dean and Stark distillation apparatus. Approximately 10 g (weighed accurately) sample of moist food,* e.g. *fresh fish, is placed in the 250 ml flask and covered with 100 ml toluene*
(Reproduced from *The Analysis of Nutrients in Foods*, by D.R. Osborne and P. Voogt, Academic. Press, 1978, p. 110, with permission from Elsevier) (see Acknowledgements)

and mussels (*Mytilus edulis*) with recoveries of better than 80%.[91] Similarly, samples of milk drinks stored in low-density polyethylene bottles were steam distilled to extract naphthalene for analysis.[92] Migration studies were carried out using peanut oil as a food stimulant, and temperature and storage trials allowed a model to be established from which the migration of naphthalene into milk could be calculated.

Comparison with other Methods

Solar dried thyme that had reached equilibrium moisture content was analysed by the Dean & Stark, oven and microwave methods to measure the initial content on a % wet weight basis, and the final moisture content (% dry wt. basis).[93] The Dean & Stark, oven and microwave oven methods gave values of 75.15 (10.00), 75.12 (11.85) and 72.31 (12.50) respectively. Extraction of moisture from the herb was deemed consistent by the Dean & Stark distillation and oven methods, but a significant difference was recorded for the microwave method.

Mineral Oil Distillation

Several references, emanating chiefly from two research groups, record the use of mineral oil distillation of nitrosamines from food samples between 1978 and

1984, establishing it as an AOAC method. For example, the technique was used along with direct distillation to confirm, by GC-TEA and MS, the presence of NPYR in six out of nine samples of fried bacon, and NDMA in 62 out of 64 beer samples.[94] A survey of 106 market samples of poultry products, Chinese foods, and herring meals for volatile nitrosamines was reported.

Development of Alternative Methods

The dry column (DC) method developed at Eastern Regional Research Centre (USA) was compared to the established MOD method.[95] A GC-MS method to replace the TEA analysis was reported in 1982.[96] A collaborative trial with the DC method was made in 1984 and the MOD, DC and LTVD methods were later (1988) compared (Appendix 1). By 1996, a SFE method was proposed (Fiddler and Pensabene, 1996, Chapter 4, ref. 161 and Appendix 1)

4 Simultaneous Steam Distillation–Extraction

Introduction

The extraction method using simultaneous steam and organic solvent distillation from separate boiling flasks leading to a common condensing surface (Figure 5.16) was introduced by S.T. Likens and G.B. Nickerson in 1964.[97] This section is called "Simultaneous Steam Distillation–Extraction (SDE)" because

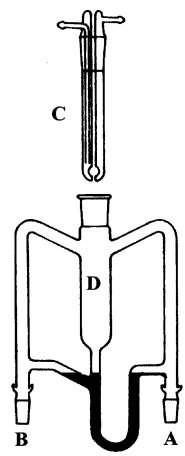

Figure 5.16 *Original Likens–Nickerson simultaneous steam distillation–extraction apparatus. The food matrix in water was placed in the distillation flask attached at point A and the organic solvent (less dense than water) was placed in a distillation flask attached at B. The cold finger condenser C was inserted in the tube D such that the condensate returned to the bottom of D, the water overflowing back to flask A and the solvent collecting on top of the water and eventually overflowing back into flask B*
(Diagram modified from reference 97 with permission from the American Society of Brewing Chemists.)

this is the name chosen by most authors. The acronym SDE is certainly in common use. However, there are many different titles used in the literature that may cause confusion. The following titles are assumed to be synonymous with the preferred title:

Concurrent steam distillation/solvent extraction
Concurrent steam distillation–solvent extraction
Simultaneous water steam distillation–organic solvent extraction
Simultaneous distillation extraction
Simultaneous distillation–extraction
Simultaneous steam distillation and extraction
Simultaneous steam distillation/solvent extraction
Simultaneous steam-distillation/solvent extraction
Simultaneous steam distillation–solvent extraction
Simultaneous steam distillation/extraction
Simultaneous steam distillation–extraction
Simultaneous steam distillation extraction
Steam distillation-solvent extraction
Steam distillation–extraction
Steam distillation extraction
Steam distillation/extraction
Steam distillation and extraction

Hopefully, SDE will be reserved for the almost universal acronym describing the Likens and Nickerson method and not used for the distillation–extraction with a single solvent.

Likens and Nickerson SDE Method

Introduction

An obvious application of the removal of volatilisable components from food is in the area of aroma and flavour analysis. Bemelmans[98] summarised the use of suitable extraction methods by saying,

> In choosing from the various methods of isolating volatiles from a food product, several parameters should be given careful consideration. Any losses of important compounds caused by inappropriate selectivity or low efficiency of the method cannot be made up for in a later phase of the investigation.

He considered factors such as concentration, boiling point range and polarity, stability (decomposition and artefact formation) of components, and ease of oxidation.

Apparatus

The Likens–Nickerson apparatus (Figure 5.16) allows the food slurries in water to be continuously extracted by steam distillation and the water vapour and

volatile compounds to be co-condensed (partitioned) with the chosen, less dense, immiscible solvent, each solvent returning to its own boiler.

The advantage of this method of extraction was given as the low ratio of the volume of solvent to the quantity of food. Likens and Nickerson used the equipment to study the hop oil components of beer.[99] Early applications were for the extraction of volatiles from potato chips, vegetables, and poultry products.

Modifications to the Original Apparatus

It is convenient to take the 1979 review by Bemelmans, 98 and the references therein, as the starting point for a discussion of subsequent modifications, application and development of the modern Likens and Nickerson steam distillation–extraction method. Maarse and Kepner[100] introduced a vacuum jacket around the return arm of the steam distillation side to minimise the premature condensation of steam and added a dry ice condenser to reduce loss of volatiles from the top of the partitioning arm (Figure 5.17).

A modification to facilitate the use of a heavier-than-water solvent, DCM, to effect the extraction of nitrosamines from meat products was developed by Groenen *et al.*[101] They also recommended the use of Vigreux indentations to increase the efficiency of the solvent transfer process. (Figure 5.18)

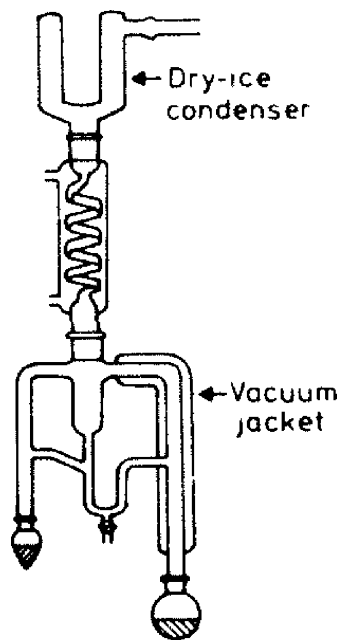

Figure 5.17 *SDE modification after Maarse and Kepner[100] who added the dry ice condenser and vacuum jacket*
(Reprint from reference 100, © (1970), with permission from the American Chemical Society)

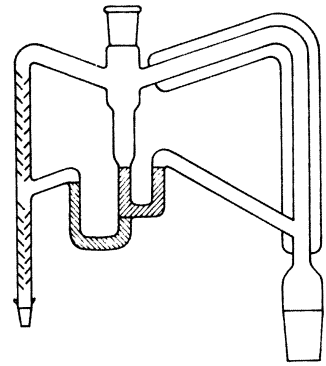

Figure 5.18 *SDE modification by Groenen* et al. *(1976)*[101]
(Diagram reprinted from page 323 of ref. 101 with permission from The International Agency for Research on Cancer, Lyon, France)

MacLeod and Cave[102] minimised the formation of emulsions during the extraction of volatiles from the difficult matrix from eggs. More efficient cooling surfaces were introduced by Flath and Forrey[103] and a smaller scale version was developed by Godefroot *et al.*[104] Römer and Renner[105] preferred an external steam generator (Figure 5.19).

A more recent development, also using the external steam generator, has improved the mixing of the steam and organic solvents such as light petroleum, and increased the condensation surface to permit the use of cooling water at room temperature.[106]

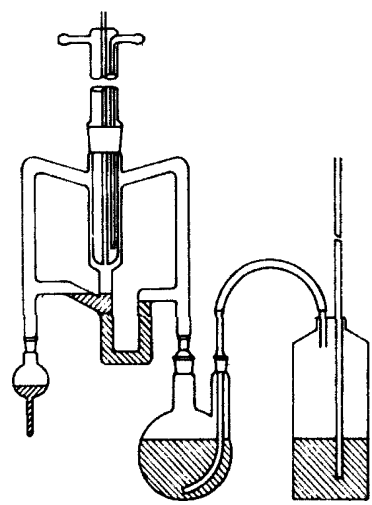

Figure 5.19 *Modified L-N apparatus after Römer and Renner (1974)*[105] *with an external steam generator*
(Diagram modified from Figure 1 reference 105, with permission from Springer-Verlag GmbH)

The developers also report an increase in sensitivity from the use of larger sample sizes. The technique was applied to the extraction of organochlorine pesticides in milk powder and other products. The authors provide full details of the apparatus and the protocol of the experiment. The SDE extractor is shown in Figure 5.20.

The main criticism of the SDE technique has been the formation of artefacts and the team from Nestle[107] introduced a modification operating at room temperature and reduced pressure, extracting volatiles from a Maillard model reaction, honey and linalyl acetate to test their modification against the standard method, and reported similar yields.

Optimisation of SDE using the sequential simplex method led Blanch *et al.* (1993) to construct a micro steam distillation–extraction device.[108] The apparatus included an enlarged surface condenser that reduced loss of high volatility compounds. Extraction solvents with higher or lower densities than the sample distillation solvent can be used. Further development of the apparatus, at reduced pressure and with inert gas purging, was reported later in the same year.[109]

The latest version of the L-N device is shown with dimensions on the DEFRA web site, archive.food gov.uk. (Figure 5.21)

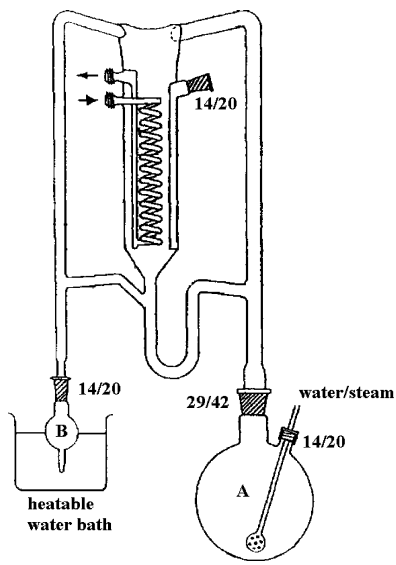

Figure 5.20 *Scale drawing. (A) Water steam extraction chamber containing milk sample. Total volume of water 700 ml, (B) Organic solvent (light petroleum bp 40–60 °C) boiler in water bath at 70 °C*
(Modified with permission from the *Journal of Chromatography A*, vol. 712, G. Filek, M. Bergamini and W. Lindner, "Steam Distillation-Solvent Extraction, A Selective Sample Enrichment Technique for the Gas Chromatographic-Electron-Capture Detection of Organochlorine Compounds in Milk Powder and Other Milk Products", pp. 355–364, © 1995, with permission from Elsevier)

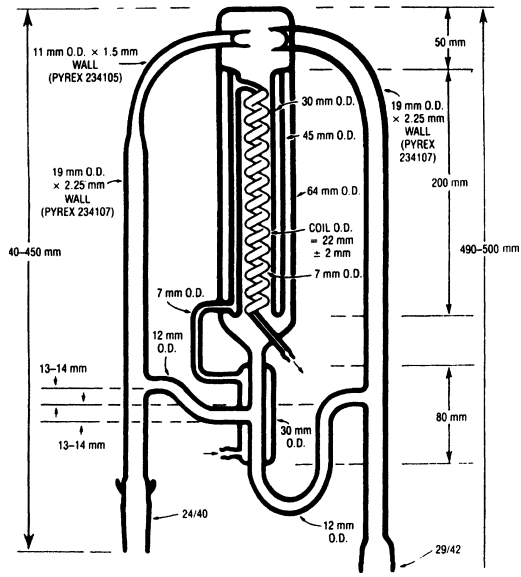

Figure 5.21 *DEFRA SDE published design with dimensions* (Reproduced from the web site)

Modified Operating Conditions

The use of reduced pressure SDE has been reported for the extraction of 16 volatiles from winter wheat oil.[110] Vacuum SDE was used in the examination of the volatiles from watermelons at 60–70 °C in a water recycling system.[111]

Aromas and Flavours

Introduction. Even though the extracted volatiles are continually distilled and returned to the boiler during the distillation, increasing the likelihood of degradation, the method is as popular today.

Fruit Extracts. A series of articles from Pino and co-workers on the extraction of volatiles from acerola,[112] strawberry guava[113] and Costa Rican guava[114] fruit using the Likens–Nickerson method enabled the isolation and identification by GC-MS and odour recognition of numerous terpenes, aliphatic esters and other aroma compounds. The volatiles from the avocado fruit were measured using a modified L-N apparatus[115] using ether and pentane solvents.[116]

Potato Flavour. To continue the work on potato flavour, the team at Reading used their modified version of the Likens–Nickerson apparatus.[117,118]

Seafoods. Comparison of the effect of two preparation methods, boiling and steaming, on the volatile components of two species of dried scallops, *Chlamys*

farreri and *Patinopecten yessoensis*, used SDE.[119] Frozen or dried scallops (45 g) were extracted with 50 ml DCM in a Likens–Nickerson (1964) type SDE apparatus (Kontes, Vineland, NJ). Extracts were concentrated in a stream of pure N and dried over anhydrous sodium sulphate.[120]

Cooking Oils. Further quantitative studies were performed by Negroni *et al.* in work on various cooking oils.[121]

Beer Volatiles. A Likens–Nickerson microextractor was used to produce the strong worty aroma associated with beer flavour and the production of 3-methylpropionaldehyde was studied as the most organoleptically active compound.[122]

Fermentation Changes. SDE and HS methods were used to monitor changes in volatile pattern during fermentation.[123] Aldehydes were reduced to alcohols, with the accompanying changes in the aroma as oily/fatty notes originating from alcohols increased. Also, hydrocarbons were removed by bacterial digestion. Other chemical changes such as hydrolysis, hydration, and oxidation, and sorption effects were discussed. The need to use extraction methods that do not change the natural chemistry is important.

Honey Flavour. 400 compounds were extracted from honey using the Likens–Nickerson extractor, among which several marker compounds for specific species were identified.[124]

Vegetable Volatiles. Very recent work on the leaves and inflorescence of cauliflower[125] identified 61 compounds listed as volatiles and semivolatiles; among the latter being the alkyl isothiocyanates, the therapeutic glucosinolate degradation compounds (Case study 2, Chapter 6 p. 244). Eighty nine volatile compounds were extracted from red fermented soybean curds by SDE.[126]

Miscellaneous. The Likens–Nickerson apparatus was used to extract estragole, saffrole, and eugenol methyl ether from food products.[127] The LODs were 10, 5, and 8 ng ml^{-1} respectively for standard solutions and foods. Sixty four volatile compounds were extracted from fermented maize dough by SDE.[128]

Off Flavours and Taints

Alkylphenols and aromatic thiols were found to be principal contributors to the tainting of rainbow trout,[129] walleye and northern pike,[130] during the spring, and geosmin and methylisoborneol caused mustiness during the late summer. SDE preceded GC-MS identification.

Pesticides

A modified SDE method was used in the preparation of water, soil, and food samples spiked with 22 pesticides and PCBs. Good or very good recoveries were obtained with 5 h distillations for food samples. The method was recommended for rapid screening and low solvent use (<10 ml).[131]

Volatile Contaminants

Gramshaw *et al.*[132] measured the migration of volatiles from thermoset polyester into belly pork by extracting the meat with the Likens–Nickerson apparatus followed by quantitative GC-MS. Subsequently, they added an internal standard, cyclohexanone, to improve the quantification of the migration of 2-cyclopentylcyclopentanone into roast chicken parts.[133]

Comparison with Other Techniques and Models

SDE Compared to Dynamic Headspace Analysis. Dynamic headspace collection was compared to the L-N simultaneous steam distillation–extraction of volatiles from cured hams.[134] In terms of reliable semi-quantitative data, the L-N method was preferred.

SDE and SFE Compared. SDE was compared to SFE for the extraction of essential oils from oregano, basil, and mint.[135]

Process Time Modelled. A model was made to describe the recovery of classes of compounds versus SDE process time. Theoretical predictions of variations in some factors are in good agreement with practical data.[136] For most substances tested, 100% recovery was obtainable in 20 min.

SDE Compared to SE and High-vacuum Distillation. SDE was compared with solvent extraction and high-vacuum distillation for aroma extract dilution analysis (AEDA) on a freshly prepared, enzyme-inactivated peach juice.[137] The SDE method yielded an overall more intense aroma extract.

SDE, SFE, VSS and Press Oil Extraction Compared. The extraction of coffee aroma was used to compare the four extraction methods (Table 5.1).[138]

Soxhlet Method as Reference for SDE Development

It is common practise to compare innovation in the area of distillation–extraction with the Soxhlet method as standard. In the development of a SDE method for chlorinated phenols, benzenes, and insecticides from water, soil and vegetable samples, recoveries of over 70% were reported.[139]

Table 5.1 *Protocol for coffee aroma extraction by four methods. In method 4, the MCT oil used was, Delios® $C_{8:0}$ (60%) and $C_{10:0}$ (40%). In method 3, 1 ml CH_2Cl_2 was added to the Carbosieve trap*
(Reprinted from *Food Chemistry*, vol. 70, C. Sarrazin, J-L Le Quéré, C. Gretsch and R. Liardon, "Representativeness of Coffee Aroma Extracts: A Comparison of Different Extraction Methods", *Food Chem.*, 2000, **70**, 99)

Parameter	*Method 1* (VSS) Water	*Method 1* (VSS) CH_2Cl_2	*Method 2* (SDE)	*Method 3* (SFE)	*Method 4* (POAE)
Wt. coffee (g)	50	50	5	6	100
Solvent 1 (ml)	100	100	50 water	5 CH_2Cl_2	100 CH_2Cl_2
Solvent 2 (ml)	–	–	2 CH_2Cl_2		20 g MCT oil
Solvent 3 (ml)	–	–	–	1 CH_2Cl_2	100 CH_2Cl_2
Dry Na_2SO_4	Yes	Yes	Yes	Yes	Yes
Conc to 1 ml	Yes	Yes	Yes	Yes	Yes
plus	35g NaCl	–	–	Vacuum stripping	–

Summary

The widely different types of flavour-producing foods that have been extracted using SDE reflects the confidence in the method and the obvious similarity between the odour of the extract and the original food source.

Vacuum Carbon Dioxide Distillation

Volatile flavour compounds were extracted from fats and oils by vacuum carbon dioxide distillation.[140]

5 Sweep Co-distillation

Introduction

Storherr and Watts introduced sweep co-distillation in 1965.[141] The principle is to use an inert gas, nitrogen, at high temperature to sweep out distillable (volatilisable) material from a sample mixed with a solid packing material in a glass tube for downstream condensation in a complex condenser. It was ahead of its time in providing a method that used only small quantities of organic solvent. Compare this method to MSPD, Chapter 4, page 124.

Development

The analysis of pesticides in meat and dairy products was among the early applications of the technique, described by the inventors,[142,143] providing the analytical foundations for the development of newer methods such as GPC and SPE.

The Storherr tube method was modified and coupled to a version of the Varian Aerograph GC.[144] After an investigation of the SCoD method, further adaptations were made to the technique to simplify it for the analysis of six organochlorine pesticides in animal fats.[145] The use of no solvent, at a distillation temperature of 230 °C, with a nitrogen flow rate of 600 ml min^{-1} in a 6.7 mm id distillation tube containing a simplified packing, and incorporating a U-tube condenser was found to be optimum. The new method gave similar recoveries to the Stoherr method, but was faster than most bisolvent partition methods and used no solvent. A silanisation of the Storherr tube packing reduced thermal decomposition and permitted recoveries in excess of 88% in the analysis of 15 pesticide residues, 4 PCB formulations, and pentachlorophenol from pork, beef, chicken, sheep, and rabbit fat.[146] An LOD of 2–5 ppb for pesticides and 10 ppb for the PCBs was readily obtained.

Applications

The technique was applied to the analysis of 26 environmental chemicals in oils (corn, rapeseed, peanut, and paraffin) and fats (beef and pork) and vegetable shortening.[147] Recoveries for most compounds were >80% with an RSD ≤10%. The method was compared to two other extraction techniques, Florisil-silicic acid column chromatography and low temperature precipitation.

The contamination of butter and margarine with phthalate esters from the aluminium foil-paper laminate wrappers was investigated using SCoD to extract the separated oil.[148]

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CHAPTER 6

Adsorption

1 Introduction and History

Adsorption processes have been a major part of food analysis since its inception. It is difficult to draw a distinction between extraction techniques and the chromatographic separation methods, both employing adsorption processes. Gas/solid and liquid/solid adsorption chromatography has been used to extract odours and colours from food samples. The methods were manual and often without on-line detectors, and therefore were basically extraction methods in columns. For example, the earliest separations of plant pigments were conducted on columns of powdered calcium carbonate (Tswett), and the separated coloured bands, fortuitously detected by the eye, earned for the technique the name of chromato-graphy. Diversification to other media such as paper (Martin and Synge, 1941)[1] and immobilised liquids – gas/liquid partition chromatography (James and Martin, 1952)[2] – has ensured that chromatography is now the most important separation principle in food analysis.

The nomenclature of solid-phase extraction (SPE) for liquid/solid partition extraction arrived after the event to neatly describe the group of applications using the percolation of solvents through columns of particulate inert material with adsorptive properties capable of reversibly retaining analytes according to their "affinity" for the surface structure. The specialisation of immunoaffinity extraction (IAE), utilising the "lock and key" selectivity of the antibody–antigen interaction, provides the high specificity sought in analytical biochemistry and food contaminant analysis. The fact that the technique is similar in practice to chemical adsorption SPE methodology promises to provide the unique "extraction" of the target analyte required for automated on-line assays.

The ease of operation of solid-phase microextraction (SPME), the combined extraction and chromatographic injection device has made the extraction and separation of volatiles a simple on-line procedure. Stir-bar sorptive extraction (S-BSE) and solid-phase dynamic extraction (SPDE) methodology recently addressed the need for a higher concentration capacity, a limitation with the microfibre used in SPME.

Perhaps the main reason for the success of SPE in its time was the reduction in the use of organic solvents compared to the methods discussed so far.

Food Colours

The analysis of natural food colours utilised columns of activated alumina powder for the separation of, *e.g.*, solvent-extracted carotenoids. The components were eluted in various solvent mixtures and collected as the separated bands, often freeing them from impurities left behind on the top of the column after development. Alternatively, after development of the carotenoids as discrete bands down the length of the column, the solvent supply was stopped, the column allowed to dry, and the bands "cut out" with a spatula and stored in separate containers. Each band was then re-dissolved in solvent and decanted from the solid phase for spectroscopic identification. Many extractions of food colorants were made using solid-phase adsorption techniques before the commercial, convenient, throwaway SPE cartridge became so popular. The Third Morton Lecture,[3] delivered in 1983 by T.W. Goodwin and entitled "Developments in carotenoid biochemistry over 40 years", reviewed the pioneering work at Liverpool on food carotenoids.

The recent application of capillary electrophoresis (Chapter 2) to the separation of natural and synthetic food colours requires only a single step SPE or, for liquid foods, a dilution and filtration as pre-treatment.

Food Flavours

Cronin and Caplan provided a valuable review of sample preparation for flavour analysis in 1984[4] (see also Snow and Slack 2002, Chapter 8, ref. 44)

Static Headspace (S-HS)

S-HS collection was practised on the gaseous phase in equilibrium with the food matrix – the headspace. It was argued that by extracting a sample carefully so as not to disturb the equilibrium, a representative (and reproducible) sample could be taken for flavour analysis. In practice perturbation was inevitable and the sample was too dilute for detection of more than the most intense components, and so dynamic headspace extraction methods were developed. Dynamic cryogenic methods of condensation extraction are covered in Chapter 5.

Dynamic Headspace (D-HS) Adsorption

Introduction. Early D-HS adsorption methods used a stream of dry nitrogen to transfer HS volatiles from above the food to a column of activated charcoal adsorbent for entrainment. When GC methods provided sensitive, on-line separation and detection able to recognise hundreds of chemical classes encountered in the concentrates eluting from these charcoal adsorption traps, it was convenient to use the GC carrier gas, normally helium or argon, also as the entrainment gas.

As the method developed, various adsorbents were introduced and commercialised. The main problem with D-HS was the entrainment of water vapour that quickly blocked the trap. Hygroscopic pre-traps filled with Na_2CO_3, $MgSO_4$, $CaCl_2$, P_2O_5, or Na_2SO_4 were used. In a series of papers by Canac-Arteaga and colleagues the topic has been modernised using Na_2SO_4, $MgSO_4$, $CaCl_2$, NaCl, and K_2CO_3 applied to dried cheese samples.[5]

Multi-bed adsorbents have been used to increase the range of volatiles trapped in a single operation.[6] Over the years, many configurations have been tried, including capillary traps coated with PDMS,[7] providing an ideal way of coupling the collection stage to high sensitivity GC-MS. Their disadvantage of low capacity was addressed in the development of multi-channel systems.[8] Open-tubular systems are compatible with CCGC columns, but for trace analysis a higher concentration factor is required and, therefore, PDMS packed adsorption traps were used in the splitless desorption mode.[9] The developers compared four adsorbents: Carbotrap 300, Tenax TA, Chromosorb 101, Lichrolut EN with the PDMS sorbent.

Artefact-free cold trapping was one of the first on-line trapping methods for volatile concentration and injection onto GC, and continues to find applications.[10]

Purge and Trap. An inert gas is passed through the sample chamber (which may be heated) and the volatiles leaving the chamber are trapped by adsorption on a solid surface, condensed on a cold surface, dissolved in a liquid solvent, in an open- or closed-loop (preferred) arrangement. The introduction of alternative adsorbents such as Tenax heralded the technology of "purge and trap" for the extraction of headspace flavour and aroma volatiles for GC and GC-MS analysis. The popularity of purge and trap techniques made a study of the mechanisms of adsorption (and desorption) important in order to avoid artefact formation through chemical decomposition. With so many chemical classes present in the extract it was, and still is, difficult to be certain that the concentrate was a true representation of the dilute, equilibrated headspace aroma. The commonly used alternatives to Tenax were Poropak and Chromosorb and these were the precursors of the SPE phases currently in use.

The most widely used purge and trap methods in flavour research were those employing adsorbents with a low affinity for water and short-chain alcohols. The adsorption process then fractionated the major components of the HS vapour, allowing longer collection times without blocking the entrainment trap. Problems of breakthrough, artefact formation and blocking provided the impetus for new materials to be developed. In the 1980s Tenax was favoured for its low tendency to form artefacts, but it suffered from a low sample capacity.

An on-line steam distillation/purge and trap GC procedure for halogenated compounds in foods gave recoveries > 80% (versus aqueous standards) from vegetable oil, flour, root beer, cream (10% butter fat) and milk spiked at 1–3 $\mu g\ kg^{-1}$ for each of 32 compounds.[11]

Desorption. Solvent extraction and evaporation (concentration) or thermal desorption in a stream of inert gas and cryotrapping (concentration) are alternatives for the preparation of the adsorbed volatiles for GC analysis. Volatiles were extracted with diethyl ether from Tenax with excellent recoveries for MS analysis.[12] In general, care was needed to avoid chemical decomposition during the desorption processes. Cryogenic trapping or cryofocusing in capillary tubes immersed in liquid N provided an efficient low volume transfer, provided the trap could be heated rapidly to release its contents over a short period of time. Filling the trap in one direction and desorbing in the other aided the cryofocusing into a small volume for further processing.

Small (1–5 mg) loadings of activated carbon provided proportionately large adsorption volumes and thus enabled high concentration capacity extracts to be made ready for direct injection into the GC inlet using rapid thermal desorption.[13,14] A microwave instantaneous desorption device was reported for the removal of tropical fruit volatiles from charcoal.[15]

Poropak Q (Waters, 80–100 mesh), Tenax GC (Enka N.V., 60/80 mesh), and Chromosorb 105 (Johns Manville, 80/100 mesh) were used to adsorb volatiles from drink juices of fruits and vegetables purged from 1 ml aliquots by helium.[16] Figure 6.1 shows an on-line transfer configuration.

Alternatively, high-pressure CO_2 Soxhlet-type extraction was used to provide solvent-free extracts.[17]

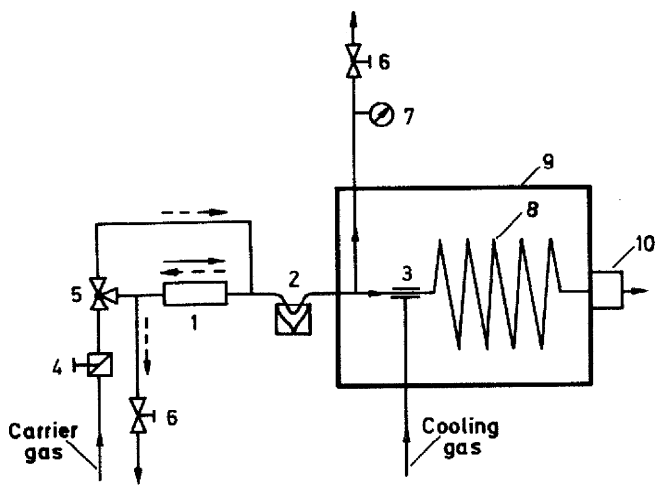

Figure 6.1 *The helium purge flow in the reverse direction dried the adsorbent in (1) prior to desorption and condensation in (2) before cryofocusing in (3) for subsequent heating for injection into the separation column (8) in the GC oven (9) and detection of the constituents (10)*
(Reprinted from S. Adam in *Chromatography and Mass Spectrometry in Nutrition Science and Food Safety*, eds. A Frigerio and H. Milon, Elsevier, Amsterdam, © 1984, with permission from Dr. S. Adam)

Restricted Access Media

Of particular note is the work on the development of restricted access media (RAM), created to tackle the problem of unwanted adsorption of say large biomolecules onto the surface of porous solid materials designed to adsorb smaller analytes such as drugs. Most of the larger molecules can be desorbed, but in practice, over a period of use, a degree of irreversible adsorption renders the column less efficient and reproducibility suffers. RAM are prepared[18] to have a surface non-adsorbent to macromolecules and an internal structure to adsorb smaller analytes. Applications of RAM are given in the section on SPE.

Cyclodextrin Extractions

Cyclodextrins have structural properties that enable them to discriminate between different isomeric forms of a chemical compound. They have been used in separation science for many years, and out of that experience their value in selective extractions has been recognised. A major review of the role of cyclodextrins in separation science was published in 2000,[19] discussing their use in TLC, GC, SFC, CCC, LC, ITP, recycling ITP, GE, IEF, preparative scale CFFE, CE, MECC, LLE, and LSE dialysis, membrane extraction, and MIPs. As such, they can be combined with other extraction methods to provide high selectivity to target analytes in food matrices.

p-*Hydroxybenzoates in Soy Sauce*

γ-cyclodextrin was used for the preconcentration of *p*-hydroxybenzoates in soy sauce. The γ-cyclodextrin formed an insoluble complex with solute molecules that were back-extracted from the precipitate into a non-aqueous solvent. Various solvents were tested to find the most effective at releasing the solute from the complex. Diethyl ether, dioxane, and EtOH gave high recoveries, and MeOH, $CHCl_3$, and DMSO gave lower values.[20]

Sol-gel Technology

Involves the formation of a solid phase (gel) from a colloidal liquid (sol). Textures available include, spherical powders, thin film coatings, ceramic fibres, microporous inorganic membranes, *etc.* IAC is perhaps the most selective extraction method in food analysis, and it employs interactions between immobilised antigens and antibodies. Sol-gel methods are used to entrap antibodies in the pores of hydrophilic glass matrices. Sol-gel columns have antibacterial stability, making them ideal columns for food extractions.

Popularity Surveys of Adsorption Methods

Two recent surveys in 2001[21] showed the popularity of 39 different chemistries among the responding analysts (Chapter 8, Figure 8.1). Nineteen categories of

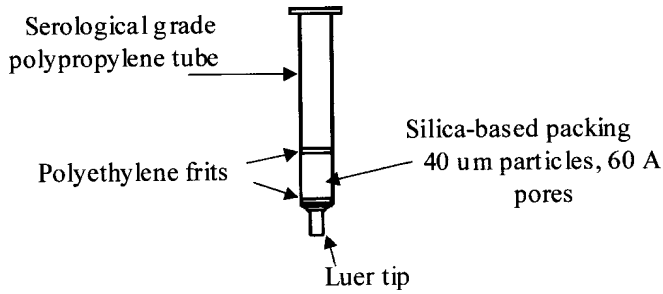

Figure 6.2 *Supelco Supelclean Extraction Tube*
(Redrawn with permission of Supelco, Bellefonte, PA from the Supelco Chromatography products catalogue)

adsorbents were recognised: other, C2 (ethyl), C4 (butyl), C1 (methyl), carbon, cyclohexyl, diol, polymer, affinity, Florisil, alumina, phenyl, cyano, cation exchange, amino, anion exchange, silica, C8 (octyl), and C18 (octadecyl) in ascending order of popularity. C18 was by far the most popular, and C8 and silica were approximately second equal.

2 Solid-phase Extraction

Introduction

Adsorption of analytes from a percolating liquid phase onto an immobile solid phase has two functions. Non-adsorbed components of the sample are extracted by the moving phase and the "cleaned up" adsorbed fraction can be concentrated by using less solvent to elute it than was used to adsorb it.

Cartridge and Adsorbents

For aqueous samples, liquid–liquid extraction is limited to the use of water-immiscible solvents and to those solvents that will not cause emulsions when shaken with water. Furthermore, the financial and environmental cost of solvent extraction has encouraged the fractionation of aqueous solutes by selective adsorption on a solid phase.

Commercially available SPE cartridges, *e.g.* Figure 6.2, are convenient and cheap and only require small quantities of solvents to extract a growing range of solutes from food matrices. They can be constructed from the body of a plastic hypodermic syringe, fitted with a frit at the bottom, and the solid absorbent packed into about a third of the volume and plugged at the top to keep the adsorbent compacted. There is space above the packing for the sample in solvent to be loaded.

The ready-made cartridge can be used in one of two ways: (a) to retain the compounds of interest and let the impurities pass through or (b) to retain the impurities while the compounds of interest are eluted for collection (Figure 6.3a–c).

(a) Retain the compounds of interest

1. Condition the tube by allowing an activating solvent, depending on the filling and the sample type, to pass through, wetting the powder and clearing air from the pores (Figure 6.3a).
2. Add the sample dissolved in a solvent to the conditioned tube. Allow solvent to flow through the tube in a drop-wise fashion, using vacuum, gravity, or positive pressure (Figure 6.3b).
3. Wash the packing to remove the impurities with the same solvent or one that will retain the compounds of interest (A), and then elute the compounds of interest with a small volume of the chosen solvent (B). Two small aliquots are better than one large one (Figure 6.3c).

(b) Retain the impurities.

If the impurities are to be retained on the tube, employ steps 1 and 2 above and then change to a solvent to dissolve the compounds of interest to liberate them from the packing material (Figure 6.3d).

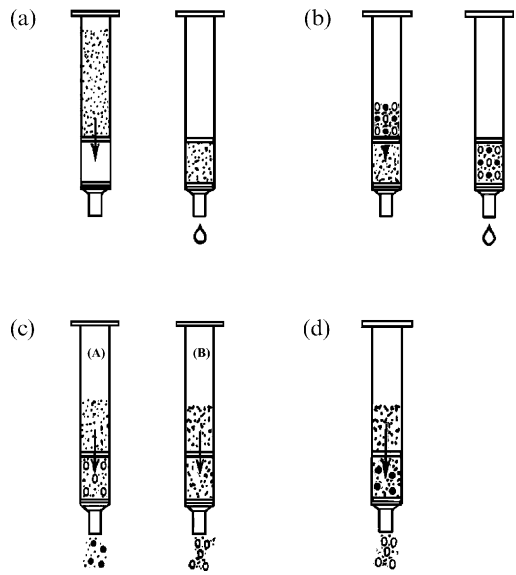

Figure 6.3 (a) *Condition the cartridge by washing the packing with an activating solvent before loading the sample*. (b) *Load the sample in a suitable solvent*. (c) *(A) Wash off the impurities and (B) elute the compounds of interest with suitable solvents. (Legend: • = contaminants, ○ = compounds of interest)*. (d) *An alternative strategy is to carry out steps 1 and 2 above, and then choose a solvent that will remove only the compounds of interest from the packing* (Redrawn with permission of Supelco, Bellefonte, PA from Supelco Chromatography products catalogue)

Adsorption Processes

Adsorption Mode

Bonded Phase Partition Mode.

1. Normal phase – a polar stationary phase will retain polar analytes from a sample matrix, allowing nonpolar analytes to pass through the column.
2. Reversed phase – a nonpolar stationary phase will retain nonpolar analytes from a sample matrix, allowing polar analytes to pass through.
3. Ion-pairing – a counter-ion is added to the sample to neutralise the analyte ions and then a reversed phase extraction can be used.
4. Ion-exchange. – using anion or cation adsorbents of a suitable strength, the ionic analyte of the opposite charge can be adsorbed in normal or reversed phase mode.
5. Immunoaffinity – the antibody–antigen interaction is used to bind a target analyte to the adsorbent while other components pass through. The sorbed analyte is then released in the pure state. This type of adsorption is very specific.

Applications

In this section on the applications of SPE it will be assumed that the necessary conditioning, washing, and reconditioning of the adsorbents will be required, but are not specifically mentioned here.

Alcoholic Drinks – Organic Acids

Twelve organic acids were extracted from 1 ml brandy and whisky samples on Chromosorb P and subsequently converted into TBDMS derivatives for GC analysis.[22] A 200 mg Chromabond C_{18} column was used to retain (*E*)-2-nonenal from a 15 ml steam distillate from beer – made up to 100 ml with water. Adsorbed components were eluted with 1 ml acetonitrile. (Chapter 5, Santos *et al.*, 2003, ref. 31, and Appendix 2.)

Colorants

A CE separation of eight colorants, natural carminic acid, and synthetic tartrazine, fast green FCF, brilliant blue FCF, allura red AC, Indigo carmine, sunset yellow FCF and new coccine, required flavoured milk samples to be diluted with EtOH (1:1), mixed for 10 min and adjusted to pH 2.0, centrifuged (1 h at 16000 rpm) in readiness for polyamide phase SPE. The adsorbed colorants were eluted in 0.5% ammonia–MeOH (1:1).[23] β-Cyclodextrin added to the running buffer gave improved resolution due to complex formation with the colorants influencing migration rates.

Cooked Meat – Heterocyclic Amines (HA)

C_{18} SPE cartridges were used to isolate 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP), the most abundant aromatic amine in cooked meat, from biomatrices with a recovery efficiency of > 86%.[24] A detection limit of 1 pg was obtained.

Home-cooked Spanish meat dishes (fried beef hamburgers, fried pork loin, fried chicken breasts, fried pork sausages, griddled chicken breast, griddled lamb steak, and griddled beef steak) were extracted by SPE and the HA separated by LC-MS/MS.[25]

Further work on home-cooked meats prepared according to recipes used in Upper Silesia, Poland, used tandem SPE (Extrelut-type columns filled with diatomaceous earth, propylsulphonic acid and chemically bonded phase-C_{18}) to extract aminoazaarene from 10 meat samples, including pork, beef, turkey and chicken.[26]

Edible Oils and Fats

Free and esterified sterols were separated from a solvent extract [1 ml diethyl ether–hexane, 20:80 (v/v)] of 31 oils and fats on neutral alumina SPE by elution of the esterified sterols in the same solvent, followed by elution of the free sterols with EtOH–hexane–diethyl ether (50:25:25, v/v/v).[27]

Honey – Tetracycline Residues

RP-LC for the separation of tetracyclines from honey used phenyl cartridge SPE. The residues included tetracycline, oxytetracycline, chlortetracycline, doxycycline, minocycline, and methacycline. Sample preparation for extraction involved using a mild acidic solvent containing EDTA to release protein-bound or sugar-bound residues.[28]

Infant Formulae – Vitamins (A, D_3, and E) and Phytoestrogens

Infant formulae were dissolved, thoroughly mixed, and centrifuged to produce a supernatant for SPE. A C_{18} adsorbent retained phytoestrogens. It was dried and then the analytes eluted with MeOH, dried with $MgSO_4$, and evaporated to dryness in a stream of N in preparation for derivatisation with BSTFA/TMCS/DTE, 1000:10:2 (v/v/w) (Kuo and Ding, 2004, Chapter 2, ref. 102).

Chromabond XTR® cartridges were used to extract vitamins A, D_3, and E from the saponified sample.[29]

Migration Volatiles from Plastics

Possible migrants-poly(ethyleneterephthalate), thermoset polyester, poly(ether-sulphone), and poly(4-methyl-pent-1-ene) – from dual-ovenable plastics were

analysed by the dynamic headspace method at 200 ºC using an ice-cooled Tenax trap and/or solvent extraction.[30]

Milk – Docosahexaenoic Acid

A three-step method was described for the direct extraction of DHA from cow's milk.[31] DHA was methylated *in situ* without the need to make a lipid extraction. The methyl ester was concentrated on a $AgNO_3$-modified silica gel column and the extract re-chromatographed by HPTLC.

Mycotoxins

Solvent extraction preceded immunoaffinity extraction (IAE) containing monoclonal antibodies against aflatoxins bound onto agarose. HPLC analysis of aflatoxins B_1, B_2, G_1, and G_2 spiked into peanuts, pistachio nuts and corn at 20 ppb level showed recoveries > 70%.[32]

Aflatoxin M_1 was adsorbed on an immunoaffinity column (containing monoclonal antibodies against aflatoxin M_1) from a hexane-washed DCM extract of cheese.[33] IAE gave a cleaner preparation than SPE. Naturally contaminated and spiked samples were extracted for HPLC analysis.

Ochratoxin A in Food and Wine

C_{18} cartridges were used to extract ochratoxin A from spiked samples of red and white wines for RP-HPLC analysis,[34] and silica phase SPE or IAC was used to clean up ochratoxin A from foods for LC-MS-MS.[35]

Patulin

Patulin is present in decaying apples. It is measured in commercial apple juices as a quality control indicator. A macroporous copolymer cartridge was used to extract patulin from apple juices, and eluted with 2% acetonitrile in anhydrous diethyl ether for RP-LC.[36]

Pigments

E 140 and E 141 are copper complexes of chlorophyll. Extraction of chlorophyll has been included in the examples of extraction protocols (Appendix 4, Scheme A4.5). A solvent extraction with DMF requires further purification with SPE. The whole preparation is performed under dim lighting in an ice bath.[37]

Pesticides

Brito *et al.* (2002) (Appendix 1) reported a simple method for the extraction of 11 pesticides from coconut water and an isotonic drink, using Sep-Pak Vac C_{18} disposable cartridges with methanol elution.

A C_8 SPE cartridge was used to preconcentrate carbamate pesticides – propham, propoxur, carbofuran, carbaryl, methiocarb, isopropoxyphenol, and naphthol – from a light petroleum–dichloromethane (1:1, v/v) extract of powdered potato samples.[38] Recoveries from spiked potato samples at 10 and 0.5 mg kg^{-1} were in the ranges 72–115 and 50–73%, with detection limits of 50–210 (FID) and 41–53 (NPD) μg kg^{-1} respectively.

Aliquots of wine (10 ml) were screened for 74 pesticide residues by loading directly onto 500 mg C_{18} bonded silica. Columns were washed with acetone and EtOH–H_2O and analytes eluted with ethyl acetate.[39]

Soymilk Flavours

Those soymilk volatiles that were adsorbed onto a C_{18} SPE column and desorbed by a 3:1 v/v mixture of hexane and diethyl ether were analysed by GC for the beany flavours in soymilk.[40] A detection limit of 0.1 ppm was obtained for the extraction of both hexanal and hexanol, whose total content was linearly correlated with the lipoxygenase activity.

Star Fruit – Natural Antioxidants

SPE provided adequately clean samples for high resolution, LC-ESI-MS-MS detection.[41] This paper explores the phenolic compounds of star fruit, including (–)-epicatechin and gallic acid in gallotannin forms. Singly-linked proanthocyanidin dimer, trimer, tetramer, and pentamers of catechin or epicatechin were reported.

Sulphonamides in Meat

Solvent extraction was followed by SPE clean up and concentration for CE analysis of eight commonly used sulphonamides. Recoveries from 80–97% were achieved.[42]

Developments

Optimisation of SPE using Solid–Liquid Partition Constants

Six SPE adsorbents were compared in a study to find the best phase for the extraction of aliphatic γ and δ lactones from wine using solid/liquid partition constants and basic bed parameters.[43] The relationship between the retention factor k and the solid/liquid partition constant K is given by Equation (6.1),

$$k = \frac{C_S m_S}{C_L V_L} = K_\varphi \tag{6.1}$$

where, C_S is the concentration of analyte in the sorbent, C_L is the concentration of analyte in the liquid phase in contact with the sorbent, m_S is the mass of

sorbent in the SPE bed, φ is the phase ratio, and V_L is volume of liquid in the SPE bed. K was measured by placing an aliquot of sorbent with an aliquot of spiked wine, rinsing solvent or elution solvent in a vial, shaking for 24 h and extracting with DCM for GC-FID or GC-MS. The authors provide details of the model, basic equations, and the algorithm for the optimisation of the method. They considered the following system parameters:

1. The nature of the sorbent, rinsing and elution solvents.
2. Bed dimensions (hold up volume and number of plates).
3. Volumes of sample, rinsing and elution solvents.

and optimisation parameters:

1. Maximum volume of sample and rinsing solvent without losses of analyte.
2. Maximum volume of rinsing solvent to pass through to eliminate interference.
3. Minimum volume of solvent to ensure complete elution of analyte.
4. Minimum volume that can be passed without elution of additional interference.

Furazolidone

Furazolidone is banned by the EU. An SPE method has been developed to replace the solvent extraction method for measuring the 3-amino-2 oxazolidone moiety in animal tissue.[44] SPE provides 99% removal of the potentially-interfering derivatising agent 2-nitrobenzaldehyde, and is conducive to automation. The extract is suitable for both HPLC and ELISA assays. The method has been validated in fortified and incurred pig liver samples.

Folates

Introduction. A report of the work done to find an alternative method to the classical microbiological assay for folates in foods provides an insight into the attention to detail necessary when a thorough-going study is made.[45] The folates are naturally-occurring B vitamins, existing in the polyglutamate form in fruits, vegetables and berries. Thus, before extraction with phosphate buffer, the polymers were enzymically deconjugated to the monomer. HPLC methods have formed the main challenge to the classical method, but the authors aver that the HPLC methods developed so far lack sensitivity and resolution. A new HPLC method is described using a Zorbax SB C_8 column, with the resolution to determine individual folate forms and, to be less time-consuming than the microbiological method that still remains the method of choice. The new method was verified using CRM 485-lyophilised vegetable material. SPE extraction of folates from plant material (raw and processed beetroots and,

potentially, other vegetables) requires pretreatment to depolymerise the polyglutamate structure of the vitamin. A representative sample from 5–7 roots was pooled, processed and frozen at –20 °C until analysed.

Sample Preparation. The minced samples were homogenised in the extraction buffer and heated in a boiling water bath for 10 min, then cooled and the pH adjusted before pre-treatment with hog kidney deconjugase to convert folate polyglutamates into monoglutamates. This involved a lengthy multistage process, including a 4 h incubation at 37 ºC, centrifugation at 27000*g* for 30 min, precipitation, further centrifugation, re-suspension and a two-step dialysis (2 h and overnight).

Sample Clean-up. SPE with strong anion exchange cartridges (Isolute, 500 mg, 3 ml, International Sorbent Technology, UK) were used in the extraction of folates from beetroots. The SPE tubes were conditioned with 2×2.5 ml methanol and water (2×2.5 ml) at a flow rate of 1–2 drops s^{-1}, and then the sample was applied at < 1 drop s^{-1} flow rate. The cartridge was washed with water and the retained material eluted with 0.1 M sodium acetate containing 10% (w/v) NaCl, 1% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol, the first 0.7 ml being discarded. The ensuing 3.8 ml of eluate contained the three folate forms, H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate. The proposed method was thoroughly researched and the developers used CRM 485 (IRMM, Geel, Belgium) for validation.

Conclusion. The paper is recommended for its rigorous coverage of the multifarious aspects of method development and it seems churlish, after reading such an exemplary analytical report, to comment that the new method still appears to be time-consuming and to contain procedures difficult to automate. (Summarised from ref. 45 with permission from Elsevier)

Cation Exchange

Aminoglycoside Antibiotics in Milk. A new method was developed and validated using ES-ITMS, a recent addition to the arsenal of MS instrumentation to be applied to regulatory methodology. However, even with the resolving power of MS-MS techniques, some preliminary extraction is necessary, and in this case the milk sample was acidified and centrifuged and the separated fat layer discarded. The supernatant layer was made neutral with sodium citrate and passed through a novel weak cation exchange SPE column. The amino glycosides, *e.g.* gentamicin and neomycin, were eluted with acidified methanol.[46] The method threshold was 0.3 mg l^{-1}.

Separation of Amino Acids and Carbohydrates. Because carbohydrates remain neutral over a range of pH at which amphoteric amino acids are

charged, a strong cation exchange resin was used to fractionate the two chemical classes in preparation for quantification by anion-exchange chromatography. Earlier work had made good progress and established the idea of retaining charged species on IE surfaces while the neutrals were eluted.[47] The SPE method was developed to avoid problems with arginine and to speed up and simplify the extraction process.[48] High carbohydrate foods such as sourdough and potato, protein-rich skimmed milk, and a fruit juice (lemon juice) were used to demonstrate the applicability of the method.

Pre-treatment of the food sample involved deproteinisation with perchloric acid (if required), dilution and mixing with 5 parts of water to 1 part of food and the supernatant after centrifugation used for SPE, as illustrated in Figure 6.4.

The method was thoroughly validated and optimised. Quantitative recovery was measured by standard addition.

Streptomycin and Dihydrostreptomycin Antibiotics in Meat. Sample preparation for extraction involved protein precipitation with dilute perchloric acid and centrifugation. Cation exchange SPE provided the final extraction and the adsorbed analytes were desorbed with phosphate buffer at pH 8.[49]

Comparison of Sorbents

Two Sorbents Compared. The simplicity of use and the higher recovery of the octadecylsilane cartridges were preferred over Florisil packed columns when

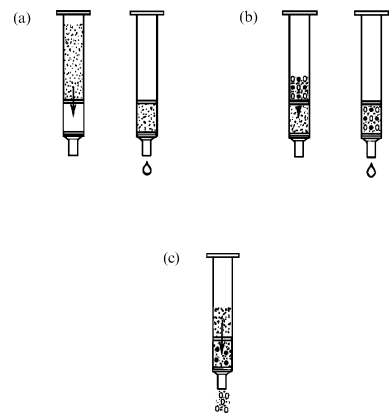

Figure 6.4 (a) *The 1 ml, 100 mg sorbent tubes (Strata SCX, Phenomenex, Torrance, CA) were conditioned with MeOH–water*. (b) *Sample was diluted 10-fold with 0.01 N HCl + 10% MeOH and 1–2 ml loaded onto the column. Carbohydrates were eluted with 1 ml water*. (c) *Amino acids were eluted with 2 ml 0.2 M* $CaCl_2$ *+ 1 ml demineralised water*
(With permission from Elsevier to reproduce detail from *Analytical Biochemistry*, vol. 310, C. Thiele, M.G. Gänzle and R.F. Vogel, "Sample Preparation for Amino Acid Determination by Integrated Pulsed Amperometric Detection in Foods", pp. 171–178, © 2002)

they were compared for the extraction of the insecticide Rotenone from raw honey.[50] The method threshold was 0.015 mg kg^{-1}.

Five Sorbents Compared. Extraction and analysis of the polar volatiles of butter is a difficult task, and the extraction stage is very important. Liquid butter at 40 °C was centrifuged at 4000 rpm for 20 min, and the fat extracted from the aqueous phase. The yield of aqueous phase was 7 ml/100 g butter.

Before developing the SPE method, several extraction methods were listed as having been considered: headspace analysis,[51] StD and SFE,[52] trapping on porous polymer,[53] SPE over resins,[54,55] purge and trap[56] and distillation–extraction.[57] These methods were all found wanting and five SPE sorbents, octyl-, octadecyl-, cyano- and amino-modified silicas, and a styrene-divinylbenzene (PS-DVB) copolymer, were selected for evaluation to optimise the extraction of polar volatiles from the aqueous phase of butter.[58]

With a wide range of polarity within the group of target flavour compounds (Table 6.1), it was necessary to test a wide range of sorbents for properties such as breakthrough and desorption (Figure 6.5), enrichment, recovery and detection limits. The enrichment value and detection limits were measured using a spiked aqueous phase from butter.

The sample volume was kept at 2 ml to minimise the chance of losses from early breakthrough. The summarised SPE details are

Cartridge volume:	1 ml
Sorbent charge:	100 mg
Rinse solvent 1/volume:	Methanol/2 ml
Rinse solvent 2/volume:	HPLC water/2 ml
Sample load:	2 ml Aqueous solution
Loading rate:	2 ml min^{-1} (with suction)
Clean-up solvent:	HPLC water/1 ml
Clean-up flow rate:	2 ml min^{-1}
Drying time/conditions:	15 min at ambient temperature under vacuum

Table 6.1 *Oil/water partition constants for five polar flavour volatiles* (Log $K_{o/w}$ data reproduced from the *Journal of Chromatography A*, vol. 844, M. Adahchour, R.J.J. Vreuls, A. van der Heijden and U.A.Th. Brinkman, "Trace-level Determination of Polar Flavour Compounds in Butter by Solid-phase Extraction and Gas Chromatography-Mass Spectrometry", pp. 295–305, © 1999, with permission from Elsevier)

Volatile flavour compound	*Log* $K_{o/w}$
Diacetyl	–1.37
Furaneol	1.07
Sotolon	0.33
Maltol	0.02
Vanillin	1.28

Desorption solvent/vol:	Methyl acetate/0.5–1.0 ml
Flow rate:	0.5 ml min^{-1}
Dry eluent:	Na_2SO_4/1 g
Sample size:	0.5–1.0 µl
Procedure time:	4–5 h (for up to 8 sample batch)
Breakthrough at 5 µg ml^{-1}:	(Figure 6.5)
Detection limits s/n 3:1:	20–100 µg kg^{-1} (full scan MS) (See caption to Figure 6.5)
	3–12 µg kg^{-1} (SIM MS) (See figure caption)
Enrichment factors:	10–15
Recovery at 400 ng min^{-1}	
Desorption volume:	0.5 ml (Figure 6.5)

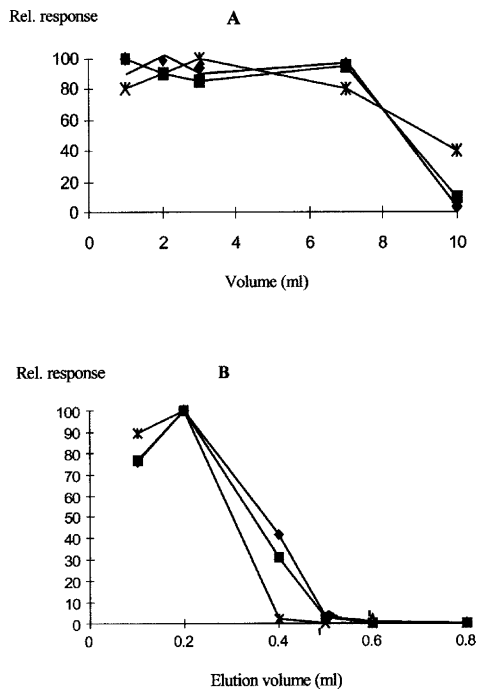

Figure 6.5 (A) *Breakthrough curves at 5 µg ml^{-1}, and (B) Desorption curves with methyl acetate for diacetyl (*), maltol (■), and vanillin (◆), on SDB-1 phase. Full scan MS limits the sensitivity because time is spent visiting all the ions in the spectrum in a continuous sweep, whereas in SIM MS only a few ions are chosen and therefore for the same scan rate, a longer dwell time allows more signal to be collected*
(Reprinted from the *Journal of Chromatography A*, vol. 844, M. Adahchour, R.J.J. Vreuls, A. van der Heijden and U.A.Th. Brinkman, "Trace-level Determination of Polar Flavour Compounds in Butter by Solid-phase Extraction and Gas Chromatography-Mass Spectrometry", pp. 295–305, © 1999, with permission from Elsevier)

(Permission granted to reproduce the data in this form from the same source as Figure 6.5 and Table 6.1).

Potato Glycoalkaloids. Five commercial SPE sorbents, C18, SCX, CN, Certify and Oasis HLB, were compared for the recovery of alpha-solanine, alpha-chaconine and alpha-tomatine (and its impurity dehydrotomatine), in extracts of potato, and the highest recovery of approximately 100% was obtained using Oasis HLB.[59]

Comparison of Commercial and Laboratory-made Cartridges

500 mg loads of Supelco LC-NH_2 and LC-18, and Merck LiChrolut NH_2 and LiChrolut RP-18, were compared with laboratory-made phases for the multiclass determination of pesticides in grapes.[60] Details are given of the manufacturing process used in the laboratory. The success of the laboratory-made 40% loaded-NH_2 cartridge compared to the commercial equivalent was attributed to its mixed-mode sorbent effect. Comparison of chromatograms from the two columns showed that while the laboratory-made column gave higher resolution for some components, the commercial column gave generally better peak shapes/performance.

Columns in Series

For Acrylamide in Breakfast Cereals. The ground powder was suspended in water, homogenised, centrifuged, and the supernatant mixed with acetonitrile to precipitate any co-extractives and centrifuged. The supernatant was evaporated under N_2 at 45–50 °C for extraction by, first, Isolute Multimode and, then, Accubond II SCX columns for LC-ESI-MS/MS.[61]

For "Difficult" Food Matrices. A novel NH_2 SPE column was introduced, along with the existing silica-based column, to deal with the extraction of lasalocid in difficult matrices such as baby foods and meat pies.[62] The new method was validated down to 10–40 mg kg^{-1} across a range of products. Recoveries were 74% at 10 μg kg^{-1} (pork sausages) and 96% at 40 μg kg^{-1} (meat pies).

For Niacin. The liberation of niacin in cereals, meat, fish, yeast, nuts, peanut butter, and sunflower seeds from the food matrix used alkaline digestion in aqueous calcium hydroxide.[63] SPE using C_{18} and cation exchange columns in series purified and concentrated the extract for CE separation. The CE method compared favourably with the AOAC colorimetric method.

For Prefractionation of Aroma Extracts from Fatty Foods. An interesting use of size-exclusion (styrene-divinylbenzene) chromatography was to fractionate

aroma volatiles and semi-volatile triglycerides from goats' cheese. The extract for SEC was obtained according to Scheme A4.2, Appendix 4. Typically, SEC has been used for the separation of a few specific compounds based on their molecular weights, whereas here the aroma volatiles of goats' cheese occupy many chemical classes and molecular sizes. The degree of separation from the triglyceride fraction depends upon the sample size, and is discussed in detail.[64]

For Cysteine Sulphoxide Amino Acids. *S*-methyl, *S*-propyl, and *S*-propenyl-*L*-cysteine sulphoxides, specific marker compounds in the genus *Allium*, were solvent extracted from commercial onion and further extracted using a Sep-Pak C_{18} cartridge. The eluate was then applied to the H^+ form of a Bond Elut cartridge, washed with 0.1 M HCl and eluted with 0.5 M ammonia[65] for derivatisation and GC-MS measurement. The amount of each sulphoxide found in commercial onion was 0.3, 3.1, and 3.0 mg g^{-1} fresh weight, respectively.

For Antibiotics in Milk and Meat Tissues. Sample pre-treatment for extraction of the antibiotic colistin involved protein precipitation with 10% TCA. Preliminary extraction with C_{18} SPE, followed by derivatisation with *o*-phthalaldehyde and 2-mercaptoethanol in borate buffer at pH 10.5 before injection of the reaction mixture into the flow-switched supply to the pre-column for a final extraction of contaminants with acetonitrile-phosphate buffer at pH 7 preceded injection onto the analytical column.[66]

Immunoaffinity Extraction (IAE) (Table 6.2)

Introduction. The specificity of antigen (analyte) adsorption through covalent bonding onto an immunosorbent surface, created by immobilising the antibody onto a suitable support medium, provides a highly selective extraction method. The preparation of antibodies for low molecular weight antigens has improved and the analyses of aflatoxins[67] and pesticides in biological samples were early applications of immunosorbent packings in standard SPE-type cartridges. On occasions, the cross-reactivity is low enough to target a single compound or, for some applications, cross-reactivity for several members of a chemical group of compounds is advantageous.

The use of more than one immobilised antibody in a single step extraction enables multiple targeting of analytes.

Antibody Production and Immobilisation. For high MW compounds, polyclonal antibodies are cheaper to produce and usually provide more cross-reactivity than monoclonals. The steps in the production of an antibody and the covalent binding to the immobilising medium are well explained by Pichon *et al.*, 1998.[68] The sorbent is chosen to have a pore size to accept the antibody and is usually hydrophilic for analytes in aqueous solvents. Immunosorbent

Table 6.2 *Examples of immunoaffinity extractions*

Analyte	*Commodity*	*Pre-treatment*	*IAE column (manufacturer)*	*Separation/detection /LOD*	*Reference*
Phenylurea herbicides	Carrots, celery, corn, grapes, onions, potatoes, and strawberries.	5 g homogenised, dissolved in 20 ml MeOH, vortexed, centrifuged, evaporated, diluted in PBS	1 g silica-immobilised antibody (generated against isoproturon in the rabbit) in syringe barrel	HPLC/diode array UV/< 25 ng g^{-1}	Lawrence *et al.*, 1996[a]
Fumonisins B_1, B_2, and B_3	Corn	50 g corn extracted in MeOH + NaCl by blending	Fumonitest-HPLC (Vicam Watertown, MA, USA)	HPLC/ /0.016 μg g^{-1} Fluorometer/0.25 μg g^{-1}	Duncan *et al.*, 1998[b]
Deoxynivalenol	Wheat	Water extract and filtered twice	DONtest-HPLC (Vicam, Watertown, MA, USA)	HPLC/UV/0.1 μg g^{-1}	Cahill *et al.*, 1999[c]
Ochratoxin A	Wheat, barley, coffee	Extracted with MeOH–$NaHCO_3$ (7:3), filtered and diluted with PBS		RP-C_{18}-HPLC/fluor/0.03 ng g^{-1}	Trucksess *et al.*, 1999[d]
Ochratoxin A	Wine	Diluted with 1% PEG 8000 + 5% $NaHCO_3$, filtered	Applied to commercial IA column	HPLC/FLD/0.01 μg l^{-1}	Visconti *et al.*, 1999[e]
Ochratoxin A	Currants, raisins, sultanas	Acidified methanol extraction, blended and filtered	IAC column	HPLC/MS/0.2 μg kg^{-1}	MacDonald *et al.*, 1999[f]
Aflatoxin B_1	Olive oil	Mixed with MeOH–H_2O (60:40), extracted with hexane and $CHCl_3$, and evaporated. Residue dissolved in DCM for silica Sep-Pak	Sep-Pak eluted with $CHCl_3$–acetone (9:1). Aflaprep IAC.	HPLC/fluorometric/ 2.8 ng g^{-1}	Daradimos *et al.*, 2000[g]

Table 6.2 *Continued*

Analyte	*Commodity*	*Pre-treatment*	*IAE column (manufacturer)*	*Separation/detection /LOD*	*Reference*
Aflatoxins B_1, B_2, G_1, G_2	Corn flour, paprika, peanuts, pistachios	Corn and paprika; 200 ml MeOH–water (8:2, v/v), nuts; +100 ml hexane or PE. Blend for 3, or shake for 30 min and filter. 20 ml filtrate diluted to 140 ml with PBS pH 7.4 and filter.	Conditioned IAC (Rhone-Poulenc Diagnostics, UK),	TLC/UV/0.1–0.2 ng g^{-1}	Stroka *et al.*, 2000[h]

[a] J.F. Lawrence, C. Ménard, M-C. Hennion, V. Pichon, F. Le Goffic and N. Durand, *J. Chromatogr. A*, 1996, **732**, 277. [b] K. Duncan, S. Kruger, N. Zabe, B. Kohn and, R. Prioli, *J. Chromatogr. A*, 1998, **815**, 41. [c] L.M. Cahill, S.C. Kruger, B.T. McAlice, C.S. Ramsey, R. Prioli and B. Kohn, *J. Chromatogr. A*, 1999, **859**, 23. [d] M.W. Trucksess, J. Giler, K. Young, K.D. White and S.W. Page, *J. AOAC Int.*, 1999, **82**, 85. [e] A. Visconti, M. Pascale and G. Centonze, *J. Chromatogr. A*, 1999, **864**, 89. [f] S. MacDonald, P. Wilson, K. Barnes, A. Damant, R. Massey, E. Mortby and M.J. Shepherd, *Food Addit. Contamin.*, 1999, **16**, 153. [g] E. Daradimos, P. Marcaki and M. Koupparis, *Food Addit. Contam.*, 2000, **17**, 65. [h] J. Stroka, R. van Otterdijk and E. Anklam, *J. Chromatogr. A*, 2000, **904**, 251.

phases may be stored under PBS solution and conditioned with distilled water before use. After the analyte solvent has passed through the column, the adsorbed material may be eluted with aqueous MeOH, for example. The application of immunosorbent extraction will be discussed later.

Sol-gel Immunoaffinity Support Phases. The application of sol-gel technology to the preparation of immunosorbent phases provides a non-destructive and non-intrusive support for the antibody used in IAE. Nitrated polycyclic aromatic hydrocarbons (NPAHs), formed in low concentration from PAHs and nitrogen oxides, are possible carcinogens and a challenge for the food analyst. In an instructive account, Spitzer *et al.* (2000) provide full details for the production, testing, and operation of a sol-gel immunoaffinity column for the extraction of 1-nitropyrene from herbs (basil, chervil, marjoram, oregano, and sage) with pre-extractions using UAE, SE (acetonitrile) and GPC (Sephadex LH-20, to extract the high MW components) in readiness for IAE.[69] The authors describe their column preparation method:

A silica sol column filling was made by mixing 0.4 ml of 0.04 M aqueous HCl, 1.5 ml double distilled water and 6.8 ml TMS under constant stirring. The mixture was cooled in ice water and sonicated for 30 min. 2 mg IgG fraction (isolated from rabbit anti-1-nitropyrene antiserum) was added to 1 ml PBS and a 1 ml aliquot of the silica sol mixed in. The gel formed in 2 min and was stored at 4 ºC for the duration of the ageing process, which was stopped after a weight loss of 50%. Silicate glass was ground in a mortar and 0.64 g used to pack a 3 ml column, washed with 20 ml PBS and stored under PBS at 4 ºC. After pre-conditioning the column was loaded by pumping at 1 ml min^{-1} 50 ml of the sample solution of acetonitrile–doubly distilled water (10:90, v/v) containing the analyte and flushed with 20 ml of the same solvent solution to remove non-specifically bound material.

The antigen–antibody complexes were dissociated with a 40:60, v/v solution of the same solvent mix. (Permission granted from Elsevier to report in detail from ref. 69).

Comparison of IAE with Other SPE Methods. Five methods were compared for the extraction of ochratoxin A from wine, must and beer in preparation for HPLC with fluorescence detection (Sáez *et al.*, 2004, Appendix 1).

1. Dilution of the sample with 20 ml aqueous solution containing 5% $NaHCO_3$, addition of 1% PEG 8000 as a filtration aid (Visconti, *et al.* 1999, Table 6.2). and pH adjustment to 8.5 with 1 M NaOH, and filtered preceding IAE.
2. Acidification with H_3PO_4 and NaCl added for SE with $CHCl_3$ in a separating funnel, centrifugation of the organic phase, and re-extraction with $CHCl_3$ (twice), combined and evaporated to dryness in a vacuum rotary evaporator (Büchi) at 40 ºC. Residue dissolved in 10 ml PBS containing 10% (v/v) EtOH for IAE.

3. Extraction was as in 1. The whole solution was loaded onto the conditioned RP C_{18} column and 2 ml MeOH–acetic acid (95.5:0.5, v/v) used for elution.
4. A phenylsilane packing was used for RP-SPE. 10 ml sample was passed through the conditioned column and the ochratoxin A eluted with 5 ml MeOH–acetic acid (95.5:0.5, v/v), evaporated to dryness and taken up in 250 µl mobile phase.
5. Oasis HLB cartridges (Waters, Milford, MA, USA). 10 ml sample was passed through the conditioned column, and the column washed with 5 ml water–MeOH (95:5, v/v), ochratoxin A was eluted with 2 ml MeOH and finished as for 4. above.

The effect of the IAE on the quality of the data is shown by comparing the chromatograms obtained using extraction protocols 1 and 4 (Figure 6.6).

Thin-film SPE

The accumulation of semi-volatile, toxic organic compounds in the food chain was investigated using thin films of ethylene vinyl acetate coated on a glass surface to sample fish tissue without the need for solvent extraction and clean-up.[70]

Molecularly-imprinted Adsorbents

Triazines from Vegetables. The application of molecularly-imprinted polymer (MIP) technology to SPE was demonstrated with the extraction of triazines from vegetable samples using a propazine-imprinted polymer.[71] Atrazine, simazine, desethylatrazine, desisopropylatrazine, and propazine were studied using a methacrylic acid-based imprinted polymer, prepared by precipitation polymerisation (propazine as the template and toluene as the porogen). Soxhlet extraction removed the template and the system was optimised for triazines in pea, potato and corn. However, interferences remained, which bound strongly to the polymeric matrix, and an extra clean-up step was put in front of the MISPE stage, successfully removing them.

Quercetin in Red Wine. Quercetin was used as the template for SPE of a red wine sample directly applied to the cartridge. The efficacy of using MIP selection was verified by comparison with non-imprinted polymer and C_{18} silane RP extraction for HPLC analysis.[72]

Off-line Automation

Automated, off-line SPE instruments,[73] after some simple sample preparation, *e.g.* homogenisation in NaOH solution, provide robotic supervision of the extraction process up to the filling of the sample vial with the target fraction

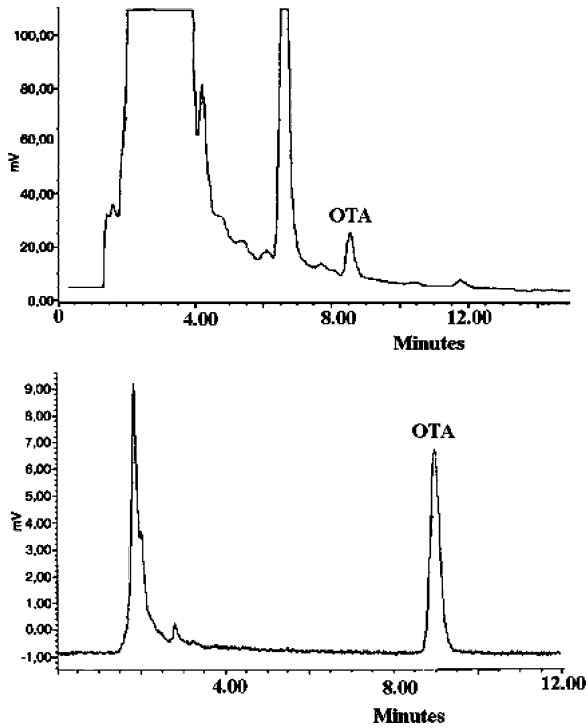

Figure 6.6 *LC chromatograms of must contaminated with 0.19 ng ochratoxin A (OTA) per ml and spiked with 1.0 ng ochratoxin A standard (top), and 0.5 ng ochratoxin A standard (bottom). Top: SPE on RP phenylsilane, and bottom: 1% PEG 9000 + 5% $NaCO_3$ solution, filtration and IAE. The IAE produces greatly increased sensitivity and high selectivity using the same chromatographic conditions*
(Adapted from the *Journal of Chromatography A*, vol. 1029, J.M. Sáez, A. Medina, J.V. Gimeno-Adelantado, R. Mateo and M. Jiménez, "Comparison of Different Sample Treatments for the Analysis of Ochratoxin A in Must, Wine and Beer by Liquid Chromatography", pp. 125–133, © 2004, with permission from Elsevier)

in readiness for injection into a chromatograph for separation.[74] Samples of microwaved meat extract, vacuum dried meat extract, grilled beef, merguez sausages, chicken-flavoured pasta and peanut butter were homogenised and then the robot supervised the DCM elution and transfer of HAAs from Extrelut to cation exchange cartridges for the extraction of polar and apolar fractions. These extracts were transferred to C_{18} cartridges, eluted with MeOH–NH_3 and collected in HPLC vials. Automated extraction instruments work in conjunction with automatic sample injection attachments to chromatographic systems.

Rapid SPE of Aflatoxins. Foods such as milk, cereals and nuts were screened for aflatoxins and the extraction stage was automated. Sample preparation, cen-

trifugation of liquids, grinding of solids, extraction with 60% aqueous MeOH, filtration and dilution remained manual, but the MeOH extract was injected into an immunoaffinity pre-column, the eluate transferred to RP-HPLC automatically for analysis at the rate of 2–4 samples per hour.[75]

Solid-phase Spectrophotometry. Food colorants, tartrazine, ponceau 4R, and sunset yellow FCF were adsorbed onto Sephadex DEAE A-25 gel at pH 2.0 and the filtered beads packed into a 1 mm silica cell for measurement in the range 400–800 nm. The remote sensing method compared favourably with HPLC.[76]

Acrylamide Analysis. A 300 mg Isolute multimode SPE column (International Sorbent Technology, Hengoed, Mid Glamorgan, UK) and the ASPEC™ XLi (Gilson, Middleton, WI, USA) was used to extract the acrylamide from coffee samples for LC- electrospray MS/MS in the positive ion mode.[77]

On-Line Automation of SPE/HPLC

The next step towards full automation is to control the extraction process and transfer the eluate automatically from the SPE column on to the chromatograph and this is achieved by using the SPE column in the place of the pre-column for HPLC. The original role of the pre-column was to protect the analytical column from unnecessary contamination, but other uses for it evolved.

Flow Switching Valves as Extraction Assistants. When a pre-column is used with a FSV it can be turned into a pre-fractionation device. It is this role that SPE sorbents as the pre-column packing now occupy in the modern multidimensional (two column mode) chromatography. The SPE pre-column placed in line with a FSV allows the process of compound concentration and extraction to occur directly from certain types of flowing liquid food matrices in the off-line mode.

The use of a FSV to automate the loading of the pre-column from a flowing liquid food matrix, and elution of analytes for injection into the analytical column is illustrated in Figure 2.8 Chapter 2 where the two FS modes are shown. Some sample preparation before on-line concentration and extraction (*e.g.* filtration) will be required if the life of the SPE pre-column is to be economical. The FSV with two pumped flow lines allows the SPE pre-column to be automatically taken off-line from the HPLC for the loading stage (pump 1 mobile phase 1). Mobile phase 1 can be chosen to have a low affinity for the analyte, leaving it to be adsorbed on the packing of the pre-column while other compounds are eluted to waste. The valve is then switched to put the pre-column in-line for the period of the injection (pump 2, mobile phase 2), and then taken out of line again after the injection (return to the load position).

This avoids unwanted slow moving fractions (end fractions) contaminating the analytical column, and allows the pre-column to be cleaned up. Figure 6.7 shows the configuration of the back-flushing load and inject modes used to increase the resolution of the injection.

An alternative arrangement using the FSV in the back flush mode, but using only one pump, facilitates a loop injection mode but, obviously, it uses a single mobile phase (Figure 6.8).

Application of FS-SPE/HPLC. The determination of PAHs in edible oils and fats (sunflower, olive, coconut, bean, fish, sesame, and palm) using an automated on-line SPE pre-column, two analytical HPLC columns, and three FSVs, admirably illustrates the application of automated, on-line extraction and chromatographic separation.[78] Preliminary sample preparation for extraction entailed simply adding 12.5% v/v isopropyl alcohol (IPA) to the oil and filtering. Assuming the required conditioning, rinsing and cleaning of the columns and syringes is handled by the program in the background, the analytical steps are: the sample loop is filled, the sample is sent to the pre-column for adsorption on the electron-acceptor phase, the unadsorbed bulk of the oil sample is flushed to waste. The flow is switched to back-flush and the PAH-rich sample is sent at high resolution to the analytical column for separation. The developers describe the optimisation of the operational conditions in terms of:

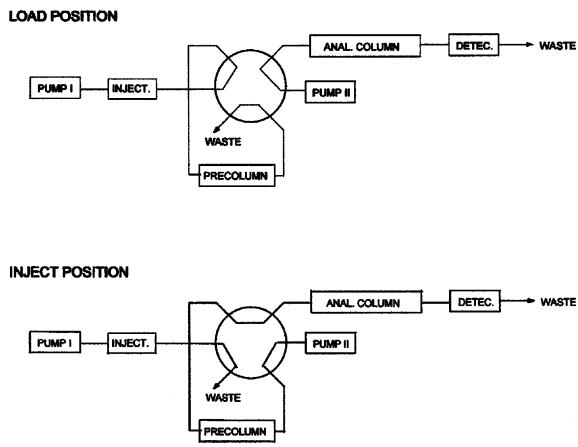

Figure 6.7 *Back-flush mode for high-resolution concentration and injection of a fraction extracted from the flowing liquid food matrix. The back-flushing mode is used to improve the resolution. By adsorbing the liquid food matrix onto the pre-column in the forward direction and injecting the chosen fraction onto the analytical column in the opposite direction, the relative velocities of the separated fractions is reversed, bringing all the fractions back to the same focal point before entering the analytical column*
(Reprinted from the *Journal of Chromatography A*, vol. 880, L. Bovanová and E. Brandšteterová, "Direct Analysis of Food Samples by High-performance Liquid Chromatography", pp. 149–168, © 2000, with permission from Elsevier)

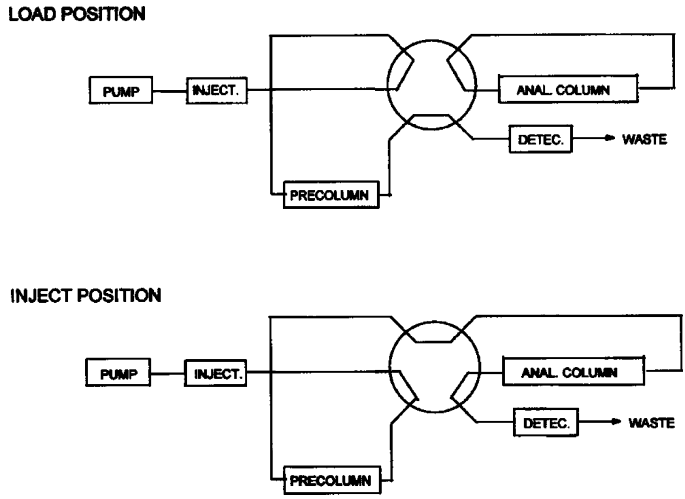

Figure 6.8 *Single pump, single mobile phase flow system with back flushing for improved resolution. In this single mobile phase mode, the pre-column elution is organised to inject the front, middle, or end cut by controlled flow switching* (Reprinted from the *Journal of Chromatography A*, vol. 880, L. Bovanová and E. Brandšteterová, " Direct Analysis of Food Samples by High-performance Liquid Chromatography", pp. 149–168, © 2000, with permission from Elsevier)

1. Polar and apolar mobile phases – IPA chosen.
2. Ratio of IPA/oil –12.5% (v/v). Precipitation occurs at > 60%.
3. Dimensions of the pre-column –80 × 3 mm. Larger columns allowed too much IPA to reach the analytical column with negative effects on the separation. These dimensions allowed 250 µl volumes to be injected, giving a LOD of 0.1 µg kg^{-1}.
4. Column temperature –20 °C. Lower temperatures cause precipitation and higher temperatures reduce the efficiency of the pre-column.
5. Mobile phase flow rate –0.35 ml min^{-1}. Higher rates over-pressurise the system after injection.
6. Minimise amount of IPA reaching analytical column – extra valve fitted so that IPA is switched to waste for the first 145 s of the back flush mode.
7. Composition of the back flush mobile phase –85:15 (v/v) acetonitrile–water. A higher % acetonitrile gives sharper peaks for PAHs eluting from the pre-column, but reduces the efficiency of the analytical column.

An arrangement using a pre-column (called a trap column), two analytical columns and two FSVs was used to determine diarrhetic shellfish poison in mussels. The sample preparation for extraction was homogenisation of a 1 g of sample with 4 ml of MeOH–H_2O (80:20, v/v) in an ultra-turrax for 3 min,

followed by centrifugation at 2980*g* for 5 min. The supernatant was extracted twice (separating funnel) with 4 ml DCM and made up to 10 ml. Then, 1 ml of the DCM extract was evaporated to dryness (under N_2) and derivatised to the ester with 9-anthryl-diazomethane at 25 ºC in the dark for 90 min. Derivatised sample (20 µl) was injected and the separation commenced on the first analytical column. Just before the analytes were due to elute the flow was switched and the analytes absorbed on the trap column. The flow was then switched to the second analytical column for final separation and detection. With this method much of the original derivatised sample is vented to waste. The automated on-line extraction and separation speeds up the analysis compared to the manual procedure.[79]

SPE affinity chromatography with FS-SPE/HPLC produced a highly selective, on-line extraction of tetracycline antibiotics from animal tissues, *e.g.* sheep liver.[80] Sample preparation for extraction was to homogenise thinly-sliced tissue with succinate buffer (pH 4) and centrifuge at 30897*g* for 15 min. The supernatant was decanted and filtered and diluted with pH 4 succinate-EDTA buffer with 3% pentanesulphonic acid solution. Various SPE "clean up" cartridges (Bond-Elut C_8 Isolute C_8 EC, XAD-2 resin) were used for the different tissues and a MeOH extract taken for HPLC. The MeOH extract was adsorbed onto the conditioned metal chelate affinity chromatography (MCAC) pre-column (in place of the sample loop) and the pre-column was washed and switched on-line to the analytical column and eluted with mobile phase for 11 min before being switched off-line. The method developers optimised the deproteinisation sample preparation and SPE procedures.

Improvements to the method were made later for application to other animal products: egg, poultry, fish and venison.[81] The preliminary treatment of the sample before MCAC-HPLC was changed fundamentally from aqueous buffer extraction to organic solvent extraction. The tissue samples were homogenised with ethyl acetate and centrifuged. The residue was re-extracted twice and the combined supernatants dried over sodium sulphate, filtered, evaporated to dryness, taken up in MeOH by vortex mixing, and filtered ready for HPLC separation. The organic solvent extraction was selective enough to permit circumvention the off-line SPE extractions of the earlier method. The solvent extraction method had higher throughput and recoveries, and lower LODs.

Application of FS-RAM-SPE/HPLC. Direct extraction from flowing food matrices by solid phase devices has been discussed, but the problem of deterioration of the SPE column after a number of injections has not been addressed. The use of RAM packing materials for the pre-column for food samples has provided a solution. Macromolecules that are normally adsorbed onto the surfaces of SPE packings cannot occlude to the RAM-treated surface, and are too large to enter the porous, hydrophobic internal structure where smaller analytes can be absorbed. The determination of chloramphenicol in animal tissue was an early use of RAM in food extraction analysis.[82]

Robotic On-line Automated SPE/HPLC

Method Development. An automated sample preparation system (ASPEC™, Gilson, Villiers le Bel, France) was coupled on-line to HPLC for the extraction, derivatisation and injection of fumonisins B_1 and B_2 from corn using purpose-made disposable extraction cartridges (DECs).[83] The DEC was optimised by testing 200, 300, 400, and 500 mg loads of absorbent (C_{18}, $C_{18\ ec}$ and SAX) packed into 3 ml tubes capped with a frit above and below the packing. Preliminary extraction in a blender was optimised by testing:

1. A 10 g aliquot of sample in 50 ml of acetonitrile–water (50:50, v/v) with C_{18} DEC.
2. A 25 g sample in 50 ml MeOH–water (75:25, v/v) for SAX DEC. The mixture was pH adjusted and diluted (for C_{18}, $C_{18\ ec}$) and filtered ready for the ASPEC™ to complete the extraction stage by controlling the following procedures:

For C_{18}, and $C_{18\ ec}$ columns

1. Condition DEC with 2 ml acetonitrile and then 2 ml water (2 ml min^{-1})
2. 2 ml extract + 6 ml water passed through column (1 ml min^{-1})
3. Rinse the needle (2 ml min^{-1})
4. Wash column with 5 ml water (2 ml min^{-1})
5. Elute mycotoxins with 2 ml acetonitrile–water (70:30, v/v), pH 5.8–6.5 (1 ml min^{-1})

For SAX columns

1. Condition with 8 ml MeOH and then 8 ml MeOH–water (3:1, v/v) (2 ml min^{-1})
2. 5 ml extract passed through column (1 ml min^{-1})
3. Rinse needle (2 ml min^{-1})
4. Wash DEC with 8 ml MeOH–water (3:1, v/v) (2 ml min^{-1})
5. Wash DEC with 3 ml MeOH (2 ml min^{-1})
6. Elute mycotoxins with 5 ml MeOH–acetic acid (99.5:0.5, v/v) (1 ml min^{-1})

The extraction with acetonitrile–water (50:50, v/v) and extraction on $C_{18\ ec}$ DEC was preferred for this analysis. (Summarised from ref. 83 with permission from Elsevier)

On-line SPE/HPLC/MS

An obvious objective in food analysis is for the extraction stage to be coupled on-line to the separation and detection stages in a rational development of fully automated analysis. Early reports of the coupling of SPE to the already coupled separation and detection stages came from environmentalists concerned about water quality.[84] They reported the use of various forms of carbon to adsorb polar pesticides from water supplies. The advantage of the on-line application was quickly recognised by other disciplines.

On-line SPE-LC-ESI-MS/MS. Using the extraction of chlormequat (CQ) and mepiquat (MQ) pesticides from tomato, pear and wheat flour as examples of food matrices, the team from Nestle, Lausanne, and Metrohm, Herisan, Switzerland, reported the development of four SCX solid phase resins for the efficient extraction of these two pesticides (Figure 6.9).[85] The higher resolution and shorter analysis time of LiChrolut SCX gave it advantages over the other three. Furthermore, the repetitive use of this cartridge did not seriously lower the efficiency for extracts of pear and wheat flour.

On-Line SPE/CE

Sample Concentration. The main limitation of CE is the low concentration sensitivity, which for trace analysis of biological samples has been addressed in

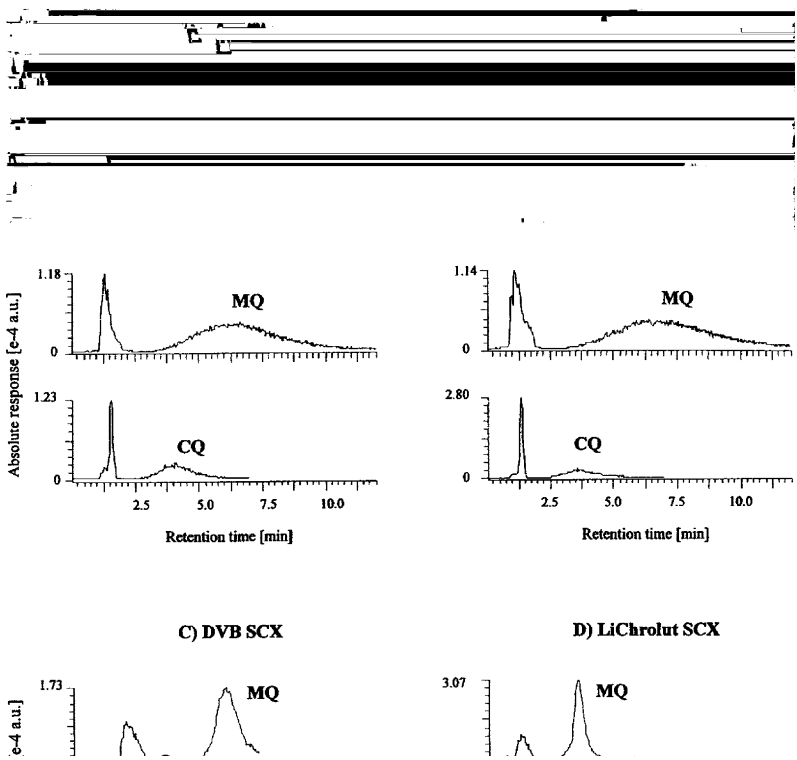

Figure 6.9 *Retention time of MQ and CQ pesticides extracted from fortified wholemeal wheat flour on Prospekt SPE cartridges and eluted for flow injection into LC-MS/MS.* (A) *BondElut SCX*, (B) *Isolute SCX*, (C) *DVB SCX*, (D) *LiChrolut SCX filling. The potential resolution of the LiChrolut SCX phase for the separation of these two compounds is well illustrated* (Reprinted from the *Journal of Chromatography A*, vol. 1020, S. Riediker and R.H. Stadler, "Analysis of Acrylamide in Food by Isotope-Dilution Liquid Chromatography Coupled with Electrospray Ionisation Tandem Mass Spectrometry", pp. 121–130, © 2003, with permission from Elsevier)

several different ways. An SPE micro-cartridge of solid phase adsorbent has been used to pre-concentrate metallothionein isoforms loaded from mobile phase 1 and then, by changing to mobile phase 2, the adsorbed and concentrated analytes are desorbed and "injected" onto the analytical column for separation.[86] A miniaturised SPE devise has been described for the on-line concentration of analytes, providing concentration factors of 7000[87] (Figure 6.10).

Optimisation

Recently, a stategy for the optimisation of critical SPE volumes was described in terms of sample load, conditioning and desorption solvents, calculated from solid/liquid extraction constants and basic bed parameters using chromatographic theory with satisfactory agreement between calculated and measured volumes and applied to the optimisation of SPE conditions for the extraction of aliphatic lactones from wine.[88]

3 Solid-phase Microextraction (SPME)

"One such technique was solid phase microextraction (SPME), a simple solventless sampling method developed in the early 1990s,[89] for absorbable and volatile samples, it integrates sampling, extraction, concentration, and GC[90] or LC[91] sample introduction into a single step." L.N. Surugau, MSc thesis, UEA, 1998 (ref. 149).

Introduction

SPME was introduced[92] as an extraction method for absorbing mixtures of volatile and semi-volatile substances from the headspace above liquid and solid samples for direct transfer to the heated zone of the GC inlet for desorption and separation by GC analysis. Later, when suitable adsorbents were introduced, the technique was extended to the sampling of liquids injected into

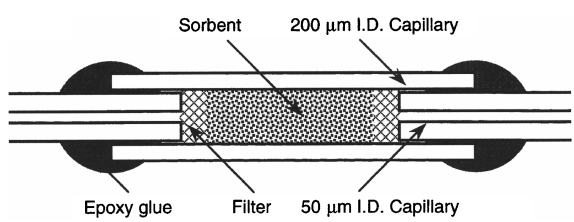

Figure 6.10 *Solid-phase concentrator*
(Permission to reproduce from the *Journal of Chromatography A*, vol. 841, M. Petersson, K-G. Wahlund and S. Nilsson, "Miniaturised On-line Solid-phase Extraction for Enhancement of Concentration Sensitivity in Capillary Electrophoresis", pp. 249–261, © 1999, with permission from Elsevier)

HPLC. The most versatile detector for GC and HPLC analysis of volatile compounds is mass spectrometry (MS) and, therefore, SPME-GC-MS and ater SPME-HPLC-MS were practical combinations that quickly gained a place in the armoury of volatile sample analysis. SPME uses a fibre coated with a stationary phase, *e.g.* PDMS, located in a stainless steel sheath, similar to a hypodermic syringe needle. The fibre is exposed for sampling and retracted for insertion through the septum of the inlet.

Flavour chemists were cautious at first because they feared that the adsorption selectivity of the fibre might exclude certain chemical classes of compounds from the mixture of volatiles and, if the chief objective was the total collection of headspace gases to study the odour quality of the sample, this device might be a problem. Not that other devices used in flavour chemistry were totally free from problems, but through experience their limitations were better understood. If qualitative measurement was sufficient then, providing the adsorption properties of the fibre were known and understood, SPME-GC-MS was fast and solventless. However, if the extraction can be calibrated with known compounds or carried out under reproducible conditions then some degree of reliable quantification is possible, using the usual internal standardisation or standard addition methods.

The special requirements for aroma analysis are discussed and SPME compared with the classical methods.

Practical Aspects

SPME Probe Construction and Desorption Chambers

Figure 6.11 shows the Supelco holder. The fibre is protected inside the steel capillary tube for insertion through the GC injection septum and for safe storage. It is exposed during sample adsorption and during thermal desorption in the heated GC inlet zone.

The SPME microprobe, with the fibre withdrawn into the barrel, handles like a GC injection syringe with the "needle" being inserted through the septum to a pre-determined depth into the heated injection port liner and then the fibre extended from the sheath into the centre of the heated zone for efficient desorption onto the separation column (Figure 6.12).

For injection onto a HPLC column a special desorption chamber has been devised (Figure 6.13). The SPME probe containing the liquid sample is inserted into the desorption chamber linked into the injection loop arm of a FSV. Solvent washing and mobile phase desorption can be arranged by flow switching. Desorbed sample from the fibre is transferred to the HPLC column for separation.

Background Literature Review

GC-MS has been singularly successful in supporting the development of instrument-based methods for the analysis of mixtures of VOCs for flavour

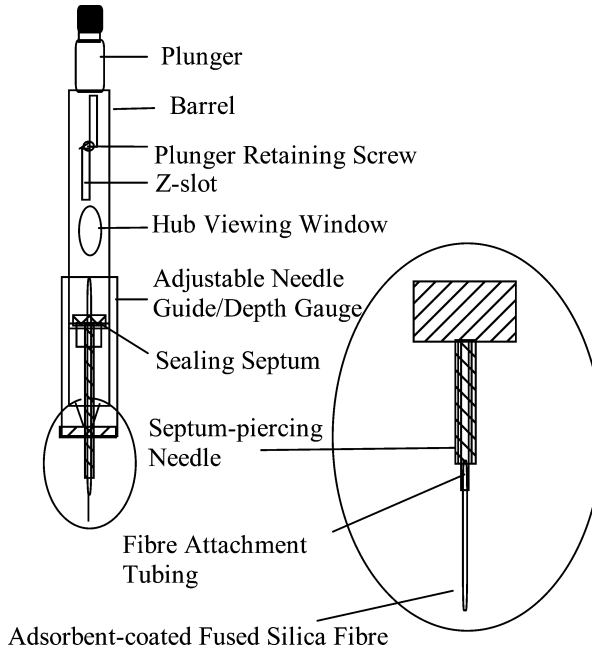

Figure 6.11 *Schematic diagram of the commercial SPME device. The plunger operated, adsorbent-coated, fused silica fibre can be withdrawn into the stainless steel septum-piercing needle for protection during the injection step, and exposed for desorption in the heated injection zone of the GC inlet*

research.[93,94] In addition, it has been used for the extraction of volatiles from model systems simulating aroma release in the mouth during eating.[95] Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was modified to handle gas phase samples, and was applied to the study of the breath-by-breath dynamics of the aroma volatiles released from food in the mouth,[96,97] but the evaluation of the total mouth environment, *i.e.* including those volatile substances not readily released from the liquid phase (saliva) into the headspace (nose space) for immediate detection at the olfactory epithelium, has not been properly studied. This is another multi-phase system needing a partition distribution approach.

Various sample preparation and sample collection techniques have been described in earlier chapters, some of which have been applied to the preparation of flavour extracts for analysis by GC-MS, including cryogenic and solid surface adsorption (*e.g.* solid phase Tenax trapping) followed by thermal desorption, steam and vacuum distillation, solvent extraction and concentration and SPE. A distinction is made in flavour research between VOC sampling methods, which emulate the process of "sniffing" or smelling odours of foods, and those that align more closely with the overall flavour in the

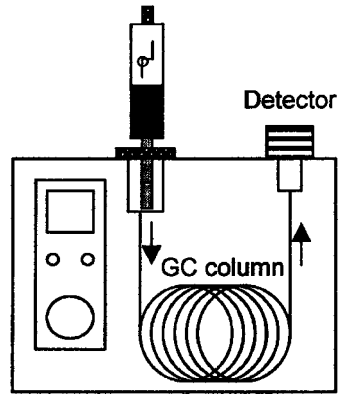

Figure 6.12 *Thermal desorption of volatiles from the coated fibre inserted into the heated injection zone connected to the GC*
(Reprinted from the *Journal of Chromatography A*, vol. 880, H. Kataoka, H.L. Lord and J. Pawliszyn, "Applications of Solid-phase Microextraction in Food Analysis", pp. 35–62, © 2000, with permission from Elsevier)

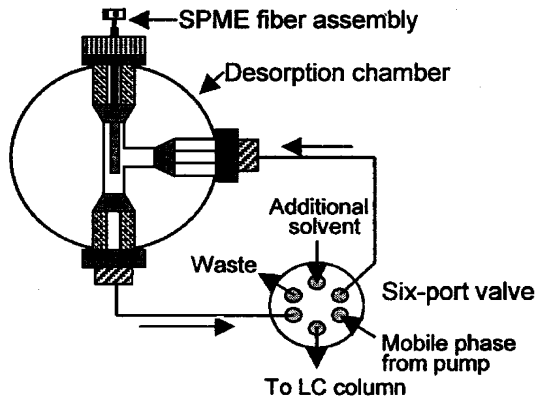

Figure 6.13 *Desorption chamber for liquid sample injection onto HPLC column*
(Modified from the *Journal of Chromatography A*, vol. 880, H. Kataoka, H.L. Lord and J. Pawliszyn, "Applications of Solid-phase Microextraction in Food Analysis", pp. 35–62, © 2000, with permission from Elsevier)

mouth. Among conventional GC extraction methods, the static headspace collection appears to be the least costly and simplest. It is also preferred for odour evaluation, while dynamic methods mimic better the processes occurring in the mouth.[98] Extraction techniques involving headspace concentration, using porous absorbents, have been widely used for the analysis of aroma compounds.[99] However, the measurement of total volatile release from a food required more than one technique and there were significant differences in

the physical and chemical phenomena involved, raising doubts about the comparative validity of the data.

All these methods suffer from drawbacks of being laborious and time consuming, but the main disadvantage of multi-step procedures was related to the fear that changes to the composition of the original flavour, *e.g.* artefact formation, might occur during distillation, irreversible adsorption or conversion during solid-phase trapping, and chemical reaction during solvent extraction. An ideal sample extraction technique should be solvent-free, simple, inexpensive, efficient and compatible with a wide range of separation methods and applications. Except for static headspace, existing methods were difficult to automate and dynamic headspace analyses, often being multi-stage, are susceptible to error.[100]

SPME addressed most of the disadvantages of the various methods mentioned earlier. It utilised a small fused-silica fibre, usually coated with a suitable polymeric stationary phase for analyte extraction from a matrix. Sampling could be carried out directly from liquids, from their headspace, or from headspace over solid samples.[101] The technique was academic until 1995 when commercial companies, *e.g.* Supelco Inc. (Bellefonte, Pa), created a convenient holder to allow the microprobe to be easily handled. Since then there has been an exponential growth in the number of papers published describing its development and applications.

Theoretical Aspects

The principle behind SPME is the partitioning of analyte between the sample matrix and the extraction medium. If a liquid polymeric coating is used, the amount of analyte absorbed by the coating at equilibrium is directly related to its concentration in the sample, as shown in Equation (6.2),

$$n = \frac{K_{fs} V_f C_o V_s}{K_{fs} V_f + V_s} \tag{6.2}$$

where n is the mass of an analyte absorbed by the coating, V_f and V_s are the volumes of the coating and the sample, respectively, K_{fs} is the partition constant of the analyte between the coating and the sample matrix, and C_o is the initial concentration of the analyte in the sample. The equation indicates the linear relationship between the amount of analytes absorbed by the fibre coating and the initial concentration of these in a sample. Because the coatings used in SPME have strong affinities for organic compounds, K_{fs} values for targeted analytes are quite large, which means that SPME has a high concentration effect and leads to a good sensitivity. In many cases, however, K_{fs} values are not large enough to exhaustively extract most analytes in the matrix. Instead, SPME, like static headspace analysis, is an equilibrium sampling method and, through proper calibration, can be used to accurately determine the concentration of target analytes in a sample matrix. As Equation 6.2 indicates, if V_s is very large ($V_s >> K_{fs} V_f$), the amount of analyte extracted by the fibre coating is not related to the sample volume

$$n = K_{fs} V_f C_o \qquad (6.3)$$

The speed of extraction is controlled by mass transport of the analytes from the sample matrix. For volatile compounds, the release of analytes into the headspace is relatively easy because analytes tend to vaporise once they are dissociated from their matrix. For semi-volatile compounds, the low volatility and relatively large molecular size may slow the mass transfer from the matrix to the headspace and, in some cases, the kinetically controlled desorption or swelling process can also limit the speed of extraction, resulting in long extraction times.

When the matrix adsorbs analytes more strongly than the extracting medium does, the analytes partition poorly into the extraction phase. Because of the limited amount of the extraction phase in SPME (compared to SPE), the extraction will have a thermodynamic limitation. In other words, the partition constant (K_{fs}) is too small, resulting in poor sensitivity. If the coating has a stronger ability to adsorb analytes than the matrix does, it is only a matter of time for a substantial amount of analyte to be extracted by the fibre coating, and only kinetics play an important role during extraction.

In headspace SPME (HS-SPME), three phases (coating, headspace, and matrix) are involved, and the chemical potential difference of analytes among the three phases is the driving force that moves analytes from their matrix to the fibre coating. For aqueous samples, the headspace/water partition constant (K_{hs}) is directly related to the analytes' Henry's Law constants, which are determined by their volatility and hydrophobicity. Although SPME is mainly an equilibrium extraction technique, it can perform exhaustive extraction. If the coating/matrix partition constant, K_{fs}, is very large ($K_{fs}V_f >> V_s$), the amount of analyte absorbed by the coating is given by Equation (6.4),

$$n = C_o V_s \qquad (6.4)$$

and exhaustive extraction is achieved.

Development

Origins and Objectives

The technique is actually an extension of laser desorption as a sample introduction scheme for GC analysis.[102] The concept of SPME was derived from SPE, *i.e.* analyte is absorbed onto a modified solid support from the sample and then is desorbed either by thermal means or by using a solvent. The method completely removes the use of solvents from the sample extraction, and the thermal desorption required is at a significantly lower input (temperature + time) than the desorption temperature used with some purge and trap devices.

Automation

Automation and optimisation of SPME has been further investigated by Arthur and co-workers,[103] by addressing factors affecting sample throughput, precision,

and accuracy of the method. They found that automated SPME allows the analysis of volatile compounds, semi-volatile compounds, and headspace samples on one instrument and is independent of the sample volume, above a minimum volume, which depends on the distribution constant.

Increasing the Sensitivity

Salting-out Effect. Practical aspects of the technique are being continually updated. The sensitivity of the method is significantly increased by addition of salt into the extraction media.[104–107] This is because the partition constants of analytes are partially determined by the interaction between target analytes and the matrix. By adding salt (*e.g.* NaCl or Na_2SO_4) to aqueous samples, the ionic strength of water can be increased, thereby increasing the partitioning of polar organic compounds into the polymer coating. Along with this, because the neutral forms of analytes are more efficiently extracted by the non-ionic polymeric coating, the pH of the aqueous sample must be adjusted to prevent ionisation of the analytes.

Acidification. For polar compounds, acidifying the sample increased the sensitivity of the SPME.

Agitation. Efficient methods of agitation shortened extraction times, especially for aqueous samples,[108–110] where it speeded up the mass transfer rate.

Heating. The release of analytes into the headspace from solid samples for SPME sampling was facilitated by heating.[111–113] Although increasing the sample temperature shortened the equilibrium time (and thus the extraction time) it also lowered the fibre–sample partition constant values, consequently lowering the sensitivity of the technique.[114] An explanation for this is that the adsorption is exothermic. Zhang and Pawliszyn (1993)[101] have suggested a practical way to overcome this problem by heating the sample matrix while simultaneously cooling the fibre coating. This creates a temperature gap between the cold fibre and the hot headspace that significantly increases the partition constants of analytes.

Special Attention to Low-boiling Volatiles. For the collection of low-boiling volatiles, which is an important consideration for flavour chemistry, Zhang and Pawliszyn[115] introduced a gas-tight syringe for SPME. The new sampling device showed an improved sensitivity.

Special Attention to High-boiling Volatiles. For the analysis of high-boiling analytes in complex aqueous or other liquid matrices, Zhang *et al.*[116] have developed a new approach where an SPME device was placed inside a cellulose

hollow membrane with a molecular weight cut-off of 18 kDa. The membrane, forming a concentric sheath around the fibre, allows target analytes, which typically have a MW of less than 1 kDa, to diffuse through while excluding high MW compounds up to several million Da. With the membrane protection, direct SPME was used successfully for extractions of large polyaromatic hydrocarbons (PAHs).

The membrane protection slows down mass transfer during direct SPME sampling, which can be improved by using higher temperatures or thinner membranes. Daimon and Pawliszyn[117] have demonstrated that coupling high temperature water extraction to the SPME method can be successfully applied to the determination of non-polar semi-volatile compounds in solid matrices.

Optimisation of the GC Injection. Modification of the GC injector improves the overall performance of SPME, *e.g.* the separation time of benzene, toluene, ethylbenzene and xylene (BTEX) components took only 8.4 s using a modified GC injector.[118] Optimising the split/splitless injection port parameters has also improved the performance of SPME.[119]

Choice of Adsorbent Coating. Using an appropriate SPME absorbent for specific analytes should increase the sensitivity of the technique to the chosen compounds. The performance of different SPME fibre coatings has been studied and reported.[120–122] In addition, Djozan and Assadi[123] have introduced porous layer activated charcoal as a new SPME coating, particularly for the analysis of BTEX components.

Log $K_{\text{fibre/gas}}$, Retention Indices and Quantification

When SRMs are Unavailable. Work by Schafer *et al.*[124] on the applicability of the index system to HS-SPME shows that the fibre–gas partition constants ($K_{\text{fibre/gas}}$) for n-alkanes (which were used for reference compounds) were related to their Kováts' retention indices. The validity of the derived linear relationship log $K_{\text{fibre/gas}}$ *versus* retention index is demonstrated for various examples. This relationship is helpful for the assessment of partition constants of substances when SRMs are not available, and also for the choice of suitable fibre coatings.

Regression Analysis and Quantification. Furthermore, quantification of analytes in the gas phase can be done without authentic substances. Bartelt[125] made a valuable contribution by calibrating a commercial SPME probe for measuring headspace concentrations of a wide range of volatile substances from several different chemical classes. He developed a regression model that allowed the quantification of the compounds belonging to the functional classes studied, for which Kováts' indices are known on a non-polar column and which reach equilibrium within the chosen sampling time.

Physical Properties of the Adsorption Layer

The knowledge of the theory of adsorption and other physical parameters relating to the coating used in SPME has been substantially extended by Martos and Pawliszyn[126] who addressed important issues such as the equilibration time, the effect of changes in fibre dimensions, detection limits, and, particularly, the measurement of fibre air partition constants of hydrocarbons (gas phase analysis).

The theory of solid-phase adsorption was extended in a study of coal wastewater when fibre/water partition constants were used to measure and compare concentrations of phenols and PAHs obtained by SPME and LLE methods.[127] Additionally, Dewulf *et al.*[128] have studied the sorption equilibrium and kinetics of eleven chlorinated C-1, and C-2 hydrocarbons and monocyclic aromatic hydrocarbons on PDMS coating. A linear regression between the logarithm of the partition constants, corrected by Henry's Law constant, and the Kováts' indices on a 100% PDMS GC column was demonstrated. In particular, the study of non-equilibrium situations has revealed that there is a linear relationship between the amount of analyte absorbed and the initial matrix concentration, if sampling conditions remain constant, regardless of whether or not equilibrium time is reached (Ai, 1997).[129]

Applications for GC and GC-MS Analysis

Fruit and Vegetables

SPME is an excellent sampling tool for VOCs from foods and food products (Wang *et al.*[113]). The characterisation of volatiles released from strawberries,[130,131] apples,[132] (see also Verhoven *et al.*[131]) vodkas,[133] wines, (see also Garcia *et al.*[109]) and for both headspace and liquid sampling of 25 common flavour volatiles from ground coffee, fruit juice beverage and a butter flavour in vegetable oil (see also Yang and Peppard, 1994[105]) have been reported. A mixture of 17 standard VOCs commonly found in orange juice flavour was used to test the performance of PDMS and PA coated fibres, and study the effect of salt addition (Steffen, 1996).

Cheese Flavour

PA fibres were preferred to PDMS for cheese flavour studies, primarily because known minor components such as volatile sulphur compounds were not detected.[134]

Mycotoxins

SPME, HS-SE and S-BSE (Section 4) were developed to examine the volatile metabolites from fungal growths.[135] A whole range of sesquiterpenes were extracted and many identified by GC-MS.

Pesticides

Fresh strawberries were crushed and centrifuged and 4 ml of the supernatant agitated while sampling 16 pesticides with a 100 μ PDMS fibre for 45 min at ambient temperature.[136] Carbofuran, diethofencarb, penconazole, hexaconazole, metalaxyl, folpet, bromopropylate, dichlofluanid, alpha-endosulfan, beta-endosulfan, parathion ethyl, procymidone, iprodione, vinclozolin, myclobutanil and chlorothalonil were analysed by SIM-GC-MS and the method was faster and more cost effective than solvent extraction.

Eight organophosphorus pesticides were extracted directly from samples of wine and fruit juices by SPME for GC-MS.[137]

Tobacco Products

Four different commercially available probes (Supelco Inc.) were tested for the qualitative and quantitative analysis of flavour additives in tobacco products.[138]

Wines and Spirits

Four different adsorbent-coated fibres [PDMS, PA, CDVB and DVB carboxen on poly(dimethylsiloxane)] were used in a study of Greek white wine.[139] Extraction time, extraction temperature, sampling mode (gas or liquid phase), and salt content were considered. PDMS was the best adsorbent for this application. The qualitative aroma composition of four Greek white wines – Boutari, Zitsa, Limnos and Filoni – was evaluated. 3-Alkyl-2-methoxypyrazines in wines were extracted by headspace SPME after the alcohol and other interferents had been removed by distillation at pH 0.5.[140]

Sandra and co-workers used both SPME and S-BSE to examine the volatiles extracted from malt whisky.[141] Again, PDMS, PA, CDVB and DVB-CAR-PDMS phases were used in the SPME work.

Natural Products

SPME has been applied to the characterisation of natural product compounds, including monoterpene hydrocarbons from conifer needles,[142] essential oils from cinnamon and cassia,[143] essential oils released by hops[144] and volatile components and their volatile decomposition products occurring in herbal medicines and herb extracts.[145]

Volatile/Substrate Interactions

SPME-GC-MS was used to study the effect of crushing and/or vacuuming spray-dried whey protein concentrate (WPC) on the adsorption of flavour volatiles and the effect that might have on the blandness of WPC.[146]

Interactions between flavour volatile compounds (hexanal, octanal, methional, 2-pentanone, 2-methylbutanal and 3-methylbutanal) and soluble dipeptides (carnosine and anserine) and protein (myoglobin) were monitored by optimised (fibre coating, sampling time, and linearity of detection) SPME.[147] The pH of the system was varied. Carnosine showed the highest affinity for all the volatiles except 2-pentanone, and its interaction with hexanol and methional were pH dependent.

Gas/Liquid Partition Constants from SPME-GC-MS Data

If the volatiles in a dynamic reflux distillation are sampled using SPME (a) in the closed space above the condensation line, the normal headspace (gas phase) sample, and (b) in the condensed vapour (liquid phase) under the condensation line, then the proportions of volatile substances are related to the gas/liquid (air/water) partition under those conditions. This process has been called gas/liquid partition analysis.[148]

Gas/Liquid Partition Analysis (GLPA) of Black Tea

If a sample of dry black tea is covered with water in a flask fitted with a reflux condenser and brought to the boil and simmered (refluxed) for 10 min and the headspace (gas phase) at the top of the reflux condenser, and condensed phase (liquid tea), sampled by the same SPME probe, dramatically different TIC chromatograms are recorded (Figure 6.14). The relative concentrations in the gas and liquid phases of each compound will be in proportion to their partition constants ($K_{a/w}$). Conversely, two measurements of area under the curve using GLPA provides a value for $K_{a/w}$, in just two GC runs, for every compound that can be identified. The conditions of the experiment must be carefully defined and the threshold value for the stationary phase will represent the "zero" concentration level, assigned a $K_{a/w}$ of 1 or 0. The GLPA of tea illustrates the general case of low-boiling, less polar volatiles in the gas phase and higher boiling, more polar and semi-volatile compounds in the liquid phase.

GLPA of Boiled Potatoes

Figure 6.15 shows a section of the gas/liquid partition extraction of volatiles from potatoes. In this case, rather than the clear separation of volatile from semi-volatile substances, the reflux distillation extracts certain chemical classes into the two phases. At the end of the process, the unsaturated aldehydes appear in the gas phase while the substituted pyranone is in the liquid phase. The saturated aldehydes cannot be separated by GLPA.

GLPA of Cooked Star Anise Fruit

Figure 6.16 shows the section of the TIC where the sesquiterpene hydrocarbons are eluting in the gas phase at retention times of 12.81, 13.29, 13.42, and

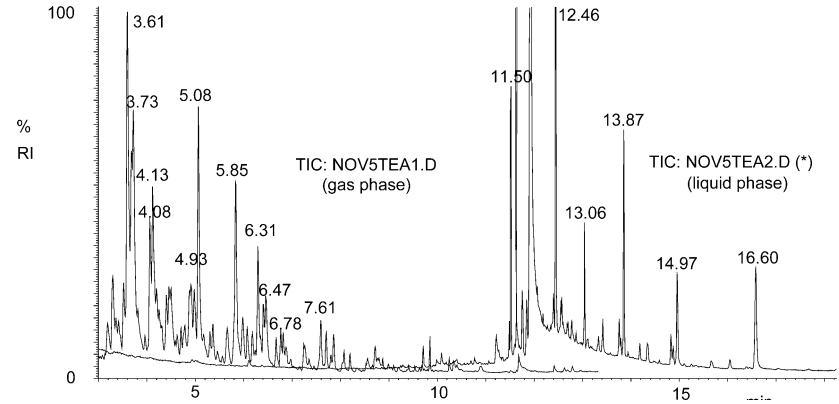

Figure 6.14 *Gas/liquid phase SPME of volatile ("smelled" headspace aroma volatiles) and condensable ("tasted" retro-nasally when the tea is drunk) components of boiling black tea. The striking difference in the composition of the non-condensable headspace vapour in the gas phase on the left and the condensed liquid phase on the right presents in one diagram the complete pattern of volatilisable material. The major peak at 11.95 min is caffeine, a water-soluble, semi-volatile compound (mp 235 ºC). Note: no caffeine is present in the gas phase chromatogram of components extracted by SPME. The air/water partitioning is driven by a combination of volatility and solubility. None of the very volatile components has a water solubility that would enable them to partition between the two phases. Some less volatile compounds partition between the two phases, but the semi-volatile (high boiling point volatiles) appear in the condensed phase only. (For identification of the numbered peaks consult Surugau 1998)*

13.55 min. They are not partitioned between the phases. Alternatively, the major component in this section of the TIC trace is 1-(4-methoxyphenyl)-2-propanone, RT = 12.87, which is largely present in the liquid phase, but a small percentage partitions into the gas phase. The other liquid phase peaks are aromatic ketones, esters, acetates and alcohols.[149] Thus, the aromatic hydrocarbons have been extracted into the gas phase while the more polar aromatic compounds have partitioned into the liquid phase.

Measurement of Oil/Water Partition Constants

In flavour research, the oil/water partition constant ($K_{oil/water}$) of a compound is taken as a measure of its lipophilicity, and more effective methods for the measurement of ($K_{oil/water}$) are being sought. In a definitive article, Pollien and Roberts (1999, Chapter 3, ref. 21) established the usefulness of SPME for the measurement of oil/water partition constants, due to its capability of measuring the concentration of the solute in both partitioning phases, thus providing a degree of certainty not found with previous single-phase measurements. This concept was paramount in the planning of the work at UEA (*e.g.* Surugau, 1998[149]) in using GLPA of food volatiles to measure their extraction during the cooking process.

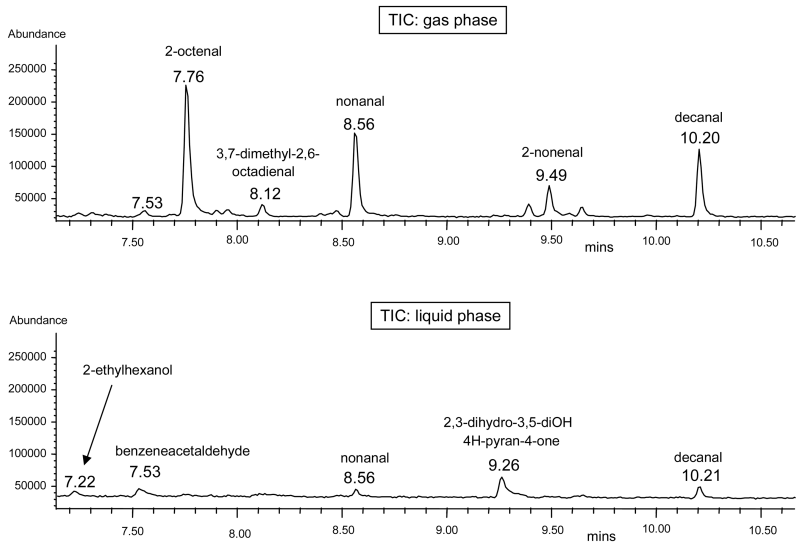

Figure 6.15 *A section of the GLPA of potato flavour showing the selective extraction of volatile compounds into the gas and liquid phases around potatoes being cooked by boiling. The TICs show the composition of the headspace (top) and the liquid in which the potatoes were boiled (bottom). Saturated aldehydes are present in both phases in the proportions of their gas/liquid partition constants under the prevailing conditions. Mono-unsaturated aldehydes (-enals) and 3,7-dimethyl-2,6-octadienal are only present in the gas phase while 2,3-dihydro-3,5-dihydroxy-*4H*-pyran-4-one is only present in the liquid phase*
(Reprinted with the permission of The Royal Society of Chemistry from *Mass Spectrometry of Natural Substances in Food* by F.A. Mellon, R. Self and J.R. Startin, © 2000)

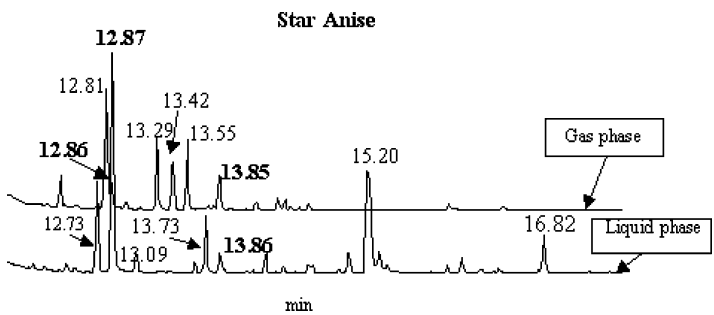

Figure 6.16 *Portions of the SPME-GC-MS TIC traces showing partitioned components at retention times: 12.86(7) and 13.85(6) min, while other components are extracted into either the gas or liquid phases*

Partition Constants Correlated with Kováts' Index

Louch *et al.*[90] developed two models to describe sorption kinetics, (a) under unstirred, Fick's law diffusion, in both the aqueous and organic phases, fitting

the observed profile and (b) for practical reasons an agitated model based on a perfectly mixed water phase and Fickian diffusion only in the organic phase. This model did not fit the experimental equilibration times, so it was assumed that the water phase was not fully mixed. Dewulf *et al.* (1997)[128] continued the work by using an SPME fibre as the organic phase to sorb out of water 11 chlorinated C_1 and C_2 hydrocarbons and monocyclic aromatic hydrocarbons. Partition constants ($K_{org/water}$) at equilibrium were measured and related to Kováts' indices (I_k). The partition constant was calculated from Equation (6.5),

$$K_{org/water} = \frac{RA}{VC_w} \tag{6.5}$$

where R is the GC response factor (ng per area unit), A is the GC area count, C_w is the solute concentration in the aqueous phase (ng ml^{-1}) and V is the volume of the SPME organic phase. Jennings[150] established that in isothermal GC ln $K_{S/M}$ bears a linear relationship to I_k. If the SPME K values are divided by H (Henry's law constant), the parameter KH becomes similar to $K_{S/M}$, therefore (Figure 6.17),

$$\ln (K/H) \sim I_k \tag{6.6}$$

Thus, partition constants in SPME can be estimated from the Kováts indices.

The importance of these physicochemical studies of the sorption equilibrium is noted (Case Study 1).

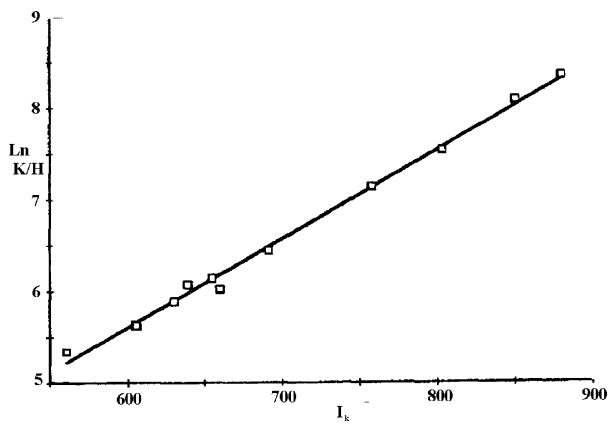

Figure 6.17 *ln* K/H versus I_k
(Reprinted from the *Journal of Chromatography A*, vol. 761, J. Dewulf, H. Van Langenhove and M. Everaert, "Solid-phase Microextraction of Volatile Organic Compounds. Estimation of the Sorption Equilibrium from the Kováts Index, Effect of Salinity and Humic Acids and the Study of the Kinetics by the Development of an 'Agitated/static Layer' Model", pp. 205–217, © 1997, with permission from Elsevier)

Case Study 1. Selective Extraction

Extraction of Volatile and Semi-volatile Substances from Broccoli

Introduction. The analysis of food volatiles has been a challenge to researchers for many years and particularly since the development of capillary column gas chromatography. The main interest is in the identification of flavour impact substances thought to be directly responsible for food flavour. From earliest times, certain vegetables and fruits have been used as both medicines and foods, and today some of them are thought to play a protective role in the etiology of various diseases, such as cancer. One group of vegetables already used for medicinal purposes in ancient times and now seen as having cancer-protective properties are vegetables of the family Cruciferae – of which the brassicas were the bane of childhood meals, "eat up your greens". The protective effects of cruciferous vegetables against cancer have been suggested to be partly due to their relatively high content of glucosinolates. However, glucosinolates are partly responsible for the bitter taste and have been "bred out" of modern varieties of rape, for example. Conversely, the bitter principle protects the plant against insect attack. Thus, so the ecological argument is complex.

Enzyme Hydrolysis. Recent studies have localised the therapeutic effects to some of the hydrolysis products of glucosinolates, namely indoles and isothiocyanates. The enzyme myrosinase, for example, found in separate plant cells, catalyses the hydrolysis of glucosinolates. When the plant cells are damaged (*e.g.* by cutting or chewing) the myrosinase and glucosinolates mix, and hydrolysis occurs. The cooking process is actively involved in the fate of the enzyme and the degradation of the cellular structure, and therefore a study was made of the optimisation of the extraction of indoles and isothiocyanates from a range of common brassica crops.

SPME. Volatile and semi-volatile glucosinolate breakdown products formed during the preparation and cooking of *Brassica* and other glucosinolate-containing vegetables were extracted by SPME. These volatile and semi-volatile substances are distributed between the gas an liquid phases and therefore an attempt at total volatile analysis (TVA) is only possible if so-called gas/liquid partition analysis (GLPA) is employed. For the first time, both condensed and non-condensed volatiles produced during food preparation can be measured by one sampling technique (Pollien and Roberts, 1999, Chapter 3, ref. 21), ensuring that the analyses were made of the total cooking process.

Earlier works have provided considerable information on the breakdown of glucosinolates but, despite advances in instrumentation, the extraction procedures employed were lengthy. It was necessary to re-examine the glucosinolate breakdown products, explore the metabolic pathways, and validate SPME as the extraction method.

During the development of GLPA, difficulty was experienced with the reproducibility of the partition distributions (Chapter 3). Nevertheless, the values obtained have been reported in the hope that future studies will help to explain the discrepancies. Furthermore, at this stage in the method development, SRMs were difficult to obtain and therefore no calibration curves were prepared.

Prior to the main experiment, preliminary experiments were conducted (one of which is reported in Case Study 1) to establish the general applicability of SPME to gas and liquid phase sampling in a qualitative survey. To authenticate the claim made for the qualitative integrity of SPME, the first preliminary experiment was to analyse the volatiles extracted from a number of foods, beverages, medicinal plants, and herbs and spices. Both non-condensable (gas phase) and condensable (liquid phase) volatiles were analysed by SPME-GC-MS and the data presented in a combined form intended to represent the TVA for the SPME method employed. The literature was equivocal about the quantification accuracy of SPME and, therefore, the second experiment was to prepare a calibration line using the SPME method for the measurement of the amount of a condensed phase volatile, caffeine, in B.O.P. black tea.[149]

The potential for SPME is the development of probes that will absorb specific compounds or chemical classes. The present day commercially available probes are designed around the chromatographic concepts of selective retention and surfaces coated with bonded stationary phases provide levels of polarity. Three adsorbents were used routinely in the analytical chemistry teaching laboratory and the experimental data acquired over a period of time can be interpreted retrospectively in terms of their performance in relation to the chromatographic data obtained on different samples.

Choice of Adsorbent

Choosing a suitable adsorbent for particular compounds is one way to improve the specific sensitivity of the technique. Figure 6.18 shows the relative performance of three types of SPME fibres.

Carbowax divinylbenzene (CDVB) was designed to be suitable for the extraction of volatile polar compounds, whereas 100 μm polydimethylsiloxane (PDMS) is a non-bonded, general-purpose phase, and PA is for polar semi-volatiles. The results show that CDVB was the best adsorbent for the compounds of interest since most of the glucosinolate breakdown products were fairly polar, due to the presence of heteroatoms such as nitrogen, sulphur and oxygen.

For GLPA, the adsorbent chosen must also be efficient in the extraction of volatiles from the liquid phase too. Two of the three adsorbents were tested for the extraction of volatiles and semi-volatiles from the cooled liquor used to boil broccoli (Figure 6.19). The chromatograms show the major peak in both extracts at 11.91–11.94, which was also a major peak in all three extracts from the headspace (Figure 6.18), but now the semi-volatile later-running components are present.

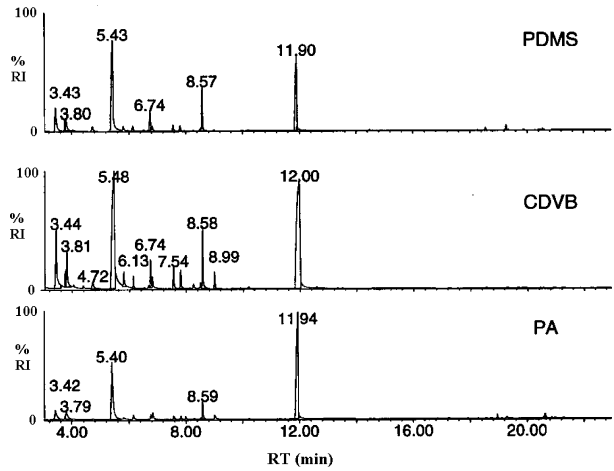

Figure 6.18 *Relative performance of three types of SPME adsorbent in sampling VOCs (headspace) from broccoli* (B. villosa)
(Reprinted from MSc Thesis, L.N. Surugau, "The Analysis of Gas and Liquid Phase Volatiles from Foods using Solid-phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC-MS), 1998, with permission from L.N. Surugau and the University of East Anglia)

Another problem associated with solid phase adsorption is illustrated by comparison of the two TIC chromatograms towards the end of the temperature program range. The peak at 19.27 min from CDVB is the 16:0 fatty acid, palmitic acid. Peaks at 18.95 and 20.64 min from PA are the 16:0 and 18:1 fatty acid methyl esters (FAMEs), raising the question whether PA has preferentially adsorbed the esters rather than the free fatty acids, or whether the PA surface has degraded the fatty acids to their methyl esters in this particular environment (or possibly all environments?). To continue with this investigation and to pursue others, the regions of these TIC chromatograms after a RT of 12 min have been enlarged in Figure 6.20.

The overview of the comparative performances of the two surfaces is that CDVB has absorbed more of a wider range of substances than PA. Continuing with the fatty acid story, the cluster of peaks on PA at 20.59, 20.64 and 20.86 min represent the 18:2, 18:1 and 18:0 FAMEs, not recorded in the CDVB extract, based on MS evidence derived from the TIC. The peak at 20.59 on CDVB was a mixture of a long-chain hydrocarbon and a known impurity from the stationary phase.

The "close up" view of a narrow region of the two chromatograms of the later-eluting volatiles in Figure 6.20 is shown in Figure 6.21. The major peak at 13.50 min in both extracts is 4-methylthiobutyl isothiocyanate and the peak at 13.91 min, from both extracts, is the same alkene, the remaining peaks are all different. The difference serves to illustrate the value of choosing the adsorbent carefully to provide the selective extraction required.

Having considered the polarity of the two adsorbents, it is interesting to compare their performance in this small region of the chromatogram. The

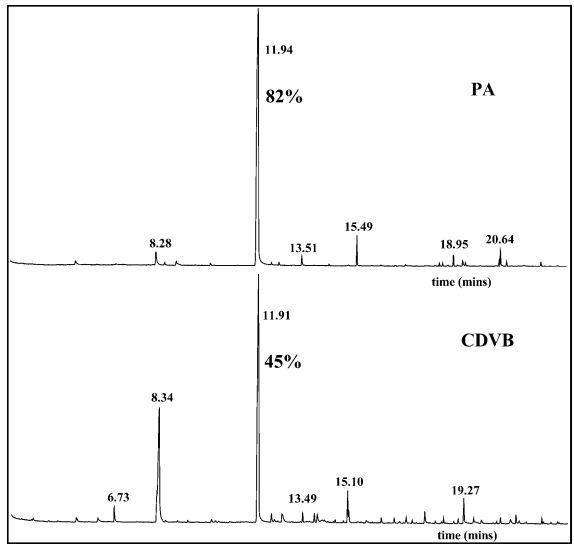

Figure 6.19 *Relative performance of two SPME adsorbents extracting volatile and semi-volatile compounds from identical samples of the liquor of boiled broccoli (*B. villosa*). Top TIC chromatogram, polyacrylate (PA) adsorbent; bottom TIC chromatogram, Carbowax/divinylbenzene (CDVB). The largest peak (11.91–11.94 min) in both chromatograms is 3-methylthiopropyl isothiocyanate, a known degradation product of the glucosinolate glucoiberverin. It makes up 82% [221 million (M) arbitrary counts] of the total extract (270 M counts) adsorbed by the PA surface, but only 45% (148 M counts) of 327 M counts on the CDVB material. Conversely, the peak at 8.28–8.34 min is the equivalent nitrile, extracted efficiently by CDVB, making up 24% (79 M counts) of the total extract, but the amount collected by the PA adsorbent constituted only 3% (8.9 M counts) of the total extract. Another isothiocyanate, 4-methylthiobutyl isothiocyanate at 13.49–13.51 min was extracted equally by both adsorbents, but in trace amounts only. The other major peaks illustrate the useful variability of the two materials in their adsorptive properties. The peak at 15.10 min (CDVB) is an unknown compound with a molecular weight of 180, while the peak at 15.49 min on PA is an unknown compound with a molecular weight of 286, both constituting about 3% of the total material adsorbed*

main component extracted by CDVB and noticeably absent from the PA extract is the peak at 12.75 min. This peak is 3-methylsulphinylpropyl nitrile, a member of the second most polar chemical class shown below in Figure 6.22. The CDVB adsorbent is described as suitable for polar analytes compared to PA, which is described as suitable for polar semi-volatiles. This classification would appear to be correct since, at 12.75 min retention, this peak is likely to be as volatile as the nearby nicotine (RT = 12.37 min) and more volatile than the C_{12} fatty acid eluting as the peak at 14.95 min. The peak shape of the nitrile is poor, in keeping with the presence of the sulphinyl group, which may mean that thermal desorption was inefficient or that the partition constant is non-linear. The peak at 12.37 min on CDVB is nicotine [3-(1-methyl-2-pyrrolidinyl)-pyridine], M = 162, and is not absorbed by PA. The smaller

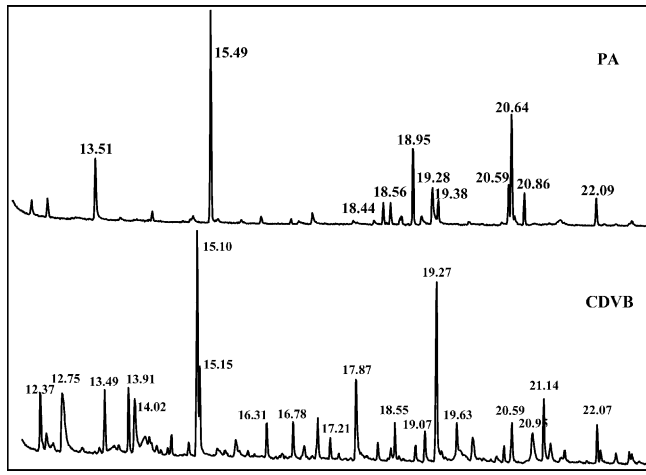

Figure 6.20 *Relative performance of two different SPME adsorbents, sampling from the liquid phase, and recording the later-eluting minor components. Top chromatogram: PA; bottom chromatogram: CDVB of the "high end" volatile and semi-volatile components extracted from simultaneous gas/liquid samples of an aqueous extract of broccoli* (B. Villosa). *A 1 g sample broccoli refluxed in 10 ml water for about 5 min. The heating was stopped and the liquor cooled and sampled by the SPME probe for 2 min, the fibre washed in HPLC-grade water and the adsorbed material thermally desorbed onto the GC-MS column for analysis.*

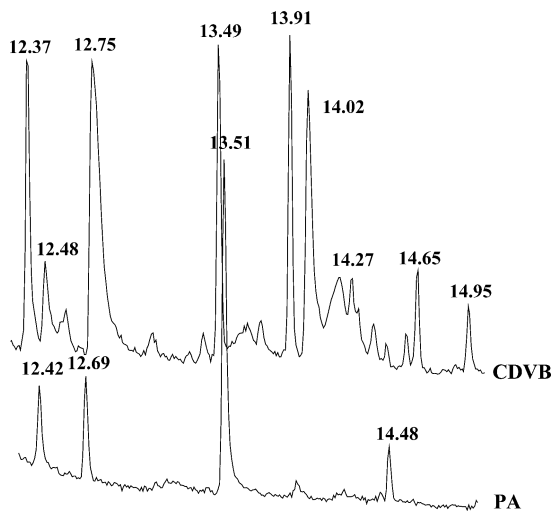

Figure 6.21 *Comparing the detail of the same small region of successive TIC chromatograms of broccoli volatiles collected from the cooled cooking liquor by SPME probes with CDVB (top) and PA (bottom) adsorbent-coated fibres*

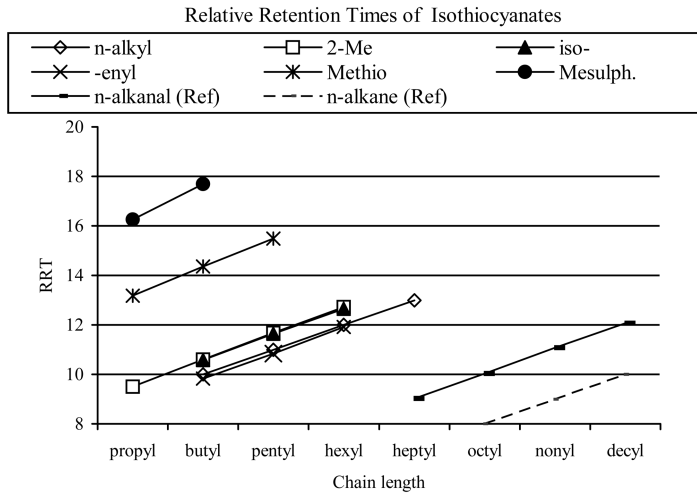

Figure 6.22 *RRTs (Kováts' Index) of isothiocyanates extracted from the aqueous phase of the cooking of broccoli (*B. villosa*). Legend: n-, 2-Me- and iso- represent the straight chain and two branched alkyl groups respectively. The abbreviation enyl: terminal mono-unsaturated alkenyl isothiocyanates, e.g. pent-4-enyl ITC. Methio; terminal methylthio group, e.g. 3-methylthiopropyl ITC, and Mesulph; terminal methylsulphinyl ITC, e.g. 4-methylsulphinylpentyl ITC. Note: For reference purposes the dotted line in the bottom right-hand corner represents the baseline* n-*alkanes, and the first line (horizontal bars) moving to the top left-hand corner represents the* n-*aldehydes that have a polarity equivalent to approximately 2 Kováts' units*

peaks at 12.48 min on CDVB and 12.42 and 12.69 min on PA are all unknown structures. The reason for showing this excerpt is to point out the useful selective extraction of these popular adsorbents. With the knowledge available from this case study, the extraction of trace amounts of nicotine should not be attempted with PA adsorbent.

Having completed the preliminary studies to optimise the methodology, it would now be possible to pursue research objectives using the calibrated probes.

Case Study 2. Isothiocyanate and Nitrile Extraction Methods

Determination of Kováts' Indices by SPME-GC-MS

Chemical compound polarity relative to the apolar n-alkanes has been expressed as the Kováts' (or other) index and the close relationship with the partition constant has been reported (Dewulf *et al.*[128]). In work on the degradation products of glucosinolates in brassicas and other vegetables, the extraction of several classes of isothiocyanates and nitriles were found to be present mainly in the liquid (aqueous) phase and only minor amounts were in the gaseous phase. From the GC retention data obtained with the homologous

series of n-alkanes as internal standards, the relative retention times (RRTs) were calculated.

Figure 6.22 shows RRTs versus chain length for the classes of ITCs present in the range of vegetables being studied. Interpretation of the positions of the ITC functional group series' on this GC stationary phase shows homologous polarity increasing in a linear fashion (over this limited middle range) and the chemical classes are in the order alkene < n-alkane < iso-alkane < 2-methyl alkane < n-methylthio alkane < n-methylsulphinyl alkane ITCs in increasing RT/polarity, moving from bottom right to top left of the diagram.

On the practical scale, the addition of a double bond in the straight chain compounds only slightly lowers the retention (polarity) and the position of the branch in the branched chain compounds does not have any effect. Conversely, the addition of the polar methylthio or methylsulphinyl group significantly increases the retention (polarity) of these compounds. It is possible to assign carbon chain length equivalents to the functional groups. The n-alkanals, added for reference purposes, have an approximate carbon equivalent of 2. N-alkyl isothiocyanates have a carbon equivalent of 6 (*i.e.* not including the carbon in the ITC functional group). The presence of the CH_3S group increases the isothiocyanate's retention by the equivalent of another 4.4 carbon atoms and the CH_3SO group of another 7.7 carbons. In other words, the 3-carbon chain sulphinyl ITC elutes with the 16-carbon chain n-alkane, indicating the extremely high polarity of these groups. The best adsorbents would be CDVB (for polar analytes) or PA (for polar semi-volatiles).

The equivalent exercise was carried out on the nitriles produced in similar enzymic reactions as the isothiocyanates. The RRTs are plotted against chain length in Figure 6.23.

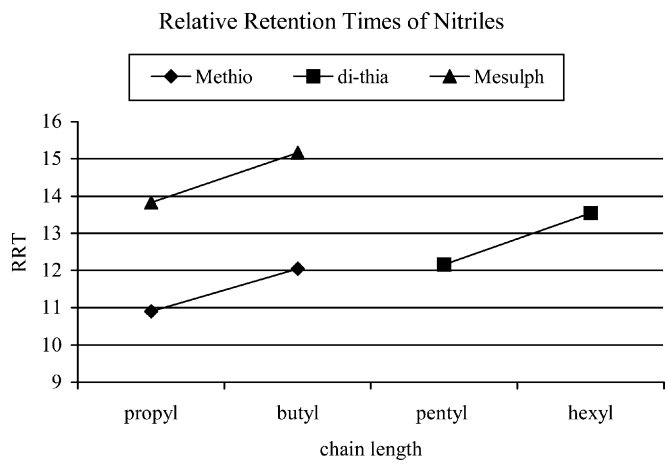

Figure 6.23 *Legend: di-thia = 2,4-dithiapentyl and 3,5-dithiahexyl nitriles. Methio = terminal methylthio group,* e.g. *3-methylthiopropyl ITC, and Mesulph = terminal methylsulphinyl ITC,* e.g. *4-methylsulphinylpentyl ITC. The smaller number of nitriles prevents an accurate homologous series from being plotted, but the two members of each group show the polarity regions equivalent to the ITCs in Figure 6.22*

Partition Constants from Binary Phase SPME (GLPA)

Finally, to bring together all the evidence for the selection of CDVB for work on the extraction of target isothiocyanates and nitriles from brassica and related vegetables, the adsorbent was used in the GLPA of the *B. villosa* sample for the determination of $K_{a/w}$ for all the identifiable compounds (Table 6.3).

Based on the information in Table 6.3, CDVB fibre extracted 21 compounds likely to be related to glucosinolate breakdown, whereas in other experiments PDMS and PA extracted 15 and 10 compounds, respectively.

Table 6.3 *CDVB adsorbent for GLPA of the VOCs from* B. villosa. *Column:* (1): *Compound (MW), abbreviated chemical name and (molecular weight);* (2) *RT, GC retention time in mins;* (3) *RRT, relative retention time (Kováts' Index);* (4) *HS:LQ for the area under the GC peaks, gas phase (HS) followed by liquid phase (LQ) in millions area units, and in brackets, the partition constant* ($K_{a/w}$). *For compounds detectable in only one phase, its peak area in the other phase was noted as zero. However, to calculate* $K_{a/w}$, *the 'zero' peak area was assumed to be at the threshold level,* i.e. *5000 area units. Large* $K_{a/w}$ *mean the particular compound was present largely in the gas phase, and* vice versa
(Modified from MSc Thesis, L.N. Surugau, "The Analysis of Gas and Liquid Phase Volatiles from Foods using Solid-phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC-MS), 1998, with permission from L.N. Surugau and the University of East Anglia)

(1) Compound (MW)	*(2) RT*	*(3) RRT*	*(4) HS:LQ*($K_{a/w}$)
Isopropyl ITC (101)	3.45	–	9.7:0(1940)
Prop-2-enyl ITC (99)	4.46	–	0.3:0(60)
1-Methylpropyl (115)	5.43	–	36:0.1(360)
2-Methylpropyl ITC (115)	5.83	–	2:0(400)
Dimethyltrisulphide (126)	6.15	9.70	1.2:0.3(4)
MMTSO (110)	6.51	–	0:0.4(0.02)
Butyl ITC (115)	6.67	9.96	0.3:0(60)
2-Methylbutyl ITC (129)	7.83	10.59	2:0(400)
3-Methylthiopropyl nitrile (115)	8.37	10.90	0.5:0.9(0.6)
Pentyl ITC (129)	8.52	10.97	0.5:0(100)
M(MT)Mdisulphide (140)	9.02	11.29	2:0(400)
4-Methylthiobutyl nitrile (129)	10.24	12.05	0:0.1(0.05)
Dimethyltetrasulphide (158)	10.49	12.22	0.03:0.04(0.8)
3-Methylthiopropyl ITC (147)	11.88	13.18	43:14(3)
2,3,4,6-Tetrathiapentane (172)	12.61	13.66	0:0.6(0.008)
Benzyl ITC (149)	12.64	13.73	0.2:0(40)
3-Methylsulphinylpropyl nitrile (131)	12.78	13.83	0.06:0.2(0.3)
4-Methylthiobutyl ITC (161)	13.51	14.36	0.07:0.5(0.1)
2-Phenethyl ITC (163)	13.94	14.66	0:0.4(0.01)
Indol-3-ylmethyl nitrile (156)	17.88	18.17	0:0.4(0.01)
4-Methoxyindol-3-ylmethyl nitrile (186)	19.92	20.12	0:0.2(0.02)

Comparison of SPME with Other Extraction Methods

MacGillivray *et al.*[151] have presented a comprehensive study on the comparison of HS-SPME and purge-and-trap methods for the determination of substituted benzene compounds in water. The technique compares favourably with the traditional method. The report by Nilsson *et al.*[114] on analysis of VOCs in drinking water was in agreement with this finding. Both the results of the analysis of volatiles in strawberries (Ulrich *et al.*[130]) and the determination of organophosphorus pesticides[152] obtained by using SPME were comparable with LLE. It is also comparable to the ELISA technique for the analysis of metolachlor in water samples.[153] Krumbein and Ulrich[154] found that HS-SPME gave comparable results to dynamic headspace trapping on Tenax TA in the analysis of tomato aroma.

The simplicity of this solventless sampling method, using, *e.g.*, PDMS absorbent on a retractable probe for direct insertion into a GC inlet, was recognised early but as with all solid-phase adsorption techniques there has been concern about its quantitative integrity. Pelusio *et al.*[155] have shown in a comparison of HS-SPME technique with traditional Tenax trapping for the analysis of volatile sulphur compounds in truffle aromas that the former was less suitable because the PDMS coating strongly discriminated against more polar and very volatile compounds (consult Case Study 1). Passive adsorption on SPME fibres was compared to dynamic adsorption onto Tenax GR and Chromosorb 103 in the analysis of amine malodours from spoiled ham and potato samples.[156] Elmore *et al.*[157] have found that, for solid samples, SPME yielded very little data compared to dynamic headspace extraction (Tenax). They recommended that for straightforward analysis of major volatile components SPME would be the method of choice, but, for trace analysis, only dynamic headspace trapping is suitable. They noted that the number of artefact peaks was greater in SPME than in the traditional method of extraction. The formation of artefacts in the application of SPME was also noted by Verhoeven *et al.*,[131] especially for flavour analysis where thermal desorption led to the formation of Maillard products[158] due to high concentrations of carbohydrates and amines in the samples. Nonetheless, the researchers reported that artefact formation could be significantly reduced by rinsing the fibre with water prior to thermal desorption.

Some of the criticism in this review has been addressed by recent developments.

Recent Progress in SPME Technology

Coupled Instrumentation

SPME coupled to liquid chromatography has been reviewed by Zambonin.[159] Application of the SPME method has been widened by coupling to various analytical instruments, including HPLC,[160] automated SPME-HPLC,[161] use of capillary electrophoresis,[162] electrochemistry,[163] capillary zone electrophoresis-microelectrospray-tandem MS[164] and inductively coupled plasma MS.[165]

Optimisation of SPME/HPLC

Four fibre coatings, CW-TPR, CW-DVB, PDMS-DVB, and PA were tested for efficiency of extraction of 9 HAAs.[166] A detailed study was made to optimise adsorption and desorption time, desorption mode, solvent composition, pH, ionic strength, and % modifier. CW-TPR coating was recommended for these amines. A new protocol was proposed for HAA analysis: hydrolysis with methanolic NaOH, adsorption on CW-TPR for HPLC-DAD analysis.

In-tube SPME-HPLC

In-tube SPME (open tubular trapping) has been developed over the past five years from the original idea by Pawliszyn and co-workers.[167–172] A small piece of fused silica capillary tubing coated with a suitable stationary phase is used to collect volatiles from the liquid sample as it is passed through the capillary tube. The processes of extraction, washing, desorption are under autosampler control. The extract is transferred to the HPLC column for separation. HPLC-ESI-MS provides the MS detection for liquid samples and Wu *et al.* (2002) described the coupling of in-tube SPME with HPLC-ESI-MS for the analysis of polar pesticides in water and wine.[173]

Because the sample preparation stage for the extraction of isoflavones from food matrices was too complicated, involving LLE, CCC or SPE, and a recently developed SPME method[174] was found to require care in handling the delicate fibres,[175] an in-tube SPME method was automated to remove the manual difficulty and to improve the throughput.[176] The method is described as being simple, fast, low cost, with reduced solvent use and easily automated. Nevertheless, there were preliminary stages: hydrolysis, partitioning with hexane and centrifugation to be added to the total time and complexity of the assay prior to the use of the in-tube SPME-HPLC procedure.

Siloxane–Water Partition Constants. The partition constants of the BTEX aromatic hydrocarbons were measured using in-tube SPME.[177]

Thin Film Microextraction

The originator of SPME has addressed the problem of low extraction rates of fibres by introducing the use of thin sheets of PDMS membrane.[178] The larger surface area to extraction phase volume ratio of the thin film over the microfibre allows larger amounts of analytes (n) to be extracted in the time scale, according to Equation (6.7),

$$n = K_{es} V_e C_s \tag{6.7}$$

where K_{es} is the partition constant between the sample (s) and the extraction membrane (e), V_e is the volume of the active extraction membrane and C_s is the initial concentration of the analyte before the extraction starts.

Thin film microextraction provides higher extraction efficiency and sensitivity and was used to extract PAHs from spiked lake water in a GC-MS-coupled experiment with low ppt detection level and a linearity of 0.996. The way

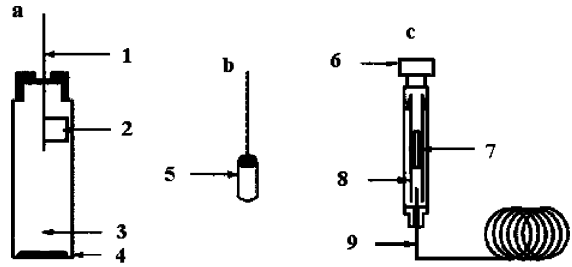

Figure 6.24 *Thin film microextraction system.* a. *1. Deactivated stainless steel rod. 2. Flat sheet membrane. 3. Sample. 4. Stirring bar.* b. *5. Rolled membrane, and* c. *6. GC septum in injection nut. 7. Rolled membrane in 8. Glass liner. 9. Capillary column*
(Reprinted with permission from *Anal. Chem.* 2003, vol 75, No. 4, p. 1002, Figure 1, © 2003, American Chemical Society)

in which the membrane is inserted into the GC injection port is illustrated in Figure 6.24.

Multiple SPME

The non-equilibrium extraction yield in multiple extraction mode can equal the yield from a single equilibrated extraction. Conversely, a higher yield can be had using multiple extractions up to the equilibrium time. Good agreement has been shown between the theoretical and experimental data.[179] The partition constant influences the effect of multiple extraction because high values allow the solution to be depleted in solute during early extractions, reducing the efficiency of subsequent extractions. Conversely, at equilibrium $>90\%$ of the theoretical cumulative yield of multiple extractions can be obtained with a single extraction with a 100 μm PDMS fibre and 1 ml sample for $K>10{,}000$. There are other factors to be considered such as the volume of the coating and the volume of the sample. The authors cover these and other theoretical and practical aspects of multiple extractions in a clear and effective manner. The theoretical cumulative yield versus number of extractions curves are shown in Figure 6.25 (A and B).

Sol-gel Technology Applied to the Production of Microextraction Fibres

The coating and conditioning procedure used to manufacture a hydroxy-fullerene (fullerol) SPME adsorbent was described.[180] A fullerene polysiloxane porous coating was bonded onto the fused silica fibre surface. The surface was stable to 360 °C and to organic and inorganic solvents. PCBs, PAHs and polar aromatic amines were tested from headspace samples and the new technology compared to conventional commercial fibres. The new surface showed higher sensitivity and faster mass transfer properties. It was also firm and reproducible, cheap, durable and simple to prepare.

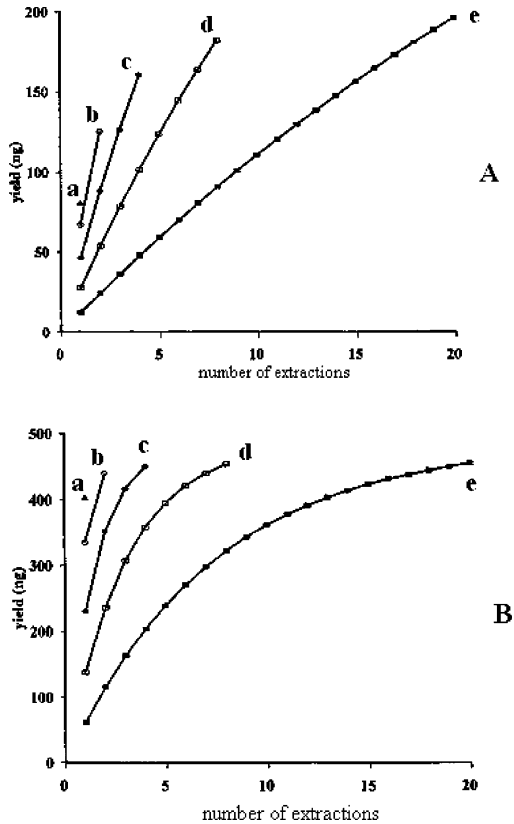

Figure 6.25 *Theoretical cumulative yield* versus *number of extractions at partition constants (K) of (A) 330 and (B) >10,000. Legend: (a) one extraction of 40 min, (b) two extractions of 20 min, (c) four extractions of 10 min, (d) eight extractions of 5 min, and (e) 20 extractions of 2 min. Based on the extraction of lidocaine from buffer (500 ng ml^{-1}) with a 100 μm PDMS fibre. The experimental time–sorption curve is shown in Figure 6.26*
(Reprinted from the *Journal of Chromatography A*, vol. 878, E.H.M. Koster and G.J. de Jong, "Multiple Solid-phase Microextraction", pp. 27–33, © 2000, with permission from Elsevier)

4 High Concentration Capacity Extractions

Stir-bar Sorptive Extraction

Introduction

The move from open-tubular cryogenic traps to the use of PDMS as an adsorbent for volatile and semi-volatile organics in open-tubular traps was developed by analytical research groups in the 1980s, *e.g.* Grob and Grob, 1983.[181] The maintenance-free, disposable adsorbent traps were more convenient.

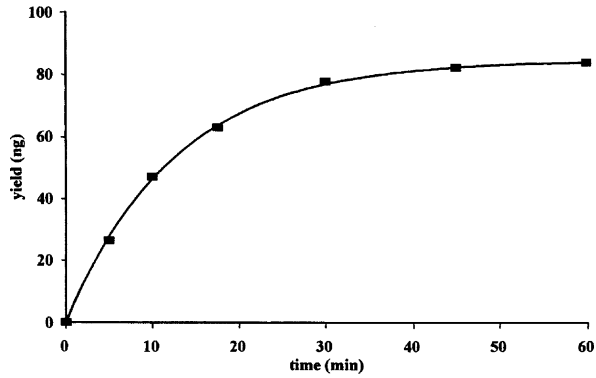

Figure 6.26 *Time–sorption curve for the extraction yield of lidocaine (ng) from borate buffer pH 9.5 (1 ml, 500 ng ml^{-1}) with a 100 μm PDMS fibre*
(Reprinted from the *Journal of Chromatography A*, vol. 878, E.H.M. Koster and G.J. de Jong, "Multiple Solid-phase Microextraction", pp. 27–33, © 2000, with permission from Elsevier)

The introduction of the SPME microprobe with its PDMS layer on a fused silica fibre (Arthur and Pawliszyn, 1990[89]) enabled the desorption/separation/detection stages for GC to put on-line. Flavour chemistry had a new, rapid, solventless method for extracting analytes from the gas or liquid phase of a food sample. However, in practice, only a small quantity of sample was collectable on the 0.6 μl volume of adsorbent on the 100 micron diameter quartz fibre used for SPME-GC. This was adequate for many experiments where either the volatile mixture contained a large number of components in similar concentrations or, conversely, only the major components of the mixture were of interest. The wide dynamic range of the classical HS methods was difficult to achieve with SPME. More adsorbent material was required.

The magnetic, glass-coated stir-bar is used in many liquid preparations as an extraction aid to reduce the time to reach equilibration. The idea of coating the outer glass casing with an adsorbent to extract target compounds from the liquid phase while stirring the liquid was simple yet ingenious.[182,183] The obvious application in food analysis was for the removal of water-soluble organic compounds such as flavour chemicals from the aqueous medium. The solutes are extracted into the adsorbent according to the magnitude of their log *P* values, and on the sample-to-adsorbent ratio. This approach provides the combination of extraction and high concentration in a single step. Along with most other aspects of analytical chemistry it is desirable to miniaturise each process to reduce the overall size of the workstations, providing the automated assays. A compromise is needed to achieve other objectives like fast reaction times.

Principles of Stir-bar Sorptive Extraction onto PDMS

The difference between S-BSE and SPME is that the amount of adsorbent used in S-BSE can be between 50 and 250 times greater than with SPME. The

principles of SPME apply to S-BSE; however, the developers of S-BSE make the very important point that the sorptive equilibrium, which is dependent on the solute partition constants, is also dependent on the phase ratio, which will be so much more important in S-BSE, and is shown in Equation (6.8). It is assumed that, as an approximation, the $K_{PDMS/w}$ partition constant can be replaced by the familiar $K_{o/w}$ in Equation (6.8),

$$K_{o/w} \approx K_{PDMS/w} = \frac{C_{PDMS}}{C_w} = \left(\frac{m_{PDMS}}{m_w}\right)\left(\frac{V_w}{V_{PDMS}}\right) = \beta\left(\frac{m_{PDMS}}{m_w}\right) \tag{6.8}$$

where $\beta = V_w/V_{PDMS}$ The recovery of solute by PDMS is the original amount (m_O) minus the amount remaining in the water phase (Equation 6.9).

$$m_{PDMS} = m_o - m_w \tag{6.9}$$

Extraction Procedure using S-BSE

The PDMS-coated stir-bar is activated in the liquid sample for 30–240 min, depending on the sample volume, dimensions and rotation speed of the stir-bar, and is optimised for each application. After use, the stir bar can be gently washed with distilled water to remove any carbohydrate, protein *etc.* adsorbed on the surface. This does not remove the more firmly bound organics occluded to the PDMS structure. These can be desorbed either with thermal energy or by dissolution in a suitable solvent. The larger volume of solute material is then reconcentrated by cold trapping.

Stir bars and the thermal desorption tubes are commercially available from Gerstel GmbH (Müllheim a/d Ruhr, Germany) and the desorption, trapping, and GC injection can be automated.

Applications

Symposium Reports. Several relevant papers were presented at the 23rd International Symposium on Capillary Chromatography (IOPMS, Kortrijk, Belgium, CDE-ROM, 2000), D34 (off-flavour compounds in dairy products), M33 (Flavour profiling of beverages), D35 (Corkiness in wine – Trace analysis of 2,4,6-trichloroanisole), M35 (Contaminants in wine), and at the 25th International Symposium on Capillary Chromatography (IOPMS, Kortrijk, Belgium, CDE-ROM, 2002) D19 (Sample preparation from heterogeneous matrices: Determination of pesticide residues in pear pulp at ppb level), P35 (Flavour analysis of Greek wine), which illustrate the applications already published in the area of food analysis.

γ-butyrolactone. γ-Butyrolactone is a natural-identical flavouring substance that has recently been restricted by the FDA. Its rapid analysis is important

and S-BSE-TD-GC-MS has been described.[184] A confectionery product matrix was used to test the method and recoveries of 85–92% for 1–10 mg l^{-1} and 92–95% for 10–25 mg l^{-1} concentrations were achieved.

Volatile Phenols in Wine. Fifteen samples were extracted simultaneously and desorbed in a thermal desorption system coupled on-line to SIM-GC-MS. The extraction method was optimised and the optimal values are shown in brackets: dilutions, 1:4, 1:10, 2:10 and 5:10, (1:4); sample volume 10, 15 and 20 ml (15 ml) of diluted wine; extraction time, 45, 60 and 180 min, (60 min); agitation speed, 600, 900, 1200 and 1500 rpm (900 rpm). The S-BSE method gave better recovery than the SPE method for the four phenols tested.[185]

Headspace Sorptive Extraction (HS-SE)

HS-SE is a simple way of using a PDMS phase-coated stir bar to increase the adsorption capacity (over that of the fused silica fibre SPME device) for volatile extraction (30 min at 25 °C) from the HS above samples for analysis (Demyttenaere *et al.*, 2004[135]). SPME, HS-SE and S-BSE were compared to examine the volatile metabolites from fungal growths. A whole range of sesquiterpenes was extracted and many identified by GC-MS.

Solid-phase Dynamic Extraction

Introduction

Static headspace (S-HS), HS-SE and HS-SPME can be used to sample from an equilibrated environment. In practice, a standard protocol is employed to avoid the long time to equilibrium experienced with many food matrices. SPDE, on the other hand, was designed to operate dynamically, withdrawing sample from the headspace, as often as required from experience, to provide a sufficient concentration for the separation and detection stage of the analysis.

Chromtech (Idstein, Germany) introduced SPDE to improve the adsorption capacity of SPME by coating the inside of a 5 cm long stainless steel needle with a 50 μm thick film of PDMS containing 10% activated carbon (Figure 6.27).

The needle was attached to a 2.5 ml gas-tight syringe so that by filling and emptying the syringe the required number of times a concentrated sample of the adsorbable material in the headspace being sampled would be retained on the inner surface of the syringe. The increase in concentration capacity was due to the larger adsorption volume of 4.5 μl compared to 0.6 μl of SPME.

Development

Using three common volatiles, β-pinene, isoamyl acetate, and linalool, the sampling temperature, number of aspiration cycles, plunger speed and

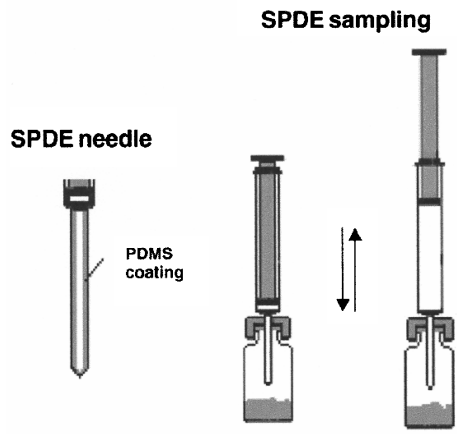

Figure 6.27 *A 5.5 cm long SPDE needle coated on the inside with a 50 μm film of PDMS containing 10% activated carbon (Chromtech, Idstein, Germany). Loading procedure from a sample vial using a gas-tight syringe*
(Reproduced from the *Journal of Chromatography A*, vol. 1024, C. Bicchi, C. Cordero, E. Liberto, P. Rubiolo, and B. Sgorbini, "Automated Headspace Solid-phase Dynamic Extraction to Analyse the Volatile Fraction of Food Matrices", pp. 217–226, © 2004, with permission from Elsevier)

volume aspired each cycle, total volume of HS sampled, desorption volume and plunger speed were optimised. Then volatiles in the headspace above commercially dried rosemary, green and roasted coffee, white and red wine and banana were sampled using HS-SPDE and analysed by GC-MS.[186]

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CHAPTER 7

Diffusion

1 Introduction

Diffusion is the main process occurring in the transport of analytes across membranes. A brief resumé of the theory of diffusion has been given in Chapter 1; for full details, textbooks on properties of matter provide the theory of diffusion of gases, liquids (and solids). In practice, other phenomena contribute to mass transfer, depending on the media through which the diffusion takes place, and upon the properties of the analytes migrating from one solution (the donor) to another (the acceptor). In modern practice, the transfer often occurs *via* an intermediate immiscible solvent phase supported in the pores of the membrane, giving rise to the notion of liquid/liquid/liquid extractions. The operations of membrane osmosis and membrane filtration that employ diffusion under pressure are included among the different ways of using membranes for separation and extraction.

Migration (Diffusion/Equilibrium) of Volatiles

The diffusion of volatile constituents from foods during storage, and the diffusion of volatiles from packaging into foods during storage and distribution, and the subsequent loss of quality on both accounts, are important issues in the food processing industry. A mathematical model for the latter process has been described using benzaldehyde as a model. Diffusion and partition constants for benzaldehyde are reported.[1]

The migration of antioxidants from polypropylene films of different thicknesses was modelled using *n*-heptane and 95% EtOH as fatty food simulants at different temperatures. All the antioxidants were extracted from the polymer by heptane but partition equilibrium was required to describe the migration into EtOH.[2]

Use of Membranes in Food Technology

Fruit Juice Clarification

A major use for membrane technology has been in the food industry for fruit juice clarification. The subject was reviewed in 2000,[3] identifying ultrafiltration,

microfiltration and reverse osmosis as applications. Aspects of the subject covered were product preparation, membrane selection and development of new membrane processes, including electrodialysis and pervaporation, and improvement in process engineering.

Pervaporation

In 1991, work was carried out on the use of collagen membranes using EtOH, iso-PrOH, ethylene glycol and acetone to test their separation properties for pervaporation[4] (Section 6). Good selectivity and surprisingly high fluxes at low operating temperatures were reported, and the dehydration of apple juice aroma was studied as an application in food technology. Non-porous membranes have been developed to extend the use of LLE.[5,6] (See also Buldini *et al.*, 2002, Chapter 8 ref. 1). The removal of unwanted odours is a second use for pervaporation, *e.g.* deodorisation of cauliflower blanching effluent.[7] The detailed theory and practice of pervaporation for aroma recovery was published in two parts by Lipnizki, Olsson and Trägårdh in 2002,[8,9] and its application to beverage processing and comparison with distillation, partial condensation, gas injection, adsorption, and SFE had been reported earlier.[10]

Reverse Osmosis as an Aid to Efficient Operation in CO_2-SFE of Essential Oils

In the extraction of essential oils by CO_2-SFE, it is necessary to extract the oil from the dense CO_2 by depressurisation and subsequent recompression to repeat the process. It was economical to add cellulose acetate reverse osmosis to the process line so that nutmeg oil could be extracted on-line.[11]

"Dipstick" Immunoassay

The simultaneous determination of atrazine and carbaryl pesticides in vegetable samples required a fast extraction followed by a multi-strip membrane test taking 10 min and achieving LODs of 10 and 200 $\mu g\ l^{-1}$ respectively.[12]

Transport Theory

With applications in the food industry in mind, the development of a unified transport model for analyte behaviour in non-aqueous media was studied.[13] Six different membranes, both hydrophilic and hydrophobic, were compared for rejection characteristics. The objectives for the work were:

1. Study the transport behaviour of organic analytes in non-aqueous media.
2. Study the separation mechanisms in aqueous and non-aqueous systems.
3. Extend existing transport theories.

A general review of transport mechanisms in polymers was made by Soney and Sabu in 2001.[14]

Analytical Extractions using Membranes

Membranes are used to select analytes in analytical separations. This can be effected in various ways. The size of the analyte molecule will determine whether it can pass through a particular membrane material. Thus, by experiment, the molecular weight cut-off (MWCO) point defining the maximum size of molecules that can cross the membrane if no other interaction occurs between the analyte and the membrane can be determined, and the membranes listed in order of exclusivity. The advantages of using membranes over solvent extraction are:

1. Elimination of the phase separation step,
2. Reduction in emulsion formation,
3. Efficiency of the high ratio of surface area to volume in most practical membrane separators.

Principles of Liquid Membrane Extraction

The detailed theory of mass transfer in supported liquid membrane extraction (SLME) is presented in a series of papers from the research team at Lund University.[15–17] The separation process depends upon differences in mass transfer rates between different analytes. The parameters affecting membrane transport rates are pressure differences in filtration (size exclusion) processes, concentration differences between the donor and acceptor streams in dialysis processes, and differences in electrical potential across the membrane where electrodialysis is concerned. In practice, there are several membrane and analyte properties that also need to be considered for the efficient separation of the target analyte(s). The correct MWCO will greatly facilitate the extraction, but the pore size, film thickness, hydrophobicity, permeability factors and simply the propensity for clogging are only some of the concerns.

Analysts may wish to use the experience in the food industry for wastewater clean up and other large-scale applications, but there is a growing literature on membrane separations for food analysis such as sorbent impregnated membranes and liquid impregnated membranes, designed to have specific functionality and they will be considered along with other membrane separation topics under the following headings.

Forms of Membrane Extraction

Supported Liquid Membrane (SLM). The two aqueous phases, the donor and the acceptor, are separated by a porous membrane containing an organic liquid impregnated into the pores (Figure 7.1). The donor aqueous phase pH is adjusted to neutralise the analytes of interest, which are then more easily extracted into the membrane. The acceptor phase is at a pH that ensures the analytes are ionised and thus less likely to back diffuse into the membrane.

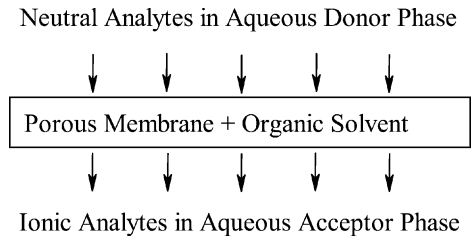

Figure 7.1 *Schematic of the diffusion of analytes through a supported liquid membrane. This arrangement is a three-phase system where the analyte is extracted from the donor aqueous phase into the organic phase in the membrane and then back extracted from the organic phase into the aqueous acceptor phase*

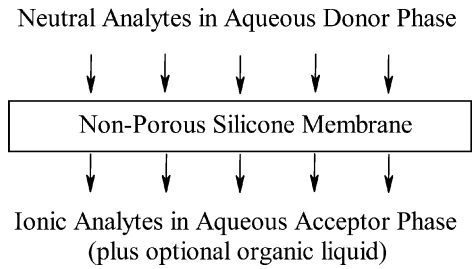

Figure 7.2 *The non-porous silicone rubber film allows neutral analytes to diffuse into the acceptor phase where they are ionised to prevent back diffusion. An organic solvent may be added to the acceptor phase to help prevent the back diffusion*

Non-porous Silicone Rubber. The two aqueous phases are separated by a non-porous membrane (Figure 7.2). The pH conditions are the same as for SLM.

Microporous Membrane Liquid–Liquid Extraction (MMLLE) (Figure 7.3) Although the number of solvents that might be used to impregnate the membrane is large, the use of undecane as a non-polar solvent and dihexyl ether

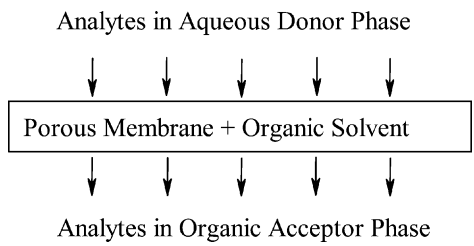

Figure 7.3 *MMLLE. A porous SLM separates the aqueous donor phase and the acceptor phase containing an organic solvent in this two-phase system. Once the analytes enter the membrane from the donor phase, molecular diffusion is a continuous process across the membrane into the acceptor phase, compared to the back extraction process in SLM*

as a more polar solvent (or a mixture of the two for intermediate polarity) are popular choices. A universal phenomenon for all three membrane extraction methods is that most of the sample medium is insoluble in the membrane and, therefore, remains in the donor phase. If this is a moving phase then the insolubles are removed continuously to waste. Conversely, the high solubility of the analytes in the SLM is important if the sensitivity of the method is to be kept high. The compromise is to avoid loss of selectivity, especially if further chromatography is not in place before detection.

Membranes as Couplers for Preparation and Separation Stages

On-line Gas-diffusion Flow Injection Analysis

Sulphur dioxide in wines was detected by an amperometric detector after extraction from the reaction chamber by a gas-diffusion unit in the FIA mode (Azevedo *et al.*, 1999, Chapter 5, ref. 65). Molecular films of tetraruthenated porphyrins were used on the surface of electrochemical detectors. Problems with the first generation electrodes were minimised by applying alternate layers of cationic and anionic porphyrins. A detailed account of the setting up of the experiment is given. Cyclic voltammetry on the EG&G Princeton Applied Research model 273 potentiostat used a three-electrode system: glassy carbon (Bioanalytical System, model TL-5), (saturated $Ag/AgCl_{KCl}$ reference), and auxiliary electrode down stream. A Teflon membrane diffused gaseous compounds from the donor flow to the acceptor stream for detection and recording (Figure 7.4).

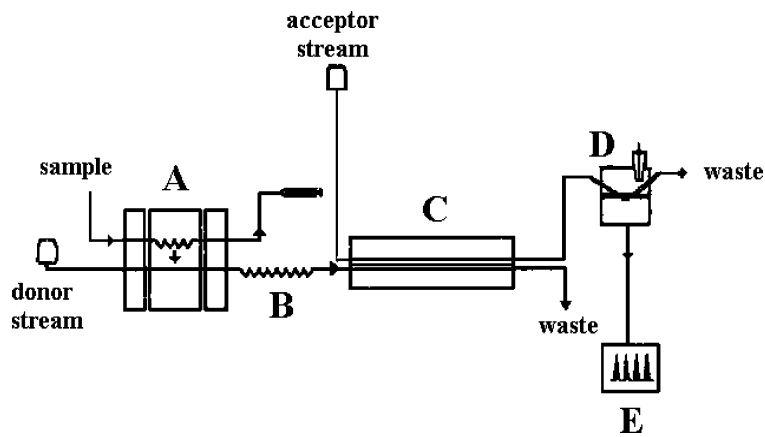

Figure 7.4 *FIA system schematic. (A) Manual injector, (B) mixing coil, (C) diffusion module, (D) amperometric detector and (E) recorder*
(Reprinted from *Analytica Chimica Acta*, vol. 387, C.M.N. Azevedo, K. Araki, H.E. Toma and L. Angnes, "Determination of Sulphur Dioxide in Wines by Gas-diffusion Flow Injection Analysis Utilising Modified Electrodes with Electrostatically Assembled Films of Tetraruthenated Porphyrin", pp. 175–180, © 1999, with permission from Elsevier)

Optimised conditions for the measurements were: injected volume 150 ml, H_2SO_4 concentration 2 mol dm^{-3} in donor stream, mixing coil 30 cm length, total volume (each side) of diffusion cell 2 ml, diffusion region 286 mm^2, applied potential +0.90 V (*vs.* $Ag/AgCl_{KCl}$), flow rates of both acceptor and donor lines 1.5 ml min^{-1}. The LOD was 3 μM SO_2.

On-line Membrane Diffusion/Chromatography

Introduction. There is keen interest in coupling and automating the extraction and separation stages, and membrane technology is being incorporated into continuous-flow workstations. Membrane extraction has been coupled on-line to HPLC, simply by using a flow-switching valve with an injection loop. Provided that the enrichment of the acceptor flow is reasonably high, a direct sampling into the loop will suffice. There is great potential in devices that can maintain high concentrations of solutes in small volumes of solvent. The *lab on a chip* approach is one to follow.

GC coupling works well with wholly organic acceptor phases when direct injection of a sample into the column (or pre-column) provides sufficient sensitivity for detection. This is more likely to work with packed columns and some degree of pre-concentration may be necessary for capillary column applications.

Membrane Extraction with Sorbent Interface (MESI). MESI originated out of work on membrane separators as GC-MS interfaces. It is also related to the use of membranes in microporous filtration, ultrafiltration, reverse osmosis, microdialysis, electrodialysis, *etc.*[18] The MESI integrated approach to the use of membranes and sorbent traps for the extraction of volatiles above aqueous systems (headspace analysis), and trapping for subsequent desorption and analysis by GC and GC-MS,[19] will undoubtedly be of interest to flavour analysts. Pawliszyn's team applied MESI to the monitoring of volatile emission from eucalyptus plants.[20] A PDMS membrane system was coupled to SPE with either PDMS or Tenax packing on-line to GC or GC-MS. The sorbent trap contents were thermally desorbed using a direct current supply. The membrane was placed in contact with the leaves and the volatile emission was monitored for 8 h.

On-line Membrane Extraction/LC. The extraction of vitamin E from butter has been automated using a silicone membrane permeation of the analyte to an on-line LC.[21]

On-line Membrane Diffusion/Electrophoresis

A very interesting development with CE, using a microcapillary hollow fibre SLM, allowed the diffusion of sufficient analyte through the fibre into the low volume of acceptor liquid for direct injection into the CE buffer.[22] A double stacking technique was used to preconcentrate the analyte for CE.

On-line Hydrolysis/Membrane Diffusion/Chromatography

Diffusion of analytes from the donor liquid to the acceptor solution, across a membrane, is an ideal isolating interface between incompatible systems. A good example was made of coupling saponification to HPLC *via* a silicone membrane for the preparation and separation of vitamin E isomers from seeds and nuts.[23] Three different approaches were compared:

1. Alkali hydrolysis, hexane extraction of the unsaponifiables, evaporation under vacuum, taken up in MeOH, and filtered.
2. Mixed with Triton X-114, MeOH and CH_3CN, water added, stirred for 30 min, diluted to 50 ml, centrifuged (3000 rpm for 10 min), filtered.
   (a) With hydrolysis. Using an on-line membrane separator and a flow-switching valve the hydrolysis mixture and the sample flow were merged and the mixture fed to the saponification reactor. The reaction products formed the donor solution. Diffusion took place with the acceptor flow (acetonitrile) stopped. The acceptor flow was restarted and sent to the sample loop of the switching valve for injection.
   (b) Without hydrolysis. The sample flow was sent along the same route but minus the hydrolysis mixture.

The preferred method was 2(b) because it took only 40 min without the saponification step.

2 Microporous Membrane Liquid–Liquid Extraction

A thin hydrophobic microporous membrane is placed at the interface between two immiscible liquid phases, one aqueous and the other an organic solvent, such that solutes can transfer across the membrane according to the laws of mass transfer[24] (Figure 7.5).

The donor solvent carries the solutes to the membrane where mass transfer occurs according to its physico-chemical properties.

The acceptor solvent is static while the donor flow carrying solute is on, concentrating the extracted substances in a low volume of the second solvent. The acceptor solvent flow is turned on to remove the solute for collection. A commercial version of the apparatus is available.[25] Variables include the nature of the extracting solvents, membrane material, and pore size since size exclusion also occurs. The combination of this technique with PHWE is discussed in Chapter 4.

Application of Membrane-based Solvent Extraction in the Food Industry

The food industry has problems with odorous effluents, and sulphur compounds are particularly difficult to deal with. A membrane-based, non-dispersive solvent (*n*-hexane) extraction process was used to recover valuable dimethyl disulphide (DMDS), dimethyl trisulphide (DMTS) and *S*-methyl thiobutanoate from simulated aqueous effluents. A hydrophobic, cross-flow hollow fibre

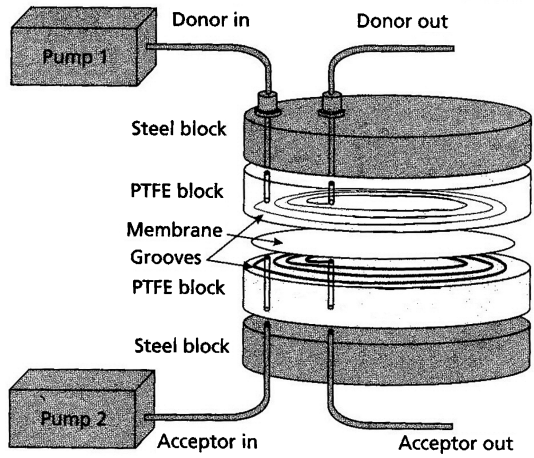

Figure 7.5 *Microporous membrane liquid–liquid extraction cell*
(Reprinted from *LC•GC Europe*, vol. 15, T. Hyötyläinen and M-L. Riekkola, "On-line Combinations of pressurised Hot-Water Extraction and Microporous membrane Liquid-Liquid Extraction with Chromatography", pp. 298–306, © 2002, with permission from Advanstar Communications (UK) Ltd.)

module Liquicel® X-40 (Hoechst Celanese) membrane was used in countercurrent mode.[26] Theoretical aspects of membrane-based solvent extraction are presented and the necessary water/*n*-hexane partition constants are calculated, from which the mass transfer coefficients were determined. Figure 7.6 shows the pilot-scale membrane extraction equipment.

Membrane-based solvent extraction was compared to pervaporation (Section 6 and Appendix 1). Further work was reported.[27] Mass transfer experiments were carried out using the crossflow hollow fibre membrane module and the same standard sulphur compounds. Selective extraction of the aroma compounds is due to their high partition constants. (See also Chapter 2 and van Ruth and Villeneuve, 2002, Chapter 3, ref. 3.)

3 Membrane-assisted Solvent Extraction

Introduction

The developers have introduced the MemASE technique as an aid to LLE that would reduce the analysis time and facilitate automation. They also provide a full review of the situation regarding membrane extraction in chemical analysis before describing their innovation. The technique, described by Hauser *et al.* (2002)[28] as membrane-assisted solvent extraction or membrane-assisted liquid–liquid extraction, was developed for in-vial (20 ml headspace vial) extraction of hydrophobic, semi-volatile organic compounds from 15 ml

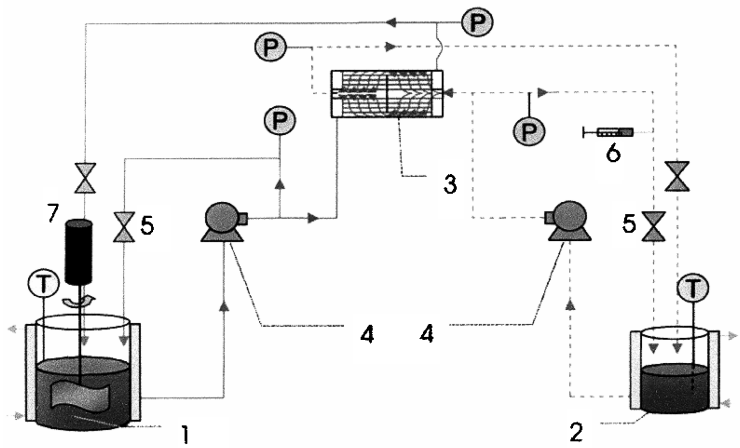

Figure 7.6 *Pilot-scale extraction device: (1) feed phase reservoir, (2) solvent phase reservoir, (3) hollow fibre module, (4) pumps, (5) by-pass valves, (6) sampling point, and (7) stirrer. 10,000 fibres were used in the X-40 Liquicel hollow fibre module*
(Reprinted from the *Journal of Membrane Science*, vol. 187, F.X. Pierre, I. Souchon and M. Marin, "Recovery of Sulfur Aroma Compounds using Membrane-based Solvent Extraction", pp. 239–253, © 2001, with permission from Elsevier)

aqueous samples into 500 µl organic solvent through a 0.05 mm thick polypropylene membrane (Goodfellow, Cambridge, UK) made into a 8 mm wide × 8 cm long tube (Figure 7.7).

Migration through the polymer wall from the aqueous matrix to the acceptor solvent was assisted by agitation at elevated temperature over the typical sampling period of 30 min.[28] The in-vial extraction can be readily sampled for GC-MS, and made part of an automated process. The low concentration of analyte in the acceptor solvent requires the large volume (100–400 µl) injection technique (Hogenboom *et al.*, 2000, Chapter 4, ref. 46) to reach acceptable LODs. The developers used the extraction of triazines as an example.

For Triazines

The acceptor solvent was hexane for the extraction of triazines and, in conjunction with LVI (10–100 µl) GC-MS, LODs of 1–10 ng l^{-1} were achieved with the 100 µl injection.[28] The large volume injector was made into a cooled injector at 20 °C using liquid N_2 coolant, the carrier gas pressure reduced to 10% of the working value, and the split vent increased to 100 ml min^{-1}. The properly cleaned membrane sacs were preferred to new membrane in terms of the leached volatile content (esters, phthalates *etc.*) in the GC-MS background. An agitation speed of 750 rpm, temperature of 55 °C, and extraction time of 30 min were found to be optimal.

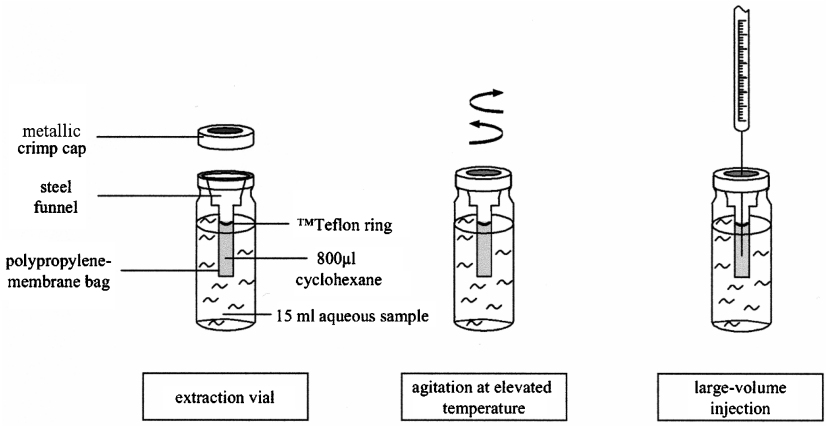

Figure 7.7 *MemASE method of extraction of hydrophobic semi-volatile compounds from aqueous samples*
(Reprinted from the *Journal of Chromatography A*, vol. 1020, M. Schellin and P. Popp, "Membrane-assisted Solvent Extraction of Polychlorinated Biphenyls in River Water and Other Matrices Combined with Large Volume Injection-Gas Chromatography-Mass Spectrometric Detection", pp. 153–160, © 2003, with permission from Elsevier)

Application to PCBs in Wine and Apple Juice

With minor changes to the conditions, MemASE found environmental and food (white wine and apple juice) applications.[29] The acceptor solvent was 800 µl cyclohexane, chosen for its low solubility in water, low diffusivity across the polypropylene membrane, and sufficient volatility to be easily vented during the LVI. No PCBs were found and, therefore, to test the extraction process the matrix was spiked with 0.5 ng ml^{-1} of each PCB: 28, 52, 101, 138, 153, and 180, and recoveries of 88–114% were reported. The method is low cost, simple to operate and uses little solvent. The membrane sacs are re-useable after cleaning. The octanol/water partition constants were given for 7 PCBs (Table 3.1).

4 Sorbent Impregnated Membranes

The specificity of an extraction across a membrane can be "tailored" by binding a chemical agent to the structure or chemically bonding an ionic group to the surface (ion exchange membranes).

Particle-loaded membranes (PLM) were developed by Lingeman and Hoekstra-Oussoren (1997) for bioanalytical sample clean-up/concentration.[30] They describe the construction of PLM and compare their operation with SPE, both on- and off-line. The PLM are evaluated for recovery, reproducibility, sensitivity and speed of analysis. Three membrane disks have the same breakthrough time as an SPE cartridge. The PLM has not been applied to food analysis to any extent.

Ion-exchange membranes have an important role in desalination, but these small pore membranes are not particularly suitable for the extraction of larger ions such as amino acids. High permeability anion-exchange membranes were developed by changing the degree of cross-linking of the basic ion-exchange polymer.[31]

5 Supported Liquid Membrane Extraction (SLME)

Planar Supported Liquid Membrane

SLME is a three-phase extraction technique where two aqueous phases are separated by a thin, porous hydrophobic membrane carrying an organic liquid by capillary action (Figure 7.8). For an analyte to cross the membrane it must dissolve in the organic liquid and, therefore, the pH of the donor liquid should ensure that the analytes presented to the membrane surface are uncharged. To ensure that the analyte does not diffuse back into the membrane, the pH of the acceptor flow is adjusted to ionise the analyte, making it less likely to back extract.

Factors Affecting Mass Transfer

If ΔC is the concentration difference of the diffusing analyte across the membrane in Equation (7.1),

$$\Delta C = \alpha_D C_D - \alpha_A C_A \tag{7.1}$$

where C_D and C_A are the concentrations of analyte in the donor and acceptor phases, and α_D and α_A are the uncharged analyte fractions in the donor and acceptor phases (Jönsson and Mathiasson, 2003[17]).

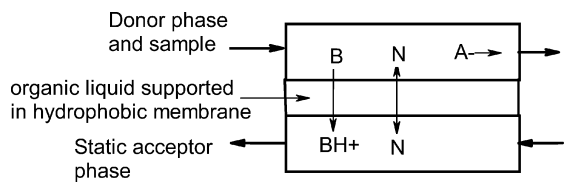

Figure 7.8 *Schematic diagram of the liquid membrane for the extraction of basic compounds (B) from the moving donor phase through the hydrophobic SLM into the static acidic buffer as acceptor phase. The neutral compound will be protonated at the interface and will be unlikely to back diffuse once charged. Because the diffusing compound is a neutral molecule, the ions in the acceptor phase do not affect the concentration gradient and the system continues to "pump" analyte across the membrane*
(Redrawn from *LC•GC Europe*, vol. 16, J.A. Jönsson and L. Mathiasson "Membrane Extraction for Sample Preparation" pp. 683–690, © 2003, with permission from Advanstar Communications (UK) Ltd.)

Hollow Fibre Supported Liquid Membrane

Liquid–Liquid–Liquid Microextraction (LLLME)

When microbore hollow fibres are used in SLM it is convenient to attach the fibre to the needle of a hypodermic syringe and conduct the filling with acceptor phase, the impregnation with organic solvent and the extraction into the donor phase, using the syringe as a handling and sampling device. After extraction of analytes into the acceptor solution in the fibre, a few microlitres were withdrawn for direct injection into the HPLC for separation.

Extraction of Acidic Herbicides from Milk.[32] SLM is also referred to as LLLME. A 3.5 cm long polypropylene hollow fibre tube (600×200 μm id wall thickness) with pore size 0.64 μm was fitted to the needle of a 10 μl syringe containing acceptor solution (plus various concentrations of NaOH solution) and 7 μl injected into the fibre. Still attached to the syringe, the fibre was impregnated by dipping it into the organic solvent for 10 s before the fibre was placed in the stirred donor solution.

SLME and Enzyme-catalysed Reactions

Lipase-catalysed reactions were coupled to SLME for the transport of organic acids through the membrane.[33] Parameters such as alcohol concentration of the feed, pH of the two aqueous phases, the nature of the solvent in the SLM and the substrate were studied.

Immuno-SLME

Immuno-SLME is based on using a SLM with soluble anti-analyte antibodies in the static acceptor phase. Uncharged analyte molecules diffuse through the membrane and form complexes with antibodies in the acceptor phase, preventing back extraction. The mass transfer will continue and a high analyte flux is experienced. The antibody–antigen interaction is used to drive the mass transfer process.[34] Optimisation studies of donor flow rate, extraction time, and type of antibody were conducted on atrazine. The SLM blocks were built at Lund University out of PEEK and PTFE, each with a machined groove $2.5 \times 0.1 \times 40$ mm of approximately 10 μl volume, and a non-porous PTFE membrane on a polyester backing, impregnated with dihexyl ether, and clamped between the blocks.

Applications

Herbicides

A fluoropore FG membrane (average pore size 0.2 μm, porosity 0.70, total thickness 175 μm, of which 115 μm was polythene backing), soaked for 30 min

in methyltrioctylammonium chloride dissolved in dihexyl ether, was used in SLME of aminophosphonates, some of which are herbicides.[35]

Caffeine in Tea and Coffee

Flow systems were designed to use SLME for the extraction of caffeine from solid and slurry coffee and tea samples.[36] In Figure 7.9(A), the flow system pumps the acceptor liquid (2.5×10^{-3} M H_2SO_4, pH ~ 2.5) into the lower chamber of the membrane unit (MU) containing the Fluoropore® FH PTFE membrane support for the *n*-undecane–hexyl ether (1:1) mixture, and on to the detector (D) and waste (W). The syringe (S) is slotted into the upper chamber above the membrane and the donor liquid is "injected" into the upper chamber for the run. In Figure 7.9(B), the above arrangement is modified so that slurry may be pumped through the upper chamber using peristaltic pump 1, while pump 2 behaves as in (A) above, supplying the acceptor liquid to the membrane.

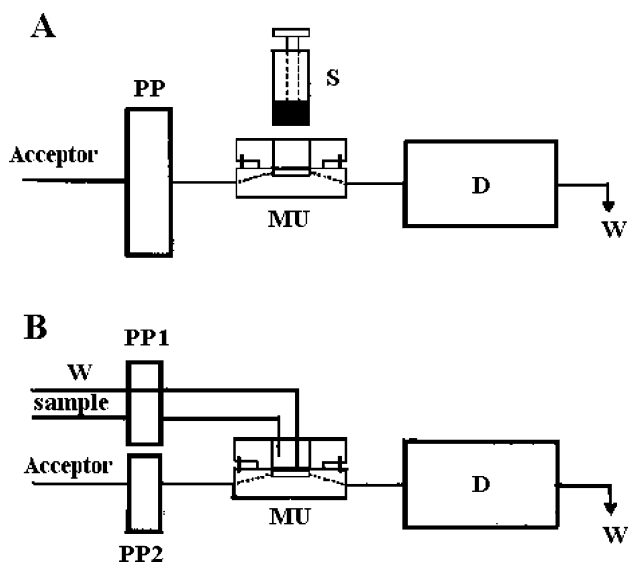

Figure 7.9 (A) *SLME unit in the flow system for the extraction of caffeine from solid coffee. PP: peristaltic pump, MU: membrane unit with a PTFE membrane clamped in circular PTFE blocks with cavities above and below the membrane, S: syringe, D: detector, W: waste.* (B) *SLME unit for handling slurry. PP1: slurry pump, PP2: acceptor flow pump*
(Reprinted from *Laboratory Automation and Information Management*, vol. 34, E. Luque-Pérez, A. Ríos, M. Valcárcel, L.-G. Danielsson and F. Ingman, "Spectrophotometric Flow Injection Determination of Caffeine in Solid and Slurry Coffee and Tea Samples using Supported Liquid Membranes", pp. 131–142, © 1999, with permission from Elsevier)

Vanillin

The apparatus described in Figure 7.9(A) was used to extract vanillin from food samples.[37]

6 Pervaporation

Principles

Pervaporation is a well-established industrial separation technique for fruit juice aroma concentration. It involves mass transfer through a non-porous polymeric membrane, *e.g.* PDMS, effecting a phase change from liquid to gas. The use of a hydrophobic membrane enables gaseous molecules (*e.g.* aroma volatiles) in aqueous solutions (*e.g.* fruit juices) to permeate the membrane, thus extracting them from the main stream (feed). Differences in the sorption and diffusion properties of the separated compounds are exploited.

Pervaporation has taken its place in the armoury of transport processes such as dialysis, reverse osmosis, and gel permeation, and its transport equations have been developed using the solution-diffusion model.[38] Karlsson and Trägårdh reviewed the use of pervaporation for the removal of organics from dilute aqueous solutions in 1993.[39] They describe the four model processes involved in the transfer of volatiles across the membrane in relation to aroma recovery. In 1994 they developed the method further with work on EtOH–H_2O solutions containing typical aroma compounds, using PDMS membranes,[40] and in 1996 they developed the method for the food processing industry.[41] The mass transfer through the membrane occurs in three steps:

1. Selective absorption of volatiles at the feed/membrane interface.
2. Selective diffusion through the membrane.
3. Desorption of the volatile permeate.

The mass transfer is assisted by the reduced pressure on the permeate side of the membrane, requiring the condenser to trap the volatiles as condensate (Figure 7.10).

Development

Four aroma compounds, low boiling diacetyl and ethyl acetate and high-boiling *S*-methylthiobutanoate and γ-decalactone were used in experiments on three kinds of commercial organophilic membranes. A silicalite-filled silica membrane for the pervaporation of LBVs showed the separation factor to be independent of the total permeate pressure. An unfilled PDMS membrane for the pervaporation of the HBVs and the filled membrane demonstrated selectivities that were highly dependent upon the total permeate pressure.[42]

The development of analytical pervaporation in food analysis was reviewed in 2000.[43]

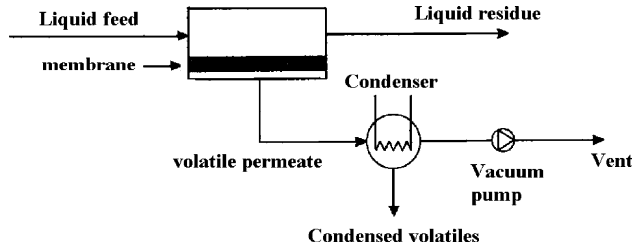

Figure 7.10 *Pervaporation process*
(Modified from *The Journal of Food Engineering*, vol. 34, H.O.E. Karlsson and G. Trägårdh, "Aroma Recovery During Beverage Processing", pp. 159–178, © 1997, with permission from Elsevier)

Applications

In the Food Processing Area

Rajagopolan and Cheryan[44] described their use of pervaporation of commercial grape essence as producing "highly enriched flavours". Flavour analysts will no doubt see the potential of developing narrow-bore micropervaporation tubes for the transfer of volatiles from aqueous (or high humidity headspace environments) to a low moisture gas line for cryotrapping, *etc.* and subsequent GC-MS analysis.

Automated Pervaporation-GC-MS

The pervaporation to GC coupling was applied to the analysis of slurry samples that erstwhile would have been sampled by purge and trap technology.[45] The experimental scheme is shown in Figure 7.11. The donor chamber at the bottom of the pervaporator unit received sample, when V1 and V2 were opened, flowing across the membrane to waste. Then valves V1 and V2 were closed for a 10 min (optimised) period during which time the permeable components passed into the upper acceptor chamber in the loop of an HPLC-type injection valve (IV). The valve IV was opened after the 10 min preconcentration period for 10 s (optimised), when the accumulated vapours were injected onto the GC from the loop. The donor chamber was maintained at 80 °C (optimised) during the static transfer (permeation) of molecules through the membrane. A cleaning cycle was used before the next sample was admitted. This fully automated pervaporation extraction was tested on the determination of acetone and acetaldehyde from samples of yoghurt, actimel and fruit juices.

Two recent papers demonstrate the potential of pervaporation-GC-MS: the technique was used to study the differences in the flavour profiles between fresh and frozen orange juices in relation to ethyl butanoate, limonene, linalool, α-pinene, geraniol, nerol and α-terpineol,[46] and the separation of 2,4,6-trichloro-, 2,6-dichloro- and 2,4,6-tribromo-anisoles from tainted wines was used to

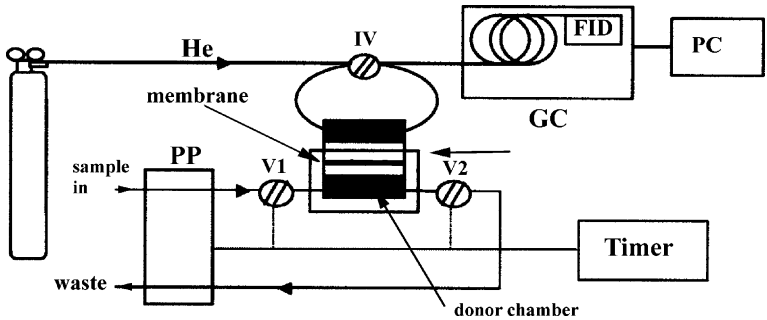

Figure 7.11 *Schematic diagram of the pervaporation-GC couple. PP, peristaltic pump; V1 and V2 on-off valves for the pervaporation unit; IV, HPLC injection valve; GC, gas chromatograph; FID, flame ionisation detector*
(Modified from the *Journal of Chromatography A*, vol. 976, E. Priego-Lopez and M.D. Luque de Castro, "Pervaporation-Gas Chromatography Coupling for Slurry Samples. Determination of Acetaldehyde and Acetone in Food", © 2002, with permission from Elsevier)

compare two procedures, *i.e.* pervaporation-GC-MS/MS and pervaporation-cryogenic trap/thermal desorption-pervaporation-GC-MS/MS.[47] The LODs for the methods were 25.8 and 4.2 ng l^{-1} respectively. For low concentration analyses the second method was preferred.

7 Dialysis

Introduction

Dialysis uses a semi-permeable membrane to bring two liquids, the donor and acceptor liquids, in close proximity without mixing. A positive concentration gradient from donor to acceptor initiates diffusion of molecules through the membrane. The rate of mass transfer is dependent on the magnitude of the gradient and on a number of other parameters, such as membrane area and thickness, analyte diffusion constant, which in turn depends upon viscosity, temperature, and analyte molecular dimensions in relation to the membrane pore size distribution. There are five modes of molecular diffusion from the donor to the acceptor liquids.

1. Static–static (modest recovery, maximum of 50%, rapid equilibrium)
2. Static–flowing/pulsed (good recovery and speed)
3. Pulsed–flowing (reasonable recovery but slower)
4. Flowing–flowing (modest recovery, good speed)
5. Counter-current flowing–flowing (higher mass transfer than 4).

A static acceptor volume is slow and inefficient for equal volumes of donor and acceptor. A flowing acceptor liquid stream increases the speed and efficiency of the analyte transfer. For large volume samples, the pulsed donor flow refreshes

the concentration gradient for each pulse. Similarly, the continuous flowing donor stream can be adjusted to provide a maximum concentration gradient across the membrane, and the use of counter-current flows exposes the donor flow to an acceptor flow with 0% analyte. Materials used as dialysis membranes for food applications include cellulose triacetate, regenerated cellulose and polyacrylonitrile metallyl-sulphonate. So far, analytes such as organic acids, amino acids, proteins, and sugars have been extracted mainly from liquid foods (Table 10, Buldini *et al.*, 2002, Chapter 8, ref. 1).

Development

The success of dialysis as an extraction method was in part due to the development of a commercial system by Gilson (Villiers-le-bel, France). They fixed on a channel depth of 0.2 mm and a 20 μm thick membrane. The channel dimension was found to be important as mass transfer resistance takes place in the donor liquid as well as across the membrane. Dialysis can often be used directly on liquid food and beverage samples with only minor preparation required for solid samples.

Collaborative Study

A collaborative study was undertaken by the Food Inspection Services, The Netherlands, on the *p*-toluenesulphonamide content of ice cream. Samples were extracted with water and the extract dialysed in a continuous flow system and the dialysate injected *via* a 500 μl loop into a LC column.[48] The CF system was used again, this time for measuring nitrate in leafy vegetables.[49]

Automated and On-line Dialysis

Combined Dialysis and Flow Switching SPE/HPLC. The Gilson ASTED system has been used for automation of on-line dialysis. Dialysis lends itself to on-line operation and examples in conjunction with flow switching devices have been published. However, analytes in the acceptor flow at concentrations below the LOD require an additional step to concentrate them as a preliminary to HPLC. SPE is ideal for this, used as the pre-column in flow switching mode, and, once it has been loaded with sample from the low concentration dialysis acceptor flow, it is switched into the appropriate HPLC mobile phase flow to allow desorption of the compounds of interest to proceed.[50] The actual sequence of switching varies with the requirements of the assay. Typically, the pre-column concentrator is switched into the analytical column mobile phase flow for long enough to desorb the compounds of interest and then switched out of line to allow unwanted components (and chromatographically immobile components) to be vented to waste. The system was used to determine amoxicillin and cefadroxil in beef muscle. The selectivity on the basis of molecular size and the low recovery, in this case 32–34%, would restrict the use of dialysis as an extraction method for general on-line application.

Direct Coupled Dialysis/HPLC. When a liquid food analyte is in a detectable concentration in the acceptor flow from the dialysis tube the SPE step is not required and direct coupling to the HPLC simplifies the method.[51] Five sugars, glucose, fructose, sucrose, maltose, and lactose, and six organic acids, citric, tartaric, malic, succinic, lactic and acetic acids, were studied from grape and apple juices, red and white wines, and cider, at LODs of 0.16–0.41 g l^{-1} for the sugars.

Dialysis/CE. A combination of a continuous-flowing (donor and acceptor) dialysis, using a planar membrane, and CE, is described and applied to the sampling of milk and orange juice.[52] Once the acceptor flow is established an aliquot is switched into the CE electrolyte flow for electrokinetic injection onto the CE column. This method has limited sensitivity since only a small proportion of the feed-flow enters the CE injector. Stacking methods are preferred in this type of application.

Counter-current Dialysis. On-line dialysis was exploited for a one-step sample preparation method for sugars (fructose, glucose, lactose, maltose, sucrose and raffinose) in beverages, again, where dilution was an asset, enabling direct HPLC of the dialysate.[53] Details of the optimisation of the process include solvent type, channel depth, flow rate, type of membrane, and direction of flow. Using a standard solution of sucrose the % mass transfer was measured and counter-current (donor and acceptor) flow was more efficient than donor and acceptor flows in the same direction. A channel diameter of 0.8 mm and a Celgard membrane, with a flow of water of 0.8 ml min^{-1}, was optimum for the extraction of 50 mg ml^{-1} sucrose.

Sample Processor Control. On-line dialysis was used with a sample processor for the pre-column derivatisation of amino acids with *o*-phthalaldehyde-3-mercaptopropionic acid and FMOC. Twenty five amino acids (including cysteine) were extracted and derivatised from foods (orange juice and red wine) using a one-step binary gradient.[54]

Applications

Riboflavin. Dialysis was combined with MAE and SPE (C_{18} minicolumn) in the extraction of riboflavin and flavin mononucleotide (FMN) in milk and ground cereals.[55] During MAE all FAD was converted into FMN and 15% FMN was converted into riboflavin. Other applications are given in a review of membrane-based extraction methods by van de Merbel (1999, Chapter 8, ref. 12).

Food Colours. The Gilson ASTED system was also used for the determination of synthetic food dyes in sugar-rich foods – boiled sweets, fruit gums, lemon curd, jelly, blancmange, and soft drinks.[56]

Aflatoxin M1. Automated on-line stopped flow dialysis of aflatoxin M1 from decreamed milk gave recoveries of >50% and measurement levels of 20 ng kg^{-1} within 20 min.[57]

Electrodialysis

By adding a pair of electrodes to the dialysis blocks, one on either side of the membrane, the potential difference as a driving force is added to the concentration gradient. In a chosen polarity, say anode in the donor liquid and cathode in the acceptor liquid, the cations in the sample will migrate to the cathode, leaving the anions moving in the opposite direction. This makes for a clear distinction on the basis of charge, admittedly with some diffusion of neutrals. The potential for coupling electrodialysis to CE is great. It has been demonstrated for negatively charged inositol phosphates with the cathode in the donor liquid and the anode in the acceptor phase. The inositol phosphates migrated to the positively charged anode and were sampled electrokinetically into the CE column.

Microdialysis

The microdialysis probe was developed for sampling from low volume physiological fluids when the diffused analytes entered a low volume, dynamic acceptor flow for external collection. Liquid food sampling takes advantage of the very low volume acceptor flow that can be coupled in static, pulsed or dynamic mode to LC for on-line extraction and separation of the chosen analytes. Microdialysis was used to analyse ascorbic acid from milk, yoghurt and fruit juices with improved sensitivity and selectivity in a straightforward preparation.[58]

Microdialysis was used to bring together a method for glucose and lactose analysis using FIA. A dual electrode amperometric biosensor equipped with a thin layer flow through cell and a Pt dual electrode was used to measure lactate and glucose simultaneously in untreated tomato juices.[59]

8 Filtration

Possibly the simplest and arguably the most common extraction method of all is filtration. With a wide range of porosities available the filter paper provides the first line of approach in the extraction of dissolved compounds from solid food matrices. The disposable syringe membrane filter, also available in a range of porosities, is used to remove solids or "clean up" solubles in crude extracts for injection onto chromatographic separation columns. Larger scale clean up may use a Büchner funnel or a filtration bed of diatomaceous earth (or the modern equivalent). Under gravity filtration the gradual blocking of the pores of the filter with the solid fraction extends the process time and more filtrate may be obtained under pressure from the feed side or under vacuum from the permeate

side. The flux of filtrate will be related to the applied pressure, sample viscosity and membrane parameters. As discussed with membrane extraction, the resistance to mass transfer of the surface on the feed side of the membrane also has to be taken into account, and when solids build up during the filtration process this adds significantly to the total resistance, especially at higher pressures. The effect of pore size, area and thickness will affect the flux. The practical compromise of MWCO values of 50–100 kDa provides a starting point, but filters by dint of their function become fouled in use and efficiency falls off.

Practically every protocol for food analysis contains a filtration step, and as on-line membrane filtration is needed for incorporation into automatic sample preparation processes, the problems with decelerating flux need to be addressed, as in fermentation filtration.[60]

Increasing the Area of Filtration

With planar filters, increasing the surface area increases the flux, but decreases the sensitivity for trace analysis due to superficial losses. The use of hollow fibre assemblies provides increased surface area with a relatively small increase in the permeate volume (van de Merbel, 1999, and references therein, Chapter 8, ref. 12).

Decreasing the Concentration Polarisation Layer

The build up of the solid layer (concentration polarisation layer) on the filtering surface, all too often, brings the process to a premature end. In manual industrial operations, the solid is scraped off and discarded. In continuous industrial processes the layer is removed by rotating blades above the filter. In continuous analytical applications, turbulence in the feed flow helps to keep the particulate matter moving past the membrane. The flow rate and the degree of perturbation may be optimised (van de Merbel, 1999, Chapter 8, ref. 12).

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CHAPTER 8

Conclusion

1 Introduction

In this final chapter, reviews of the subject since 2000 have been overviewed (Section 2) to make sure that an up-to-date opinion on extraction methods has been included, and any "developing news" that appears to have relevance has been collected together in Section 3; the concluding discussion in Section 4 brings together a state of the art appraisal at the time the manuscript was submitted.

2 Recent Reviews

Introduction

An extensive review of sample preparation and extraction techniques in food analysis was published in 2002.[1] Newer aspects of methods, wet digestion (inorganic), solvent extraction, sorbent extraction, and membrane separation, were discussed; 215 references were provided.

In the popular series of articles in *LC-GC Europe*, R.E. Majors analysed the results of a survey of chromatography users conducted by LC-GC.[2] Figure 8.1 shows the frequency of use of sample preparation and extraction techniques by the analysts who responded to the survey. Almost 40 techniques are listed, most of which have been discussed in this book, either as procedures necessary to prepare samples for extraction or as extraction techniques. Others, such as weighing are accepted as essential ancillary procedures in chemical analysis, and yet others, such as digestion, are seen as analyte release processes aiding the extraction.

The use of sample preparation and extraction techniques for food flavour analysis was reviewed by Wilkes *et al.*, 2000.[3] They listed a number of processes that might be involved with the preparation of volatile samples:

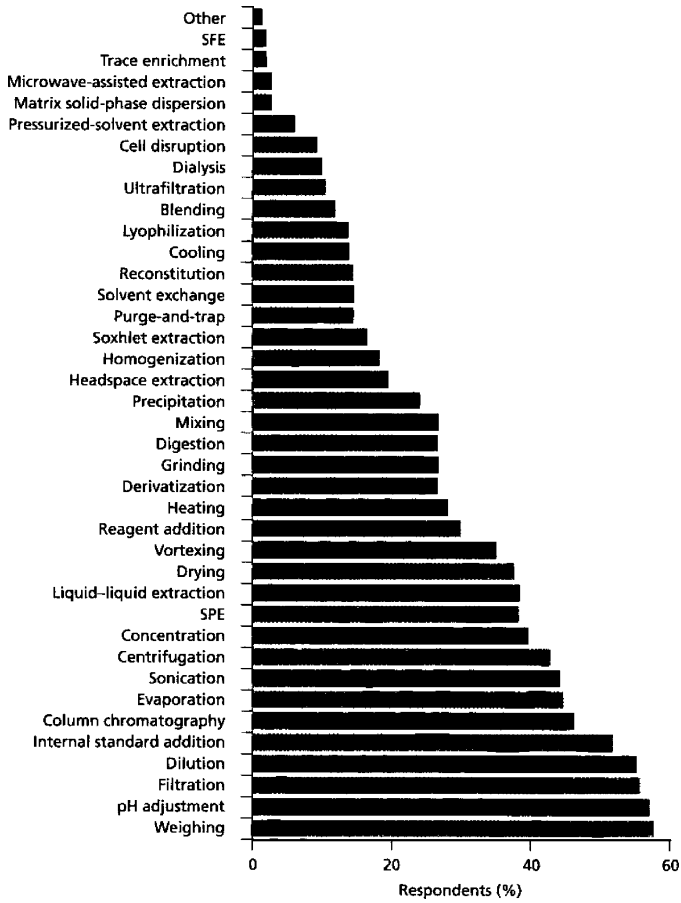

Figure 8.1 *Popularity of sample preparation techniques with analysts who responded to the survey by LC-GC*
(Reproduced from *LC•GC Europe*, vol. 16, R.E. Majors, "Trends in Sample Preparation", pp. 71–81, © 2003, with permission from Advanstar Communications (UK) Ltd.)

Mincing	Purge and trap
Homogenisation	Heating
Centrifugation	Microwave-mediated distillation
Distillation	Purge with inert gas
Simple solvent extraction	Trap with Tenax or C_{18} (SPE)
SFE	Thermal desorption
PFE	Cryofocusing
MAE	Elution with ethyl acetate (SE)
SOX	SPME (PDMS adsorption or electrodeposition)
Methylation	Thermal desorption
Derivatisation	SE

They included among the commodities: fish, cheese, sausage, olive oil, orange juice, dehydrated potatoes, dairy products, sea foods, garlic, spices, fish spices, herbs, coffee, peanuts, candy, mushrooms, beverages, and honey, using 157 references.

Although the separation and detection techniques that normally follow the extraction stage have not been discussed here, a review with 200 references by Reid (1986) discusses the preparation and extraction techniques used prior to mass spectral analysis.[4] Although the MS detection system has become much more sophisticated since then, the review makes the very important and timeless comment that "the mode of operation of the machine can influence the amount of clean-up necessary before a sample is presented for analysis."

Preparation Techniques

Biochemical Release Methods

The different approaches of the Prosky and Englyst teams to the analysis of dietary fibre are well documented here and elsewhere. In a recent review, Prosky explains his case.[5] Dietary fibre is generally accepted to be, as defined by Trowell, "the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to (hydrolysis) digestion by the alimentary enzymes of humans." The author discusses in detail the functional food properties of dietary fibre.

Microwave-assisted Extraction (MAE)

Sparr Eskilsson and Björklund reviewed the position of MAE for analysis covering basic principles, instrumentation, development and optimisation, and applications by chemical class. The sections on organic pollutants, pesticides, phenols, and natural products are useful in food analysis. They also compare six analytical methods: MAE, FMASE, PLE, SFE, SOX, and UAE – using SOX as the standard – for extraction time, sample size and solvent use, giving a valuable review of their advantages and disadvantages.[6] The main advantage of all the modern techniques compared to the classical Soxhlet extraction is the major reduction in extraction time, and there are significant savings on solvent consumption.

Extraction Techniques

Accelerated Solvent Extraction

The theoretical concepts and practice of ASE in relation to other techniques, *e.g.* SOX, and its advantage of ease of automation versus cost is discussed.[7]

Direct Injection of Liquid Food for HPLC "Extraction"

The analysis of liquid food samples using HPLC with direct injection was reviewed.[8] Making an injection of the liquid food directly into the separation

stage means that the top of the separation column (or precolumn if one is used) will "extract" any immobile interferents, leaving the mobile analyte and other mobiles, potentially interfering components to be resolved as fractions on the column as the chromatography progresses. In this review, reference is made to the direct injection of wine in the analysis of resveratrol and phenolics. Other liquid foods required minimal pretreatment before injection: dilution and filtration of wine for sorbic acid; filtration for catechins from tea and phenolics from brandy; dilution and centrifugation for thiabendazole from juice; sonication and filtration for 4-vinylguaicol and ferulic acid from beer; dilution, centrifugation and chilling for 4-vinylguaicol and ferulic acid from worts; and sonication for prenyl flavonoids from hops and beer. The purist's concept of "direct" injection is clouded by the need for pre-separation treatments in most cases. Nevertheless, extremely valuable data on the use of SPE in the column-switching mode is contained in tabular form.

Pressurised Liquid Extraction (PLE) and Sub-Critical Water Extraction (SWE)

PLE and SWE are new techniques developed for environmental samples but now being applied to food matrices. This thorough review compares the new techniques with SOX (Ramos *et al*. (2002), Chapter 4, ref. 142).

Matrix Solid-phase Dispersion

Two reviews appeared in 2000 by the originator of the technique a decade earlier.[9,10] In those 10 years over 70 papers dealing with applications in food analysis have been published. The author's claim that a wide range of food matrices can be processed using MSPD is verified by the list shown in Table 8.1.

Diffusion

Diffusion Constants by Reversed-flow Gas Chromatography. A major review of the use of reversed-flow GC for measuring diffusion constants contains a valuable review of the history of measurement, theory and GC practice.[11]

Membrane Extraction Methods. The important consideration for the use of membranes for extraction is the dramatic reduction in the consumption of organic solvents. An excellent review of membrane-based extraction methods in relation to the on-line coupling to separation techniques deals with dialysis, electrodialysis, filtration, and membrane extraction.[12] Another useful collection of membrane methods, including liquid membrane extraction, MMLLE, polymeric membrane extraction, and MESI, are described and the methods compared.[13] Liquid membrane extraction is reported in the "Sample Preparation Perspectives" column in LC-GC.[14] Analytical pervaporation – the

Table 8.1 *Applications of MSPD to various food matrices. Consult Barker (2000)[9] for the source references to the original papers*
(Reprinted from data in Table 1 of the *Journal of Chromatography A*, vol. 880, S.A. Barker, "Applications of Matrix Solid-phase Dispersion in Food Analysis", pp. 63–68, © 2000, with permission from Elsevier)

Analyte	*Commodity*	*Analyte*	*Commodity*
Alkylphenethoxylates	Tissues	Pesticides	Beef fat
Aminoglycosides	Cow's kidney		Catfish
Antibacterials	Foods		Crayfish
Benzimidazoles	Animal tissues		Fish
	Cow's liver		Fruit, Vegetables
	Cow's milk (2)		Milk
	Pork		Oranges
	Veal		Oysters
β-Agonists	Cow's liver (2);		Vegetables
β-Carotene	Medical foods		Fish
Carbofuran	Corn		Fruit, vegetables
Chloramphenicol	Milk		Citrus fruits
Chlorsulfuron	Milk	Pyrethroids	Vegetables
Chlorsulon	Milk (2)	Sulfa drugs	Chicken
Clenbuterol	Cow's liver	Sulfadimethoxine	Catfish (3)
	Liver	Sulfamethazine	Animal tissues
Drug Residues	Animal tissue (4)		Porcine tissues (2)
	Foods	Sulfonamides	Animal tissues
	Milk		Infant formula
Furazolidone	Chicken		Meat, milk
	Milk		Milk
	Pork		Salmon (2)
Ivermectin	Fish		Pork
	Milk		Tissues, milk, eggs
	Liver	Tetracyclines	Bovine, porcine tissue
Moxidectin	Bovine tissues		Foods
Nicarbazin	Animal tissues	Vitamins	Milk
Oxamyl, methomyl	Fruits		Medical foods
Oxolinic acid	Catfish		Infant formula
Oxytetracycline	Catfish		
PCBs	Fish		
PCBs, Pesticides	Fish		

integration of evaporation and gas diffusion in a single process – is reviewed in relation to its role in the enological laboratory.[15]

A review of porous membrane media for microdialysis is recommended reading for its potential in food analysis.[16]

Microwave-assisted Soxhlet Extraction (MA-SOX)

The combined MA-SOX technique was applied to the extraction of PAHs, *n*-alkanes, and pesticides.[17]

Osmosis

The osmotic concentration of liquid foods goes back to the use of animal tissues (*e.g.* intestines) as semipermeable membranes for the dehydration (concentration) of fruit juices. Water molecules diffused from the dilute juice into saturated brine until equilibrium was reached. The development of cellulose acetate membranes for reverse osmosis saw the establishment of direct osmosis as a modern extraction process. Temperature driven (membrane distillation) or concentration driven (osmotic distillation) was developed for fruit juice processing with hydrophobic PVDF or PTFE membranes. A recent review of osmotic concentration of liquid foods suggested that direct osmosis was preferred for economic and practical reasons.[18]

Solid-phase Extraction

The separation of lipids using SPE was effective with edible fats and oils, fatty foods and biological samples.[19] Rossi and Zhang reviewed automated SPE,[20] highlighting the availability of 96-well parallel-processing workstations and extraction plates. They discuss on-line SPE, development of automated methods, giving examples. The analysis of liquid food samples using HPLC with automated SPE extraction techniques, either off- or on-line, was reviewed (Bovanová and Brandšteterová, 2000[8]).

Solid-phase Extraction and other Techniques

The principles of SPE and its historical development are reviewed. SPME and other extraction techniques are also covered, and with 352 literature references is a useful paper summarising progress to 2000.[21] A review of instrumental methods for trichothecene analysis contains a useful section on the comparison of SPE columns (charcoal-alumina, Mycosep, Silica, Florisil, and combinations) used in the extraction of the analytes from cereals and other foodstuffs (Langseth and Rundberget, 1998, Chapter 4 ref. 171). The authors also compare SE methods.

Solid-phase Microextraction

Pawliszyn and Co-workers Review SPME.[22] The review by the originator provides an excellent overview of the technique, and the following précis serves to present the essentials of their experiences, and to act as a thumbnail sketch for those wanting a rapid appraisal of the state of the art in SPME.

(Summarised from the *Journal of Chromatography A*, vol. 830, H. Kataoka, H.L. Lord and J. Pawliszyn, "Application of Solid-phase microextration in food analysis", pp. 35–62, © 2000, with permission from Elsevier)

The Case for SPME. In their introduction the authors recognise the importance of the sample extraction step in the total food analysis, whether it be for

quality assessment or for contaminant or adulterant identification. They also affirm the need to reduce the time taken in sample preparation, and in the cost of large volumes of solvent. They claim superiority for SPME on all counts and add the improvement in detection limits. The SPME process is illustrated in clear diagrams and the reader is referred to literature covering partition equilibrium, quantification, types of medium that can be sampled, sampling technique for GC and LC desorption and separation.

SPME for GC and LC Coupling. The differences between the use of the fibre probe SPME for GC insertion and the in-tube SPME collection for LC are discussed. Caution about possible blocking of the capillary tube with particulate matter is noted and the fact that particulate matter is not such a problem with fibre SPME, because particles can be easily removed from the fibre with a distilled water wash before insertion in the GC inlet. The problem for the fibre sampling process is the irreversible adsorption of biomolecules, proteins for example, which change the nature of the surface, rendering the probe unusable in a very short time. Presumably, precipitation or ultracentrifugation may be needed as preliminary clean-up methods. Band broadening is not a problem with the in-tube, decoupled desorption method but may affect the on-line fibre desorption process. The two extraction modes – fibre and in-tube adsorption – provide several alternative strategies for sampling food matrices.

GLPA. Fibres can be used with liquid and gaseous environments and in general extract low-boiling, volatile substances from the gas phase and higher-boiling, semi-volatile compounds from the liquid phase (Chapter 6, Case Studies 1 and 2). In the LC coupled mode the fibre can be used to extract thermally labile compounds, as can the in-tube extraction. There will be exceptions, based on particular chemical properties such as unusually high or low polarity.

Choice of Adsorbent. There are now a number of adsorbents commercially available on fused silica fibres. On the chromatographic principle of affinity between substances of similar polarity, the choice of adsorbent allows preferential extraction from mixtures of chemical classes in food matrices. Again, in general, the thinner coatings adsorb more of the semi-volatile material than the thicker coatings. The thicker the film the greater the adsorbed mass – and hence the higher the sensitivity – but, the thicker film increases the equilibrium time. PDMS is considered to be a general-purpose, rugged surface, while the more polar PA is suited to the extraction of phenols and alcohols. The mixed phase coatings are suitable for both volatile, low molecular weight and polar analytes.

Adsorption of Analytes. Apart from the polarity and the thickness of the adsorbing surface, extraction time and analyte concentration also enter into the equation. Practical expedients include efficient agitation of the sample to provide optimum contact between the surface and the sample molecules and the

creation of the right chemical environment in terms of the pH, salt content, and temperature. Agitation rate and partition constant are the most important properties. There is no need to wait for the equilibration of the fibre in the matrix because there is a linear relationship between the amount of analyte absorbed and its initial concentration. However, this means that reproducible experimental conditions have to be maintained across a series of comparative studies.

The rules of "salting out" apply to the enhancement of extraction efficiency by the addition of NaCl, $NaHCO_3$, Na_2CO_3 and NH_4SO_3, and acidic compounds are extracted from low pH and basic compounds from high pH solutions. Volatile acids or bases are added for liquid sampling (but fibres can be damaged in extreme pH environments) and non-volatile acids or bases for headspace sampling. If it is permissible to increase the temperature of the matrix, then extraction time can be shortened.

In-tube extraction can take advantage of multiple passes of the sample matrix over the absorbing surface. The optimal length is 50–60 cm for extraction efficiency without band broadening. There is a limiting adsorption because further passes of solvent remove solute from the surface. The optimal flow rate is 50–100 $\mu l\ min^{-1}$.

Desorption of Analytes. Gas phase thermal desorption (in the GC injection port) depends on sample volatility, thickness of the fibre coating, injection depth, injector temperature and desorption time. A narrow bore liner is available to reduce the expansion volume area to improve the injection efficiency. Other aspects of good GC practice should be observed.

In-tube desorption can be either static or dynamic, using a minimum of solvent. For strongly adsorbed solutes the static method allows stronger solvents or longer soak times to be employed, for more complete removal. The in-tube fused silica capillary can be coupled to the LC and the sample desorbed directly onto the head of the column. The originator made a further review of the subject for a wide range of applications, including food and flavour analysis.[23]

Other Reviews. Originally, SPME was coupled with GC and GC-MS, but liquid phase samples are now processed with couples to LC and HPLC/MS.[24] Food and biomedical analyses were reviewed by Ulrich, 2000.[25] Food was included in a review of SPME developments in the field of environmental analysis. By the year 2000, 400 articles had been published on the subject, and 27 variations of fibre coating and size were available. Septum-less injections with 23 gauge fibres were possible and a portable field sampler was being used.[26] For the analysis of biological samples SPME was being combined with LC and CE.[27] A recent review discusses the suitability of SPME for MS analyses.[28]

Soxhlet Extraction

The review of Soxhlet extraction treats this most ancient of methods with due respect since it is cited frequently still as the yardstick for modern method

development.[29] The reviewing group prefer the term "leaching" for its physico-chemical precision rather than solvent extraction. They compare SOX to the newer extraction methods of SFE, MAE, and MASE. The updating of SOX to Soxtec® System HT and Soxwave-100 commercial systems, and FMASE, is also reviewed, and the modern SOX was thought to equal recent alternatives.

Supercritical Fluid Extraction

SFE of essential oils is a major consideration, but many other classes of compounds in biological sources are soluble in supercritical CO_2. The review analyses the solubility of essential oil constituents, using them to discuss the theoretical aspects of their isolation and fractionation.[30] Herbal applications closely related to food interests are reviewed. SFE is gaining acceptance, as other solvent methods become unacceptable. Optimisation of the extraction processes is a key issue.[31] The analysis of pesticides by SFE included food applications (fruits and vegetables).[32] High water content samples, polar pesticides, and MRAs were emphasised. SFE for herbal and food and agricultural applications was reviewed, covering sample preparation, selection of modifiers, collection methods, on-line coupling, mechanical problems, and optimisation. Modelling was described for optimising extraction procedures.[33]

Combining Extraction and Preparation Techniques with Capillary Electrophoresis

A thoroughgoing review with 457 literature references, discussing the various methods of combining preparation and extraction techniques with separation by CE, was published in 2001.[34] The methods covered and the main points for food analysis were:

1. Continuous flow systems (CFSs). Four coupling modes were described, off-line, on-line, at-line, and in-line, automating dissolution, filtration, derivatisation, matrix isolation, analyte concentration, and solvent exchange.[35] CGE-ITP was used off-line with a CFS for total titratable acid in roasted coffee.[36]
2. Leaching. Polyphenols were extracted from tea leaves with water using an on-line CFS (Arce *et al.*, 1998, Chapter 4, ref. 47).
3. Dialysis. A fully automated dialysis-SPE-CE system for pharmaceuticals has potential for food analysis.[37]
4. *SPE.* Numerous applications of SPE to clean up samples for CE were reviewed: heterocyclic amines from fried meat and fish; biogenic amines, and *cis*- and *trans*-resveratrol from wines; maleic hydrazides from potatoes and onions; cyclopiazonic acid from milk; multiple herbicides from soybeans; metallothioneins from sheep liver (Beattie *et al.*, 1995, Chapter 6, ref. 86); tetracycline antibiotics from milk; cytokinins from beet and wheat; domoic acid from shellfish; anthraquinone pigments from jelly and juice; and organic acids from wines.

5. MECC. Sodium cholate MECC to characterise hapten–protein conjugates was used for the analysis of soysaponin I in peas and soybean.[38] Zwitterion-coated capillaries reduced protein interaction.
6. Membrane separations. Membrane filtration was used off-line for the preparation of a 41000 MW protein from cod muscle tissue, and vegetable extracts were filtered through a membrane in the analysis of ascorbic acid. Caseins and large peptides from cheese were fractionated through a cut-off membrane for MECC analysis. A micro-membrane was used to filter additives and a cation-exchange membrane was used to select anions from food samples.
7. Solvent extraction. Off-line solvent extraction preceded CE for: polyphenols from tea; histamine, glycine, and 5-hydroxymethylfurfural from foodstuffs; biogenic amines were acid extracted from foods for MECC analysis; organic SE of hydroxycinnamic acids from roasted coffee for CZE analysis; vitamin C extracted from lettuce with dilute oxalic acid; chloramphenicol extracted in ethyl acetate from milk; and flavanoids extracted from sugar cane in acetonitrile–water.

The Extraction of Chemical and Generic Classes of Compounds

List of Specific Topics

Acrylamide extraction and clean-up; the cause of most variation among methods.[39]
Alkaloids (bioactive), carbolines and tetrahydrocarbolines.[40]
β_2-Agonists from meat and milk powder; 119 references.[41]
Garlic extract (aged) and antioxidation.[42]
Flavonoids, SPE from wines for HPLC and CZE separation.[43]
Food flavours, static and dynamic headspace extraction, SPME, and newer methods.[44]
Headspace techniques, *e.g.* purge and trap and SPME, and general review; 85 references.[45]
Herbicide residues in cereals, fruits, and vegetables.[46]
Heterocyclic amine food mutagens/carcinogens.[47] SPE used.
Molecular imprinting technology for polymers with biomimetic receptors;[48] 98 references.
Mycotoxins possessing carboxyl groups.[49]
Ochratoxin A in foodstuffs.[50] Partitioned into aqueous sodium bicarbonate.
PAHs in edible fats and oils.[51] Off-line LC-LC.
Phenolic acids in foods.[52]
Pesticides in honey and other bee products.[53]
Phenols (bioactive) from fruit and vegetables[54] and food and plants.[55]
Sulphur compounds in wine aroma. L-LE, static and dynamic HS, and SPME.[56]
Tomato carotenoids.[57]
Tetracycline antibiotics in foods;[58] 180 references.
Thiamine by HPLC methods. Sample extraction methods emphasised.[59]

Alkylresorcinols

The extraction of alkylresorcinols (ARs) from cereal grains was reviewed for analysis by TLC, CC, GC, GC-MS and HPLC. With log $P_{o/w}$ in the range 8.5–13.4 ARs are insoluble in water. Solvent extraction of 0.1–5 g samples of un-milled grains with acetone, MeOH, or ethyl acetate for 16–24 h, or SOX (acetone or cyclohexane) for 2 h were the preferred methods.[60] Multiple extractions are necessary for <5 ml g^{-1} while one-step extractions are adequate for 40–50 ml g^{-1} volumes. Milling reduces extraction times but increases co-extractable interferents for the chromatographic stage, as does the use of MeOH and 80% EtOH.

Allergens

The need for pure allergens for diagnosis and therapy is partly behind the development of protocols for protein extraction from foods. Purification methods and the extraction of allergens from animal and vegetable foods are reviewed.[61] Methods include ion-exchange, gel filtration, and RP chromatography (102 references). Electrophoretic methods were favoured for the analysis of allergens (30 references).[62]

Antibacterial Drug Residues

Clean-up procedures, UAE, LLP, SFE, IAE, and MSPD, for the extraction, deproteinisation, and concentration of antibiotics (aminoglycosides, chloramphenicol, sulphonamides, tetracyclines, macrolides, beta-lactams, *etc.*) from foods of animal origin are reviewed with 97 references.[63]

Antioxidants

In the light of the interest in natural antioxidants (*e.g.* polyphenols) for protection from disease, the extraction processes are reviewed.[64] Attention is focused on their extraction from inexpensive sources.

Biogenic Amines

Heteroaromatic amines are reviewed in relation to their extraction and preconcentration from several food matrices.[65] The sorbents and solvents used in the sample treatment are discussed: 123 references are quoted. Biogenic amines in general were reviewed in relation to their role in cellular metabolism, and their isolation and characterisation. Recent advances in extraction from, and analysis in, plant tissues were included.[66]

Bioactive Proteins and Peptides

Food chemists are asked to devise automated and continuous extraction methods for bioactive proteins and peptides that could be returned to functional

foods or nutraceuticals.[67] Bioactive peptides derived from milk, corn, fish, wheat gluten, casein, rice and soybean protein are mentioned. Column chromatography and solvent extraction methods are extant.

Carotenoids

The isomeric cis–trans forms of the complex group of carotenoids found in both animal and vegetable foods means that special extraction methods are required for their quantitative analysis. The conjugated double bond system is particularly sensitive to conversion by light, heat, oxygen and acids. Thus, special handling arrangements, and the fact that standard compounds are difficult to obtain, make the assay difficult.[68] Carotenoid esters are also reviewed.

Dioxins and Furans

Firestone reviewed the extraction of dibenzo-*p*-dioxins and dibenzofurans from foods and biological tissues in 1991.[69] Acid or base hydrolysis preceding SOX, LLE, SPE, and other column extraction procedures, H_2SO_4 partitioning, Florisil, silica gel, and alumina chromatography, GPC, SEC, LC fractionation with NP and RP columns, activated carbon adsorption, are covered, along with immunoassays (109 references). A recent review of chlorinated dioxins in ground beef emphasised the synergism of ELISA/GC/MS, direct sample introduction GC/MS/MS, automated clean-up, and SFE methods.[70]

Fat-Soluble Vitamins

SE, SFE, and SPE are reviewed for the extraction of fat-soluble vitamins from, *inter alia*, human foods (Luque-Garcia and Luque de Castro, 2001, Chapter 4, ref. 36). They concluded that SE, SPE, and SFE were the main extraction methods presently in use, and automation was seen as the main trend in method development to reduce handling and optimise analysis time.

Flavanoids

New techniques are required for the extraction and measurement of flavanoid composition of various foods to aid the elucidation of their protective properties. The areas of extraction already employed are listed as disruption of the food matrix, SE, enzyme hydrolysis, and acid hydrolysis, followed by HPLC.[71]

Contaminants from Packaging

A valuable review brings together most of the analytical work on the subject and includes the clean up methodology, SEC, SE, saponification, SPE, SFE, SFE-SFC, P & T, GPC, and MAE, with particular reference to the use of on-line extraction–separation–detection methods.[72] It also covers the diffusion, solvation and dispersion of migrants from film into food.

Patulin

Incidental information about extraction methods is given in a review of three separation techniques for the analysis of the mycotoxin patulin.[73] SE (ethyl acetate) and SGC preceded TLC in the AOAC method (974.18), with a LOD of 20 μg l^{-1}. Developments included diphasic dialysis with a LOD of 50 μg l^{-1}. GC analysis used derivatives: TMS ether, HFB, and acylation, but underivatised patulin could be chromatographed under electronic pressure control conditions. With MS detection the LOD for derivatised patulin was 10 μg l^{-1} and the underivatised patulin had a LOD of 4 μg l^{-1}. However, in a FAPAS trial,[74] most laboratories used HPLC methods with SE (ethyl acetate), diphasic dialysis and SGC. An interesting alternative was the use of solvent partitioning with sodium carbonate, for the removal of phenolic interferents.[75] The review itemises the modern SPE methods developed to improve the removal of interferences for work in specific matrices.

Pesticides

There are over 500 registered pesticides, including insecticides, herbicides, fungicides, and similar products. The legislative requirements of many countries have driven the development of analytical methods for their detection in the environment. Consequently, the literature abounds with sensitive assays, usually based on chromatography-MS, and recent interest has moved towards MRMs in order to reduce the enormous workload.

Ten extraction techniques (LLP, SOX, ASE, MAE, SPE, SPME, MSPD, SFC, USE and GPC) used in analytical methods for the estimation of pesticides in foods are reviewed with an historical introduction and 66 references.[76] SFE was found to be a rapid and universal method with low solvent consumption, but the need for ultrapure CO_2 was a limitation, and difficulties may be experienced with moist foods. With high lipid content matrices, GPC using Bio Beads SX-3 and solvent mixtures such as cyclohexane–ethyl acetate or cyclohexane–DCM was popular. Ease of automation was also noted. Sweep co-distillation has the advantage of low capital cost and reduced solvent consumption, and can handle a broad range of pesticides and substrates.

Three extraction techniques, SFE, MSPD, and sweep co-distillation, placed into the context of LLP, SPE, GPC and adsorption column chromatography are reviewed with 99 references, for the extraction of pesticide residue samples of plant origin.[77] Useful tables of commodity classification (Section 4, Chapter 1), GPC clean-up methods (and references), and isolation and clean-up techniques are given.

Phytoestrogens

Genistein, daidzein, lignans and their derivatives in foods are covered in a review of measurement methods made in 2002. LODs from different laboratories vary widely: the best being 0.002 pmol per assay for daidzein by radioimmunoassay.[78] Some 90 papers were used to comment on extraction methods, LODs, *etc.*, and to conclude that an interlaboratory trial was needed.

Polychlorinated Biphenyls (PCBs)

Introduction. Workers at the Joint Research Centre, Ispra, Italy, published a review of extraction methods for the rapid analysis and screening of PCBs in food matrices, with emphasis on the seven indicator PCBs. Faced with the classical methods of extraction by cold column, or partitioning, or cold centrifugation, under reflux or by SOX, they discuss the use of SFE, MAE, and ASE for solid samples, SPE and SPME for liquid samples and the new extracting syringe (ESy) method, and alternative immunoassay and bioassay methods.[79] Furthermore, the extraction step is accompanied by a clean-up procedure such as LLP, column chromatography on activated Florisil, or GPC. The authors are concerned about the "bottleneck" commonly caused by the sample preparation in the total analytical procedure, *e.g.* Majors, 1991,[80] the excessive use of solvents, long extraction times, and labour costs.

Summary of Findings. MAE has not reached its potential for PCB extraction from food matrices, whereas SFE is well established and has potential for combination with adsorption methods for further clean-up and consecutive fat estimation. The co-elution of lipids with PCBs requires further clean up and the laboratory has developed the use of sulphuric acid treated silica gel, added to the extraction thimble to produce a clear fat-free extract.[81] SPE displays increased sensitivity and reproducibility and is faster than liquid extraction methods; although clogging of filters and breakthrough were reported disadvantages. SPME was valuable for PCB analysis and automated SPME[82] was seen as even more competitive. The ESy method was included because its potential for PCB analysis was foreseen. This article extends the debate reviewed in 1999.[83]

Polyphenols

Chapter 1 of *Methods in Polyphenol Analysis* covers all the major extraction methods in detail,[84] providing an up-to-date account of the options open to the analyst for these analytes. MAE, PLE, SFE, SPE and SE are discussed, along with details of the auxiliary operations, centrifugation, *etc.* for anthocyanins, flavan-3-ols, proanthocyanidins, and other classes.

Proteins

A review gives valuable information about the practical detail of Kjeldahl nitrogen determination, in particular for milk protein but also for other dairy products in relation to the evaluation of test results, bearing in mind the use of the Kjeldahl method as a "gold standard" in food analysis[85] (30 references).

Volatile Substances

A major review by Stevenson *et al.* (1996) included a section on classical and new sample preparation and extraction methods for subsequent GC analysis of

volatile compounds originating from foods, in particular dairy protein products such as whey protein concentrate powder.[86] Various combinations of steam distillation, vacuum distillation, LLE, freeze concentration, SPE, SPME, P & T, and SDE are discussed. The development of SDE is covered in 10 references, over the period 1973 to 1995. A recent review of preconcentration and enrichment techniques, mainly for dairy product analysis, is recommended for its thorough treatment of the use of dynamic headspace and SPME (including SPDE and S-BSE) methods.[87]

The extraction of volatile substances from fruit juices on an industrial scale was reviewed for six extraction processes:

1. Vapour–liquid extractions by distillation/evaporation
2. Vapour–liquid extractions by partial condensation
3. Gas injection extractions
4. Adsorption
5. SFE
6. Pervaporation

This paper provides a useful source of the equations linking Raoult's law and Dalton's law and expressing the relationship between vapour and liquid at equilibrium.[88]

Automated Preparation and Extraction Methods

Direct Analysis of the Food Matrix

Minimal sample preparation is required if flow-switching valves are used to manage the automated extraction. Conditioning, washing, adsorption and desorption of the analytes from a pre-column, and flushing unwanted fractions to waste and transferring the analytes to the analytical column for detection and quantification are typical processes controlled in the direct analysis of the food matrix. The review by Bovanová and Brandšteterová (2000)[8] makes a good introduction to this topic. The pre-column is normally an SPE adsorbent, and automated off-line systems are commercially available. Automated off-line SPE, on-line SPE coupled to HPLC, and on-line dialysis are discussed.

Methods for Dioxin Analysis

A recent article by Focant *et al*., 2004, reviews SFE, MAE, PLE and SPE and then discusses the automation of sample preparation around PLE and SPE for dioxin analysis by GC-MS[89] (Section 3).

Automated Preparation, Extraction and Separation Methods

Methods for Protein Preparation for Proteomics

Genomics and Proteomics. The rapid advancement in genomics has facilitated, *e.g.*, the mapping of approximately 30000 genes of the human genome in

2000. Proteomics is the equivalent study of the proteins produced by a cell, which may be ten times more in number than the genes and involves a dynamic pattern of chemically modified protein molecules manufactured to deal with the changing requirements of the cell. The analytical requirements are extremely demanding, especially since the products of post-translational change have very different chemical properties. But before the separation of the protein mixture is started it is important to ensure that the sample is free from contaminants.

Sample Purification. Proteins isolated from biological sources can contain other natural cell constituents such as polysaccharides, lipids, and nucleic acids. Also, during the preparation buffers, preservatives and salts can be present. A review by Wells and Weil (2003)[90] lists the following purification processes:

1. Desalting
2. Concentration
3. Centrifugation
4. Dialysis
5. Filtration and ultrafiltration
6. Precipitation
7. Lyophilisation

and emphasises the point that proteins are easily degraded and care must be taken to avoid conversion. A typical series of steps in the extraction of the protein from the tissues or cells would include:

1. Lysis of the cell material (UAE, enzyme digestion, laser capture microdissection or mechanical release).
2. Extraction of nucleic acids (pptn or UAE).
3. Extraction of lipids (excess detergent or pptn).

Separation and Identification. There are several approaches to the characterisation of protein mixtures. Most modern methods use gel electrophoresis, in its various forms (polyacrylamide, 2D), to separate and visualise the solubilised mixture. The individual proteins are digested with enzymes of specific activity to create a family of peptides of known terminal amino acid residues. The peptide mixtures are separated and the individual peptides sequenced by Edman degradation or FAB or MALDI mass spectrometry.

On-line Separation and Identification. Recent developments have concentrated on the use of LC/MS techniques such as ESI that put the separation method on-line with the detector and exploit the identification capability of the mass spectral data. Once the information overload of the automated system had been addressed, attention turned to the remaining manual part of the process, the extraction. Gel electrophoresis gave excellent spatial separation but missed some proteins. Therefore, other electrophoretic methods were considered.

On-line Extraction, Separation and Identification. CE has entered the arena, especially combined with sample concentration techniques such as on-column "stacking" and on-column micro SPE "trapping", to raise the molar concentration to cope with the picomole level requirements of samples eluted from GE columns and plates. Isotachophoresis has laid dormant for some years but now CITP is being coupled to the separation stage to provide some preliminary separation into a continuum of concentration-based stacked bands (the longer the band the higher the concentration or, conversely, trace components are concentrated into narrow bands). Similarly, IEF produces highly concentrated zones of peptides or proteins separated according to their isoelectric points along the separation column filled with ampholytes. The latest technology uses ampholyte-free IEF. Thus, if capillary flow switching can be carried out without loss of resolution, the extraction (concentration) stage can be put on-line. Multicolumn separation stages make a continuous and automated protein analysis possible. The present state of the art for nutritionally relevant proteins was reviewed by Kvasnička (2003).[91]

Automation of Protein Sample Preparation. Many of the steps in the automation of the preparation of peptides from proteins for proteomic research have been automated with workstations. The routine taken over by liquid-handling instruments includes the tedious pipetting, washing, desalting, concentration of peptides before "spotting" a sample into the MALDI matrix on the target plate. There are now instruments that will supervise the removal of the protein spot extracted by 2D GE, undertake the enzyme digestion, and prepare and load the MALDI plate. The review by Wells and Weil (2003)[90] lists the manufacturers of equipment for these processes. The whole proteomics endeavour is generating rapid advancement in the technological support required for complex, biologically-sensitive sample handling at ultra-high sensitivity, and on small footprints.

3 Recent Developments

Acrylamide Analysis

The review by Wenzl *et al.* (2003)[93] avers that the extraction and clean-up for acrylamide are the sources of most variation among analytical methods. Roach *et al.* (2003) used room temperature aqueous extraction followed by SPE in preparation for LC-MS/MS.[92] Recent work by Hoenicke *et al.* (2004) provides an up to date approach using LC-MS/MS and GC-MS/MS and describes the sample preparation necessary for LC-MS/MS when either rapid sample throughput (method 1) or robust reliability (method 2) is the objective.[93] Method 1 is applicable to easily managed matrices such as crisps, chips, cereals, bread and roasted coffee, while method 2 is required for cacao, soluble coffee, molasses, and malt. Both methods depend upon the fact that acrylamide is very soluble in water and less soluble in organic solvents, and the aqueous expansion of the

matrix was improved by defatting. Optimisation trials found that most matrices were extracted in 15 min, but to ensure complete extraction of the more intransigent materials a time of 30 min, was used throughout. The weight of water added was adjusted to produce homogeneous swelling. The use of PLE or SOX (MeOH) was not successful in their hands.

Method 1. 2 g homogenised and standardised sample was defatted with 80 ml iso-hexane and 20 ml water added and the sample ultrasonicated for 30 min at 60 ºC. The sample was purified (acetonitrile/Carrez I/Carrez II), centrifuged and filtered.

Method 2. 2 g homogenised and standardised sample was dissolved in 50 ml water and sonicated, purified (iso-hexane in place of acetonitrile), and centrifuged as for Method 1. The aqueous phase was saturated with NaCl and extracted twice with 50 ml ethyl acetate.

The Carrez precipitation was a sufficiently effective clean-up procedure, making the more time consuming use of SPE unnecessary. Some extra precautions were taken for difficult or low level matrices. GC-MS/MS with two mass transitions was effective down to 5 $\mu g\ kg^{-1}$ without the need for derivatisation, exemplifying the use of instrumental methods of resolving potential interferences.

Sixty two analytical laboratories using seven different measurement techniques and various extraction procedures participated in an ILC study of acrylamide in crispbread and butter cookies.[94] One sample was near to the LOD and one was at a higher level. A lack of agreement prompted a closed examination of the methodology, including extraction solvent, quantity weighed, calibration method, clean up and experience of the participating laboratories.

Extracting Syringe Method

Norberg and Thordarson introduced the method in 2000.[95] It is essentially a direct inlet for coupling LLE to GC. It has not been used for food analysis yet, but would appear to have the potential.

Automation

A review article by Focant *et al*. 2004 (Section 2)[89] summarises SFE, MAE, PLE and SPE before bringing together in one series of experiments the coupling of two extraction techniques, PLE and SPE, for the automatic extraction of dioxins from biological matrices. The automated commercial instrument can clean up 10 samples containing up to 1 g lipids in <2 h. It uses a combination of three SPE cartridges and four solvents (Figure 8.2). The illustration shows a five-fraction collector used for dioxin separation, and after a series of operations PCDDs and PCDFs, cPCBs, PCBs and PBDEs were collected in fractions 1 and 2. This clean-up methodology is multi-column solid-phase "chromatography"

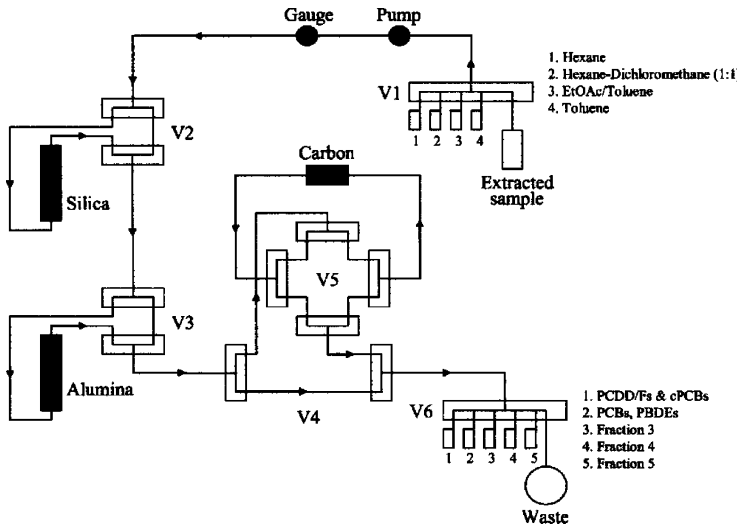

Figure 8.2 *Schematic of a commercial (Fluid Management Systems Inc.) automated SPE extraction procedure for dioxins. The system can remove up to 1 g lipid in <2 h and different fractions can be collected,* e.g. *PCDDs, PCBs,* etc.
(Reprinted from *Talanta*, vol. 63, J-F. Focant, C. Pirard and E. De Pauw, "Automated Sample Preparation-Fractionation for the Measurement of Dioxins and Related Compounds in Biological Matrices: A Review", pp. 1101–1113, © 2004, with permission from Elsevier)

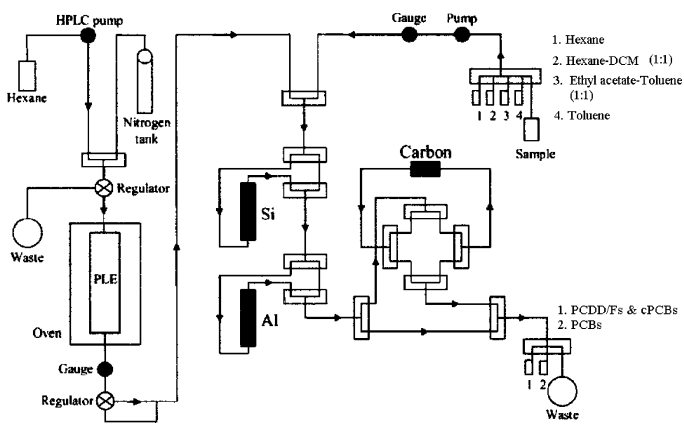

Figure 8.3 *Schematic of the integrated SPE and PLE processes*
(Reprinted from *Talanta*, vol. 63, J-F. Focant, C. Pirard and E. De Pauw, "Automated Sample Preparation-Fractionation for the Measurement of Dioxins and Related Compounds in Biological Matrices: A Review", pp. 1101–1113, © 2004, with permission from Elsevier)

and the employment of PLE to produce the sample containing dioxins from the food matrix for further separation is shown schematically in Figure 8.3. The potential here for automatic sample extraction and chromatographic

fractionation of many food matrices and analytes can be readily exploited and no doubt the next step, probably already under development, will be to couple the final fraction collector to the LC/MS stage for high resolution separation and detection.

Field Flow Fractionation

Field flow fractionation (FFF) refers to the separation of particles by molecular size. It is a sieving technique applied to molecules, particles and cells over the whole MW range. Separation occurs when a field or gradient is applied perpendicularly to an unobstructed thin channel through which a solubilised sample flows (Figure 8.4).

Molecularly-imprinted Polymers

A review in 2000 outlined new configurations and applications for MIPs. For example, bulk polymers imprinted with β-lactam antibiotics can be used as a stationary phase for the separation of β-lactam antibiotics.[96] The preparation of polymers is discussed. Applications to food analysis are expected to continue to increase.

MSPD for PCBs in Fat

There are many occasions when the extraction of pesticides from fatty tissues has been given special attention. SOX, MAE, CO_2-SFE, and PLE have been used and further clean-up, *e.g.* SEC, added. In this recent article[97] the use of MSPD as a one-step extraction purification technique for PCBs from butter, chicken and beef fat is described as an alternative that has the advantages of

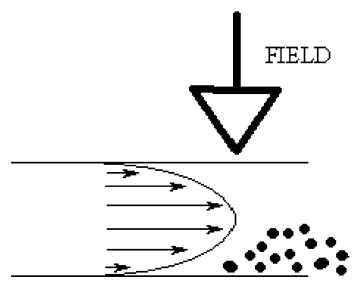

Figure 8.4 *Laminar flow velocity of the moving solvent front varies from a maximum at the centre to zero at the channel wall. The strength of the applied field inversely determines the thickness of the layer at the wall. The nearer the wall the particles are driven by the applied field the slower they move, creating a gradient with some particles moving faster than others. Unlike chromatography there is no partitioning between the phases*

being a low cost and simple method, requiring only the resolution of GC-ECD for the separation/detection stage. Florisil as adsorbent was mixed with the sample and the mixture added to the top of a Florisil SPE cartridge, and the pesticides removed with a solvent, *e.g.* hexane.

Pittsburgh Conference 2003

R.E. Majors in his "Column Watch" article[98] tabulates 21 sample preparation products (Table 2 of the article), most of which fit into the SPE category. The manufacturers' names and the technical details of 8 SPE cartridges, 2 protein removal cartridges, 2 digestion tubes for in-line enzymatic hydrolysis using immobilised papain and pepsin, 2 scavenger products, a disposable Büchner funnel, which combines SPE and filtration on a larger scale than the standard SPE cartridges, 6 and 8 mm SPE disks, and 4 new 96-well plates are included.

Scavenger Extractors

The idea of adsorbing target compounds is not new but the application to "mop up" surplus reactant molecules, *e.g.* those used in combinatorial syntheses, through covalent bonding, may find uses in food analysis. Scavenger products are available (consult Majors, 2003) with a range of different chemistries, which lend themselves to on-line clean up processes, and the removal of excess derivatising agent, for, say, esterifying fatty acids that would have been useful in this laboratory in the past. Scavenger extraction can be carried out in 1, 3, 6, 10, 25 and 75 ml cartridges or 48- or 96-well plates.

Food Sensor Network

A meeting at the Leatherhead Food International was held in February 2004 to launch the Food Sensor Network applied to food safety, quality and functionality. Thirty nine companies had registered by June 2004, and the website www.foodsensor.org has been set up to communicate with industry and research interests. Further information is available from Catherine Entwisle (centwisle@leatherheadfood.com).

Sulphite Measurement

The chemiluminescent emission from sulphite-induced autoxidation of the Ni(II)/tetraglycine complex in the presence of luminol was used to measure sulphite extracted from samples of wine and juices in a gas diffusion cell.[99] The LOD was 2.8×10^{-8} mol l^{-1} with a RSD of 4.6%.

Report on HTC-8

Heather Lord and Janusz Pawliszyn reported at the 8th International Symposium on Hyphenated Techniques on Chromatography and Hyphenated

Chromatographic Analysers, Bruges, Belgium, 4–6 February 2004.[100] Of interest was the discussion on chemical cytometry for the microseparation of single cells, their lysing and separation of the contents by CE. Porous and non-porous membranes on-line to GC for breath analysis, SPME of the volatiles emitted by living plants, and SFE/MS for rapid (10 min) analysis of plant extracts were also discussed.

Training for Collaborative Studies

In the validation of methods for mycotoxins in foodstuffs, Gilbert and Anklam prepared videos of the methods with special attention to the critical steps, and held workshops for participants. "Impressive" performance characteristics led to the adoption of six procedures by AOAC International as First Action Methods and seven methods by CEN as European standards.[101]

Application of SDME

SDME was reviewed by Psillakis and Kalogerakis, 2003 (Appendix 1) and is finding applications in food analysis, such as the determination of phthalate esters in food simulants.[102] The technique of suspending a microdrop of water-immiscible solvent into a stirred aqueous matrix provides a simple, virtually solvent-less and low cost means of extraction. The potential of having a wide range of liquid adsorbents to choose from for selective extraction of target analytes, easily retracted into the syringe needle for GC injection, would seem to equal that of SPME, while offering greater versatility than SPME.

4 Concluding Statement

The general conclusion from reading the reports of method development over the past ten years is that more often than not the extraction stage is the most problematic in the remaining process of total automation of food analytical methods. In addition, much of the "front-line" development has been directed towards the automation and putting on-line of those extraction techniques, and the related preparation methods, that are amenable to it.

Several research groups are using the concept of total assay resolution in their protocols to avoid unnecessary effort in effecting a separation in any one area of the total procedure. This is also an extremely valuable exercise in making the method cost effective. The use of chromatography–MS in all its diverse forms is providing high resolution that allows shortcuts to be made in the preliminary stages of the sample work-up where this sophisticated instrumentation is available. Where low-resolution equipment has to be used for this stage, the skills of the analyst are challenged to combine the modern extraction methods to optimise analyte resolution so that the resolving power of the total assay is adequate. Already, there are many examples of the move towards this goal.

The most promising area of high sensitivity, high resolution separation is in the use of capillary electrophoresis in its various forms. An interesting revival

of isotachophoresis is in its use as a preconcentration device on-line with a high-resolution form of CE such as CZE or, in future, CEC.

Many of the classical methods of extraction are not directly amenable to automation, but the principles can be incorporated into small-scale versions such as micromembrane techniques. While the demand to reduce solvent use has relegated many of the techniques discussed in Chapters 4 and 5, it is surprising how many modified "miniaturised" versions continue. Not all food analysts have access to expensive instrumentation and examples of ingenious, low-cost methods are being developed.

The vanguard, however, equipped with the latest separation and detection instrumentation, and having access to, or the ability to develop, robotic methods, are moving the whole process towards an era of extractionless technology, where remote sampling, robotic preparation and/or on-line extractions are the order of the day. The rapid expansion of the technology to perform microfluidics "on a chip" raises the question, "How long will it be before nanotechnology, analysis in sub microgram volumes of liquid, enters the food analytical laboratory"? But, as miniaturisation progresses more attention has to be paid to the homogeneity of the sample taken for extraction.

The literature "in press" at this time is burgeoning. Work has appeared on acylated anthocyanins, lycopene in tomatoes, and lycopene measured by SFE-HPLC, pesticides in fruits, PLE followed by MECC, trienzyme release of folate, dioxins and dioxin-like PCBs, zearalenone in cereals by ASE, EtOH and supercritical CO_2 extraction of essential oils, *etc.*

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APPENDIX 1

Comparisons of Extraction Methods

During the research for the chapters on specific methods of extraction – the main objective for this monograph – it became apparent that many authors evaluated and compared methods in the course of establishing the most suitable method for their application. Examples of these comparisons have been collected and collated in chronological order to provide a database. Consult Glossary of Abbreviations. Reference is made to the source material *e.g.* (Chapter 4), or (Table only) if no further reference is made to the method comparison.

Methods	*Commodity/compound*	*Reference*	*Comment*
1. SOX 2. SE ($CHCl_3$–MeOH–H_2O)	Cholesterol in ready-to-serve foods by GC	J.R. van Delden, J.L. Cozijnsen and P. Folstar, *Food Chem.*, 1981, **7**, 117. (Chapter 2)	No significant difference was found between the two extraction methods. Thus a simple homogenate is sufficient for GC analysis
1. MOD 2. TEA 3. DC	*N*-Nitrosopyrrolidine in fried dry cured bacon	R.A. Gates, J.W. Pensabene and W. Fiddler, *J AOAC*, 1984, **67**, 236. (Chapter 5)	The official AOAC MOD-TEA method was being replaced by the dry column method developed at ERRC, USA. Artefacts found with the MOD were not found with DC
1. Gerber method 2. SOX	Fat in Vegetables, *e.g.* tree nuts	I. Rosenthal, U. Merin, G. Popel and S. Bernstein, *J. AOAC*, 1985, **68**, 1226. (Chapter 1)	Volumetric acid digestion in Gerber tube
1. StD 2. SPE	Sorbic acid and other preservatives in foodstuffs	H. Terada and Y. Sakabe, *J. Chromatogr. A*, 1985, **346**, 333. (Chapter 5)	In combination with ion-pair HPLC
1. SPE 2. LTP 3. SCoD	Evaluation of sweep co-distillation	J. Mes and D.J. Davies, *Int. J. Environ. Anal. Chem.*, 1985, **19**, 203. (Chapter 5)	Beef and pork fat, corn, peanut, rapeseed and paraffin oils and vegetable shortening were used in the evaluation of SCoD for the recovery (>80% for most) of 26 environmental chemicals

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Soak in Hex 2. 3× Hex Dist 3. Soak in acetone–H_2O	Ethylene dibromide in grains	M. Clower Jr, J.P. McCarthy and L.J. Carson, *J. AOAC*, 1986, **69**, 87. (Table only)	4 days soaking in hexane released least EDB; acetone soak released the same as or up to 25% more than triple co-distillation
1. MAE 2. SOX 3. Shake flask	General foods extraction	K. Ganzler, A. Salgó and K. Valkó, *J. Chromatogr.*, 1986, **371**, 299. (Chapter 2)	Ground sample mixed with MeOH or MeOH–H_2O (polar), or hexane (non-polar)
1. MWD 2. ICM	Bisulphite in potato products	J.G. Moylan, F.W. Bowes and W.J. Pappin, *J. AOAC*, 1986, **69**, 11. (Chapter 5)	Below 60 ppm both methods lacked accuracy
1. FIA 2. MWD 3. C(PR) 4. Enz SO	Methods for sulphites in foods	Sullivan *et al. J. AOAC*, 1986, **69**, 542. (Chapter 5)	With an LOD of 0.1 ppm the FIA method was a vast improvement on current methods
1. SE 2. SGC 3. SE of residue 4. Dep LLP 5. Dep SE 6. SPE	Aflatoxin M_1 in liquid milk	M.J. Shepherd, M. Holmes and J. Gilbert, *J. Chromatogr.*, 1986, **354**, 305. (Table only)	Six published methods were compared for analysis time, recovery, and cost. HPLC chromatograms were published of each method to compare the quality of the final extracts obtained. The SPE method was found to be the superior method
1. Kjeldahl 2. Kjeltec 3. Hach	Total N in various foods	K.L. Watkins, T.L. Veum and G.F. Krause, *J. AOAC*, 1987, **70**, 410. (Chapter 1)	Kjeldahl > Hach > Kjeltec for Total N. The higher sensitivity of the Hach method was noted
1. DPP 2. MWD	Sulphites in foods	Holak and Patel, *J. AOAC*, 1987, **70**, 572. (Chapter 5)	Free and total sulphite in shrimp, orange juice, peas, dried apricots, and DH potatoes compared to MWD

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Kjeldahl 2. Dumas	Protein nitrogen in infant food	G. Bellomonte, A. Costantini and S. Giammarioli, *J. AOAC*, 1987, **70**, 227. (Chapter 1)	Comparable results. Dumas method faster, easier and less pollution
1. Sulfitest 2. MWD	Qualitative test for sulphites in foods	Nordlee *et al.*, *J. Allergy Clin. Immunol.*, 1988, **81**, 537. (Chapter 5)	Evaluated against the MWD method
1. MOD 2. DC 3. LTVD	*N*-Nitrosopyrrolidine in fried dry-cured and pump-cured bacon	A.J. Malanoski, W.J. Smith and T. Phillipo, *J. AOAC*, 1988, **71**, 504. (Table only)	TEA was used for all three methods and all three methods were found to be equivalent
1. HS-LC 2. MWD	Sulphites in various foods	J.F. Lawrence and R.K. Chadha, *J. AOAC*, 1988, **71**, 930. (Chapter 5)	LOD of 1 $\mu g\ g^{-1}$. MWD as standard
1. AcE-IExC 2. MWD	Vegetables with natural sulphites, *etc.*	H.J. Kim, *J. AOAC*, 1989, **72**, 266. (Chapter 5)	AcE and AcD studied. MWD as standard
1. μ Kjeldahl 2. Lowry 3. Bradford 4. biuret	Total N in *phaseolus vulgaris* beans.	O. Paredes-López, F. Guevara-Lara, M.L. Schevenin-Pinedo and R. Montes-Rivera, *Plant Foods For Human Nutrition* (Dordrecht, Netherlands), 1989, **39**, 137. (Chapter 1)	The micro Kjeldahl method gave different results from the other 3 methods
1. HS-LC + IEC 2. DI-LC + IEC 3. DI-LC + IExC 4. Op-MWD	Sulphite in 5 foods.	J.F. Lawrence, R.K. Chadha and C. Ménard *J. AOAC*, 1990, **73**, 77. (Chapter 5)	All methods similar for wine, lemon juice, and raisins. IMP and onion flakes gave problems. MWD method as standard
1. Mod MWD + HPLC 2. MWD	Sulphite in foods	L. Pizzoferrato, E. Quattrucci and G. Di Lullo, *Food Addit. Contam.*, 1990, **7**, 189. (Chapter 5)	Potential interference is avoided by the high resolution of the HPLC method. MWD as standard

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. FIA 2. MWD	Sulphite in shrimps, potato, dried pineapple, white wine	J.J. Sullivan, T.A. Hollingworth, M.M. Wekell, V.A. Meo, H.H. Saba, A. Etemad-Moghadam, C. Eklund, J.G. Phillips and B.H. Gump, *J. AOAC*, 1990, **73**, 35. (Chapter 5)	Adopted official first action for determination of greater than or equal to 5 ppm total sulphite. MWD as standard
1. AcD-IExC 2. AcE-IExC 3. Mod-MWD	Sulphite in grapes	H.J. Kim, K.R. Conca and M.J. Richardson, *J. AOAC*, 1990, **73**, 983. (Chapter 5)	IExC used ECD. MWD gave false positive. Reduce distillation time to 60 min. IExC method is rapid, sensitive, interference-free
1. StD 2. MD	Fluoride in foodstuffs.	H. Koga, Y. Tanabe, M. Hinoide and Y. Takaesu, *Dental Sci. Reps.*, 1990, **90**, 979. (Chapter 3).	Similar results on shrimp analysis
1. IExC-ECD 2. MWD	Sulphite in various foods.	H.J. Kim, *J. AOAC*, 1990, **73**, 216. (Chapter 5)	AlkHyd and IExC compared with MWD. Initial sulphite OK, but IEC higher for spikes. (Appendix 3)
1. SFE 2. SOX	PCB congeners in lyophilised fish tissue.	S. Bøwadt, B. Johansson, P. Fruekilde M. Hansen, D. Zilli, B. Larsen and J. de Boer, *J. Chromatogr. A*, 1994, **675**, 189. (Chapter 4)	LOD of 0.5–2 ng g^{-1} and SDs of <5%
1. IAE 2. SPE	Aflatoxin M_1 in cheese	S. Dragacci, E. Gleizes, J.M. Fremy and A.A. Candlish, *Food Addit. Contam.*, 1995, **12**, 59 (Chapter 6)	Immunoaffinity extraction gave cleaner preparation. Recoveries of around 75%; LOQ 0.02 μg kg^{-1}
1. *p*-Rosaniline 2. MWD 3. ENZ	SO_2 loss from bottled and canned beer	D.R. Ilett and W.J. Simpson, *Food Res. Int.*, 1995, **28**, 393. (Table only)	pH was raised in the presence of Hg ions to stabilise SO_2 ions, and prevent recombination with carbonyls. (3) Beer treated with bentonite to remove interferents

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Kjeldahl 2. Dumas	Total N in milk	E. Jakob, C. Sievert, S. Sommer and Z. Puhan, *Z. Lebensm. Unters-Forsch.*, 1995, **200**, 239. (Chapter 1)	LECO FP-428 autoanalyser was used for the Dumas method. For milk there was a good correlation
1. SE + HPLC 2. StD + HPLC 3. StD + VIS	Benzoic and sorbic acids in fruit and vegetables.	A. Montaño, A.H. Sánchez and L. Rejano, *The Analyst*, 1995, **120**, 2483. (Chapter 5)	1. Extraction with 60% MeOH and isocratic RP-HPLC, 2. StD and isocratic RP-HPLC 3. StD and spectrophotometry
1. SFE 2. SE 3. SOX	Organochlorine pesticides in eggs	Y.Y. Wigfield, J. Selwyn, S. Khan and R. McDowell, *Chemosphere*, 1996, **32**, 841. (Chapter 4)	Further clean up required C_{18} and Florisil SPE
1. SFE 2. SPE 3. MOD 4. LTVD	*N*-Nitrosamines (NPYR and NDMA) in fried bacon, *etc.*	W. Fiddler and J.W. Pensabene, *J. AOAC Int.*, 1996, **79**, 895. (Chapter 4)	SFE was superior to the other methods. Significant differences ($p < 0.05$) reported
1. Caffeine 2. SPE/HPLC	PAHs in edible oils and fats.	F. van Stijn, M.A.T. Kerkhoff and B.G.M. Vandeginste, *J. Chromatogr.*, 1996, **750**, 263. (Chapter 6)	The reference caffeine complexation method gave lower values for the 3- and 4-ring compounds in 10 olive oil samples
1. SDE 2. SOX	Insecticides, benzenes, chlorinated phenols in vegetables.	M. Veningerová, V. Prachar, J. Kovačičova and J. Uhnák, *J. Chromatogr. A.*, 1997, **774**, 333. (Chapter 5)	Review of current procedures
1. StD 2. SE (MeOH) 3. SE (Pet. ether)	Essential oils from pelargonium leaves.	M. Lis-Balchin, G. Buchbauer, T. Hirtenlehner and M. Resch, *Lett. Appl. Microbiol.*, 1998, **27**, 207. (Chapter 5)	Solvent extraction gave more potent extracts than steam distillation

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Kjeldahl 2. Dumas	Protein values of dairy produce	P.G. Wiles, I.K. Gray and R.C. Kissling, *J. AOAC*, 1998, **81**, 620. (Chapter 1)	Dumas method gave better statistics and should be recognised
1. Kjeldahl 2. Dumas	Animal nutrition studies	R.D. Etheridge, G.M. Pesti and E.H. Foster, *Animal Feed Technol.*, 1998, **73**, 21. (Table only)	Dumas method used the Leco CNS 2000 analyser. Of interest were egg yolk and milk samples. N values were comparable to Kjeldahl values
1. SPE 2. HPLC (Std)	Remote sensing of food colorants	L.F. Capitán-Vallvey, *et al.*, 1998, (Chapter 6)	Gel beads were used to absorb tartrazine, ponceau 4R, and sunset yellow from food samples for transfer to a spectroscopy cell
1. Aq Solvent 2. Org solvent	Antibiotics from animal tissues	A.D. Cooper, J.A. Tarbin, W.H. Farrington and G. Shearer, *Food Addit. Contamin.* 1998, **15**, 645. (Chapter 6)	Ethyl acetate was the best extractant. Enzyme digestion and UAE were compared as aids to extraction
1. Englyst 2. Prosky	Dietary fibre in Hungarian foods.	M. Kontraszti, G.J. Hudson and H.N. Englyst, *Food Chem.*, 1999, **64**, 445. (Chapter 2)	The different procedures lead to the extraction of indigestible polysaccharides and lignin (Prosky) and NSP (Englyst)
1. SFE 2. StD 3. SE (Hex) 4. SE (Alc)	Essential oils from fennel seeds	B. Simándi, A. Deák, E. Rónyai, G. Yanxiang, T. Veress, E. Lemberkovics, M. Then, A. Sass-Kiss and Z. Vámos-Falusi, *J. Agric. Food Chem.*, 1999, **47**, 1635. (Chapter 4)	Yield of extract was 1. 10.0%, 2. 3.0%, 3. 10.6%, and 4. 15.4%. However, the sensory quality was found to be better for SFE than the SE methods
1. B&D 2. Mod B&D 3. SOX	Total lipids in sea foods	F. Smedes, *Analyst*, 1999, **124**, 1711. (Chapters 1 & 4)	Modified B&D method was to replace the use of $CHCl_3$. The Soxhlet method gave low recoveries (see Table 4.2) called "extractable" not total lipids

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Soxflo 2. SOX	Crude fat in foods	R.H. Brown and I. Muellar-Harvey, *J. AOAC Int.*, 1999, **82**, 1369. (Chapter 5)	Soxflo gave economies in time, energy, water, and solvents were significantly environmentally friendly
1. SFE 2. ESE 3. SPE	Pesticides in baby foods by ELISA and GC-MS end methods.	J.C. Chuang, M.A. Pollard, M. Misita and J.M. Van Emon, *Anal. Chim. Acta*, 1999, **399**, 135. (Chapter 4)	SFE-ELISA gave general poor recoveries, SFE-GC-MS incurred losses during the FD stage, SPE-GC-MS gave >80% recoveries in non-fat foods, and ESE-ELISA gave quantitative yield of atrazine from fat and non-fat foods
1. StD 2. HS-GC	Pungent principal of *Alpinia galangal* (L.) swartz rhizomes	X. Yang and R.G. Eilerman, *J. Agric. Food Chem.*, 1999, **47**, 1657. (Chapter 5)	The pungent principal – galangal acetate – used in beverages was not extracted by StD, but was a major volatile in HS-GC
1. pptn-TCA 2. Dialysis	Enterotoxins in cheeses	A. Meyrand, V. Atrache, C. Bavai, M.P. Montet and C. Vernozy-Rozand, *Lett. Appl. Microbiol.*, 1999, **28**, 411. (Chapter 2)	TCA pptn method was preferred to the reference dialysis method
1. AcHyd 2. SE (ether) 3. SPE 4. SFE/CO_2 Mod SFE/CO_2	Fat content of cereal foods and cake mix	W. Zou, C. Lusk, D. Messer and R. Lane, *J. AOAC Int.*, 1999, **82**, 141. (Chapter 2)	Acid hydrolysis gave higher crude fat values, and after FAME gave higher triglyceride means. SFE/CO_2 method was modified with 15% EtOH. SFE and SPE were preferred to SE
1. Kjeldahl 2. Lowry 3. Mod Lowry	Pea protein hydrolysate	M. Soral-Smietana, R. Amarowicz, A. Swigo and L. Sijtsma, *Int. J. Food Sci. Nutr.*, 1999, **50**, 407. (Chapter 1)	Kjeldahl and Lowry methods gave similar results but the modified Lowry was at odds with the other two methods

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. ASE 2. SOX	PAHs in smoked foods	G. Wang, A.S. Lee, M. Lewis, B. Kamath and R.K. Archer, *J. Agric. Food Chem.*, 1999, **47**, 1062. (Chapter 4)	ASE has simplified protocol, reduced solvent use and analysis time
1. SDE 2. SE HVD	AEDA on enzyme inactivated peach juice.	C. Derail, T. Hofmann and P. Schieberle, *J. Agric. Food Chem.*, 1999, **47**, 4742. (Chapter 5)	SDE gave 30 *cf.* 24 odour active regions
1. NIR 2. Kjeldahl 3. NIR 4. Folch	Proximate analysis of protein (1–2) and fat (3–4)	K. Almendingen, H.M. Meltzer, J.I. Pedersen, B.N. Nilsen and M. Ellekjaer, *Eur. J. Clin. Nut.*, 2000, **54**, 20. (Chapter 1)	NIR gave accurate values for fat content but less accuracy was obtained for proteins
1. IA (Ochraprep) 2. IA (Ochratest) 3. SE ($CHCl_3$)	Ochratoxin A in wines	M. Castellari, S. Fabbri, A. Amati and S. Galassi, *J. Chromatogr. A*, 2000, **888**, 129. (Table only)	The IA methods (Ochraprep and Ochratest) were found to be comparable to the standard solvent extraction with chloroform
1. SFE 2. SOX	Total lipids by on-line SFE-piezoelectric detection.	L. Manganiello, A. Rios and M. Valcárcel, *J. Chromatogr. A*, 2000, **874**, 265. (Chapter 4)	The advantage of automation led to a throughput of 6 samples per hour
1. B&D 2. Mod B&D 3. R-G 4. SOX (hot) 5. SOX (Std)	Fat content of various foods	P. Manirakiza, A. Covaci and P. Schepens, *J. Food Comp. Anal.*, 2001, **14**, 93. (Chapter 1)	Optimisation and evaluation. Against producer's figures, all methods gave reasonable results for chocolate and dried milk powders, B&D and Mod B&D best for liquid milk and eggs, and Soxhlet for margarine
1. AcHyd + SAP 2. SAP alone	Free, esterified, and glycosidic sterols in foods	J. Toivo, K. Phillips, A-M. Lampi and V. Piironen, *J. Food Comp. Anal.*, 2001, **14**, 631. (Chapter 2)	Excellent report on optimisation of extraction procedure

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. MBSE 2. Pervaporation	Sulphur compounds from wastewaters.	F.X. Pierre, I. Souchon and M. Marin, *J. Membrane Sci.*, 2001, **187**, 239. (Chapter 7)	Mass transfer flux: MBSE > Pervaporation
1. AcHyd only 2. AlkHyd only 3. EnzHyd then AcHyd 4. AcHyd then EnzHyd	Microbiological and HPLC methods compared for niacin released from cereal-based foods by hydrolysis. Also comparison of three hydrolytic methods.	C. Rose-Sallin, C.J. Blake, D. Genoud and E.G. Tagliaferri, *Food Chem.*, 2001, **73**, 473. (Chapter 2)	Concluded that the 1 M HCl AcHyd used in the microbiological method released non-bioavailable niacin. AlkHyd released less niacin than AcHyd and additional EnzHyd made no difference. 0.1 M HCl is sufficient to release niacin from cereal-based food
1. SDE 2. SE 3. LLE	Flavour authenticity of extraction methods preceding GC/Pyr-IRMS	K. Hör, C. Ruff, B. Weckerle, T. König, P. Schreier, *J. Agric. Food Chem.*, 2001, **49**, 21. (Table only)	The three extraction methods were reported to have negligible effect on the δ(2)H values in (2)h/(1)h IRMS studies
1. AlkHyd + SE unsaponifiables 2. AlkHyd + MD 3. MD-HPLC	Vitamin E isomers in seeds and nuts	M.M. Delgado-Zamarreño *et al.*, *J. Chromatogr. A*. 2001, **935**, 77. (Chapter 7)	All 3 methods gave good results for α-tocopherol, but method 3 was best for β-, γ-, δ-tocopherol
1. UAE 2. SOX	Saponins of ginseng	Wu *et al.*, *Ultrasonics, Sonochem.* 2001, **8**, 347. (Chapter 2)	Direct probe and indirect (cleaning bath) sonication using different solvents were compared with SOX. UEA was 3× faster at a lower temperature
1. LLP 2. SPE 3. SFE	Fat-soluble vitamins.	Luque-Garcia and Luque de Castro, *J. Chromatogr. A*, 2001, **935**, 3. (Chapter 4)	Review article

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. SOX 2. Blender 3. MAE 4. PLE 5. SFE	Incurred and added pesticides in 4 dietary composites varying in fat and water content.	L. Rosenblum, S.T. Garris and J.N. Morgan, *J. AOAC Int.*, 2002, **85**, 1167. (Table only)	Incurred pesticides: Chlorothalonil, chlorpyrifos, DDE, dicloran, dieldrin, endosulfan I, malathion, *cis*- and *trans*-permethrin, and trifluralin. Added pesticides: α- and γ-chlordane, hexachlorobenzene, and fonofos
1. MASE 2. SOX	Total fat in cheese.	J.L. Luque-Garcia, J. Velasco, M.C. Dobarganes and M.D. Luque de Castro, *Food Chem.*, 2002, **76**, 241. (Chapter 2)	MASE gave significant economies in time and solvent re-use
1. SOX 2. MAE	Fenitrothion in beans	R.G. Diagne, G.D. Fosterand and S.U. Khan, *J. Agric. Food Chem.*, 2002, **50**, 3204. (Table only)	Co-extractives removed by further cleanup. MAE was shown to be an alternative to SOX
1. SFE 2. SDE	Essential oils.	M.C. Diaz-Maroto, M.S. Pérez-Coello and M. D. Cabezudo *J. Chromatogr. A*, 2002, **947**, 23. (Chapter 5).	SFE gave better RSDs, and avoided thermal degradation and solvent contamination
1. Kjeldahl 2. Dumas	Protein nitrogen	M. Thompson, L. Owen, K. Wilkinson, R. Wood and A. Damant, *The Analyst*, 2002, **127**, 1666. (Chapter 1)	Non-equivalence between the 2 methods noted. Dumas method results 1.4% higher than Kjeldahl
1. SFE 2. MAE 3. SPE 4. SPME 5. ASE	PCBs in food matrices.	Björklund *et al.*, *Trends Anal. Chem.* 2002, **21**, 39. (Chapter 8)	Review article. Emphasis on modern methods of rapid analysis
1. SOX 2. UAE 3. SFE/CO_2	Isoflavones from soybean flour.	M.A. Rostagno, J.M.A. Araújo and D. Sandi, *Food Chem.*, 2002, **78**, 111. (Chapter 4)	Total isoflavone extracted, Soxhlet: 213 μg g^{-1} UAE: 312 μg g^{-1} SFE (CO_2): 86 μg g^{-1}

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. LLE 2. SPE	Pesticides in coconut water	N.M. Brito, S. Navickiene, L. Polese, E.F.G. Jardim, R.B. Abakerli and M.L. Ribeiro, *J. Chromatogr. A*, 2002, **957**, 201. (Table only)	Hexane–DCM for LLE and Sep-Pak Vac C_{18} eluted with MeOH for SPE
1. MSPD 2. SBSE	Pesticides from oranges.	C. Blasco, Y. Picó and G. Font, *J. AOAC Int.*, 2002, **85**, 704. (Chapter 4)	MSPD superior for recovery and RSD values
1. SE 2. SDE 3. ASE 4. SFE	Markers in essential oil of lemon grass.	B.T. Schaneberg and I.A. Khan, *J. Agric. Food Chem.*, 2002, **50**, 1345. (Table only)	SE+UAE (with non-polar solvent) gave similar result to SDE
1. MIP/SPE 2. Non-MIP/SPE 3. C_{18} SPE	Quercetin in red wine	A. Milinelli, R. Weiss and B. Mizaikoff, *J. Agric. Food Chem.*, 2002, **50**, 1804. (Chapter 6)	The three extraction methods were compared using HPLC analysis
1. UAE 2. SOX 3. MAE 4. SFE	For leaching processes	J.L. Luque-Garcia and M.D. Luque de Castro, *Trends Anal. Chem.* 2003, **22**, 41. (Chapter 2)	Potential of UAE shown
1. Dilute with PEG 8000 and $NaCO_3$ + IAE 2. Acidic MeOH + IAE	Ochratoxin A in wine grapes using a modified Visconti method.	R. Serra, C. Mendonça, L. Abrunhosa, A. Pietri and A. Venâncio, *Anal. Chim. Acta*, 2004, **513**, 41. (Table only)	The two pretreatment extraction methods (Visconti *et al.*, 1999, and MacDonald *et al.*, 1999, Table 6.2) were compared for use prior to immunoaffinity extraction
1. SDME 2. LPME 3. SPME	Review of liquid phase microextraction	E. Psillakis and N. Kalogerakis, *Trends Anal. Chem.*, 2003, **22**, 565. (Table only)	1. A single microdrop of organic solvent is the acceptor suspended in aqueous donor. 2. a SLM technique 1. Was difficult to operate, SPME was less versatile than LPME (see Chapter 7 for SLM details)

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. SOX 2. B&D 3. Smedes	Effect of different extraction methods on $\delta^{13}C$ measurements in lipid and lipid-free fractions of fish.	Ch. Schlechtriem, U. Focken and K. Becker, *Isotopes Env. Health Studies*, 2003, **39**, 135. (Chapter 5)	B&D method caused systematic errors in the $\delta^{13}C$ values when chloroform was used as solvent
1. SAP + LLE 2. MAE	TCBs in fish	G. Wittmann, T. Huybrechts, H. van Langenhove, J. Dewulf and H. Nollet, *J. Chromatogr. A*, 2003, **993**, 71. (Chapter 2)	No statistical difference was found between the recoveries of both methods, but MAE was faster and used less solvent
1. PLE 2. Mod Folch 3. AcHyd	Total lipid and fatty acid composition of poultry meat	T.G. Toschi, A. Bendini, A. Ricci and G. Lercker, *Food Chem.*, 2003, **83**, 551. (Chapter 4)	Good agreement for the optimised PLE method with standard modified Folch and acid hydrolysis
1. Cold EtOH SAP (1) 2. Cold MeOH SAP (2) 3. Hot SAP 4. Transesterification	Cholesterol oxidation products by GC and GC-MS. Four release methods compared	S.J.K.A. Ubhayasekera, T. Verleyen and P.C. Dutta, *Food Chem.*, 2004, **84**, 149. (Chapter 2)	EtOH saponification (1) and the transesterification (4) methods gave the best results with spiked additions, but only method 1 was suitable for low level spikes of 5 μg. The released compounds were concentrated on SPE columns by solvent fractionation
1. MSPD 2. SPE	Dithiocarbamates in EU food composition groups.	C. Blasco, G. Font and Y. Picó, *J. Chromatogr. A*, 2004, **1028**, 267. (Chapter 4)	MSPD with ENVI-carb dispersant and DCM–MeOH elution was preferred to SPE based on improved recoveries
1. IAE 2. SE + IAE 3. SPE C_{18} 4. SPE PS 5. SPE Oasis HLB	Ochratoxin in wine, must and beer	J.M. Sáez, A. Medina, J.V. Gimeno-Adelantado, R. Mateo and M. Jiménez, *J. Chromatogr. A*, 2004, **1029**, 125. (Chapter 6)	A précis is provided in Chapter 6. IAE methods show greatly improved sensitivity and selectivity
1. UASE 2. ISO Method	Fat content of oleaginous seeds	J.L. Luque-Garcia and M.D. Luque de Castro, *J. Chromatogr. A*, 2004, **1034**, 237. (Chapter 5)	Details of both methods and the *raison d'etre* are carefully reported

APPENDIX 1 *Continued*

Methods	*Commodity/compound*	*Reference*	*Comment*
1. Kjeldahl 2. MAE Kjeldahl 3. MAE-UAE Kjeldahl	Digestion of solid and liquid food samples for the extraction of edible oils and Kjeldahl nitrogen	S. Chemat, A. Lagha, H.A. Amar and F. Chemat, *Ultrasonics Sonochem.*, 2004, **11**, 5. (Chapter 2)	Combined MAE-UAE digestion was compared to classic Kjeldahl and MAE methods for cow's milk, rice, corn, flour, beef, corned beef, chickpea, and powdered milk. All gave similar results, but MAE-UAE halved the digestion time
1. Magnet stirring 2. UAE 3. PLE	Catechins from tea leaves and grape seeds	Z. Piñeiro, M. Palma and C.G. Barroso, *J. Chromatogr. A*, 2004, **1026**, 19. (Chapter 4)	PLE was preferred, but recovery started to fall to <95% at 130 °C. MeOH was better than EtOH, ethyl acetate or water

APPENDIX 2

Combinations of Preparation/Extraction Methods

During the research for the chapters on specific methods of extraction, it became apparent that many developers combined methods to achieve the objectives for their application. The source chapter for further reading is given in brackets (*e.g.* Chapter 3). The note "(Table only)" appears in column 3 when no further reference to this record is made in the text. This is not a rigorous collection, only some examples of combinations collated to provide a database. Consult Glossary of Abbreviations.

Combined methods	*Analyte and commodity*	*Literature reference (Chapter)*	*Comment*
1. SE 2. SPE	Aflatoxins in palm kernels	S. Nawaz, R.D. Coker and S.J. Haswell, *The Analyst*, 1992, **117**, 67. (Chapter 4)	A crude SE with acetone–water (80:20, v/v) is cleaned up using SPE for HPTLC analysis. The method was evaluated against the British Standard Method
1. Dialysis 2. SPE	Amoxicillin, cefadroxil in beef muscle	N. Snippe, N.C. van de Merbel, F.P.M. Ruiter, O.M. Steiger, H. Lingeman, and U.A.Th. Brinkman, *J. Chromatogr. B*, 1994, **662**, 61. (Chapter 7)	On-line dialysis gives dilute extractant; flow switching to SPE concentrates analytes for injection on to HPLC. Flow switching removes unwanted fractions from SPE eluate
1. SOX 2. LLP 3. GPC	Pesticides from foods	L.S. Sheldon, J.T. Keever, J.M. Roberds, J.B. Beach, and J.N. Morgan, *J. Exposure Anal. Environ Epidemiol.*, 1997, **7**, 37. (Chapter 5)	An LOD of 1 ng g^{-1} was sought and at this time 10 to 100 ng g^{-1} was typical
1. SE 2. SGC	Urishiol in Australian native cashew	P.B. Oelrichs, J.K. Macleod, A.A. Seawright, and J.C. Ng, *Nat. Toxins*, 1997, **5**, 96. (Table only)	Urishiol causes dermatitis and has to be extracted to enable the cashew to be used as a food source
1. StD 2. SPME	Nitrosamines in smoked hams	N.P. Sen, S.W. Seaman, and B.D. Page, *J. Chromatogr. A*, 1997, **788**, 131. (Chapter 5)	Headspace of StD distillate sampled by polyacrylate SPME probe

APPENDIX 2 *Continued*

Combined methods	*Analyte and commodity*	*Literature reference (Chapter)*	*Comment*
1. MSPD 2. ASE	Vitamin K_1 in medical food	G.W. Chase, Jr., R.R. Eitenmiller, and A.R. Long, *J. Food Comp. Anal.*, 2000, **13**, 765. (Chapter 4)	Adding ASE allowed the MSPD eluate to be automatically quantified by RPLC
1. MSPD 2. SWE 3. SPME	Atrazine in beef kidneys	M.S. Curren and J.W. King, *J. Agric. Food Chem.*, 2001, **49**, 2175. (Chapter 4)	XAD-7 HP dispersant for MSPD cleanup. Carbowax/DVB fibre for SPME from SWE at 100 °C and 50 atm
1. PLE 2. SPE	Polyphenols in hops	M. Papagiannopoulos and A. Mellenthin, *J. Chromatogr. A*, 2002, **976**, 345. (Chapter 4)	Used ASE200 (Dionex, Germany) with acetone–water (4:1 v/v) at 60 °C for 10 min, with and without pentane pre-extraction
1. SOX 2. SE 3. GPC 4. SPE	Nitrated PAHs in biotic matrices	B. Dušek, J. Hajšlová, and V. Kocourek, *J. Chromatogr. A*, 2002, **982**, 127. (Chapter 5)	Methods for the extraction of ultra-trace levels of nitro-PAHs in various biotic matrices were developed. SOX and/or SE was enhanced by UAE, and GPC and SPE were used for clean up
1. StD 2. SPE	(*E*)-2-nonenal in beer	J.R. Santos, J.R. Carneiro, L.F. Guido, P.J. Almeida, J.A. Rodrigues, and A.A. Barros, *J. Chromatogr. A*, 2003, **985**, 395. (Chapters 5 & 6)	StD may cause some conversion of (*E*)-2-nonenal precursors. Not suitable for wort analysis
1. StD 2. SPME	Essential oil markers	M.R. Tellez, I.K. Khan, B.T. Schaneberg, S.L. Crockett, A.M. Rimando, and M. Kobaisy, *J. Chromatogr. A*, 2004, **1025**, 51. (Chapter 5)	Headspace of StD in reflux mode sampled by PDMS SPME probe. Diagram of the apparatus in Chapter 5
1. Equilibrium dialysis 2. SPME	Volatility of flavour compounds in the presence of non-volatiles.	D-M. Jung and S.E. Ebeler, *J. Agric. Food Chem.*, 2003, **51**, 200. (Table only)	Equilibrium dialysis preceded HS-SPME-GC-MS in studying the effect of involatile matrix components, β-lactoglobulin and (+)-catechin, on the volatility of flavour volatiles

APPENDIX 2 *Continued*

Combined methods	*Analyte and commodity*	*Literature reference (Chapter)*	*Comment*
1. SE 2. SPE 3. Prep-HPLC	Vitamins D_3 and 25-OH D_3 in pork cuts	I. Clausen, J. Jakobsen, T. Leth, and L. Oveson, *J. Food Comp. Anal.*, 2003, **16**, 575. (Chapter 2)	Dissection into lean, lard, intermuscular fat, rind, mixed parts. Saponification to release unsaponifiable fraction. C_{18} analytical HPLC
1. SE 2. SPE	Sulphonamide in meat by CE	M-R.S. Fuh and S-Y. Chu, *Anal. Chim. Acta*, 2003, **499**, 215. (Chapter 6)	8 sulphonamides solvent extracted prior to SPE cleanup and sample concentration for CE
1. SE 2. GPC	Bitter taste in carrots by HPLC	A. Czepa and T. Hofmann, *J. Agric. Food Chem.*, 2003, **51**, 3865. (Table only)	Alongside taste dilution analysis six suspect contributors were extracted and falcarindiol targeted
1. StD 2. SPE	(*E*)-2-nonenal and β-damascenone in beer	L.F. Guido, J.R. Carneiro, J.R. Santos, P.J. Almeida, J.A. Rodrigues, and A.A. Barros, *J. Chromatogr. A*, 2004, **1032**, 17. (Table only)	The steam distillate was passed through a Sep-Pak plus C_{18} RP cartridge and eluate taken for HPLC analysis
1. AlkHyd 2. Distillation	Naptalam and its metabolite 1-naphthylamine in foods	B.L. Worobey and J.B. Shields, *J. AOAC*, 1987, **70**, 1021. (Table only)	LC/ECD used to determine the analytes after hydrolysis with 30% NaOH to hydrolyse naptalam to 1-naphthylamine for distillation. The distillate was used directly for analysis
1. EnzHyd 2. Gravimetric	Dietary fibre in foods	L. Prosky, N.G. Asp, T.F. Schweizer, J.W. De Vries, and I. Furda, *J. AOAC*, 1988, **71**, 1017. (Chapter 2)	TDF, IDF and SDF measured using phosphoric acid in place of HCl, otherwise, the method was as reported earlier
1. MAE 2. Dialysis 3. SPE	FMN in milk and cereals	G.M. Greenway and N. Kometa, *The Analyst*, 1994, **119**, 929. (Chapter 7)	Cereal was ground. MAE followed by dialysis of extract. Trace enrichment with a C_{19} minicolumn. Results agreed with AOAC and HPLC methods
1. EnzHyd 2. SPE (silica)	Vitamin K_1 in canola oils	Z.H. Gao and R.G. Ackman, *Food Res. Int.*, 1995, **28**, 61. (Table only)	Enzyme hydrolysis of the triglycerides was followed by silica SPE and RP-HPLC

APPENDIX 2 *Continued*

Combined methods	*Analyte and commodity*	*Literature reference (Chapter)*	*Comment*
1. AlkHyd 2. SPE	Niacin in cereals, meat, fish, yeast, nuts, peanut butter, sunflower seeds	C.M. Ward and C. Trenerry, *Food Chem.*, 1997, **60**, 667. (Chapter 6)	Alkaline digestion with aqueous calcium hydroxide. SPE using C_{18} and cation exchange in series
1. AcHyd 2. SPE	Furosine in 48 foods	A. Tirelli, *J. Food Protection*, 1998, **61**, 1400. (Table only)	Acid hydrolysate subjected to SPE. Eluate dried and redissolved for CE analysis. Results agree with HPLC method
1. Enzymic 2. Chemical	Dietary fibre in Hungarian foods	M. Kontraszti, G.J. Hudson, and H.N. Englyst, *Food Chem.*, 1999, **64**, 445. (Chapter 2)	The Englyst NSP method uses EnzHyd to remove starch and then AcHyd to release the sugars for derivatisation (volatilisation) and GC analysis
1. Enzymic 2. Gravimetric	Dietary fibre in Hungarian foods	M. Kontraszti, G.J. Hudson, and H.N. Englyst, *Food Chem.*, 1999, **64**, 445. (Chapter 2)	The Prosky method uses AcHyd to release dietary fibre and gravimetric measurement of protein and ash to calculate carbohydrate content "by difference"
1. SPE 2. SFE (CO_2)	Triazine herbicides in eggs	J.W. Pensabene, W. Fiddler, and D.J. Donoghue, *J. Agric. Food Chem.*, 2000, **48**, 1668. (Table only)	10 triazines were first collected on Florisil from eggs fortified at the 100 ppb level. A serial SFE (CO_2) was effective using only 8 ml solvent per analysis
1. AcHyd 2. SAP	Free, esterified, and glycosidic sterols in foods	J. Toivo, K. Phillips, A-M. Lampi, and V. Piironen, *J. Food Comp. Anal.*, 2001, **14**, 631. (Chapter 2)	For cholesterol no AcHyd is needed. For others, the combination is superior to SAP alone
1. MAE 2. SOX	PAHs, n-alkanes, and pesticides	L.E. Garcia-Ayuso and M.D. Luque de Castro, *TrAC Trends Anal. Chem.*, 2001, **20**, 28. (Chapter 8)	Similar to Soxhlet method in performance, but faster and with low solvent consumption

APPENDIX 2 *Continued*

Combined methods	*Analyte and commodity*	*Literature reference (Chapter)*	*Comment*
1. UAE 2. MSPD	PCBs and FAMEs	P. Sandra and F. David, *J. Chromatogr. Sci.*, 2002, **40**, 248. (Table only)	FAMEs were obtained by UAE followed by MSPD, and the FAMEs and PCBs were analysed by GC. Rapid throughput for this combination was stressed
1. MAE 2. SPME	Dichlorvos on vegetables	Y-I. Chen, Y-S. Su, and J-F. Jen, *J. Chromatogr. A*, 2002, **976**, 349. (Table only)	The HS above a MAE of an aqueous solution of chopped vegetable was sampled by SPME for GC analysis
1. MAE 2. SOX	Lipids from pre-fried and fried foods	J.L. Luque-Garcia, J. Velasco, M.C. Dobarganes, and M.D. Luque de Castro, *Food Chem.*, 2002, **76**, 241. (Chapter 2)	Focused MA-SOX optimised. Faster (<1 h from 8 h) and cleaner (>75% solvent recycled)
1. SAP 2. SPE	Vitamins A, D_3, and E in infant formulae	O. Heudi, M-J. Trisconi, and C-J. Blake, *J. Chromatogr. A*, 2004, **1022**, 115. (Chapter 6)	High-resolution LC-APcI-MS in SIM mode detection enabled use of a simple matrix
1. SE (acidic) 2. SPE(phenyl)	Tetracyclines in honey	P. Viñas, *et al.*, *J. Chromatogr. A*, 2004, **1022**, 125. (Chapter 6)	Mild acidic solvent containing EDTA. SE releases protein- or sugar-bound residues
1. AcE 2. RP-IRLC 3. SPE	Sulphite in dried garlic	G.A. Perfetti and G.W. Diachenko, *J. AOAC Int.*, 2003, **86**, 544. (Chapter 5)	AcE to inhibit allicin formation

APPENDIX 3

Interlaboratory Collaborative Trials

This appendix lists interlaboratory collaborative trials in chronological order. Because these data are largely cross-referenced, the literature reference can be found in the chapter dealing with the main extraction method used and shown in column 3. Where no reference occurs in the text, the (Table only) note is shown in column 3.

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
Fluoride	12	R.W. Dabeka, A.D. McKenzie and H.B. Conacher, *J. Assoc. Off. Anal. Chem.*, 1979, **62**, 1065. (Chapter 3)	A microdiffusion method was used to extract fluoride from infant foods. 12 samples (1 replicate) of infant foods, milk, pears, and peas containing 0.2-5 ppm F were analysed. Mean CVs of 7.06% for 3 sets of blind duplicates and 21.6% for determination of 12 samples were reported. Variance analysis for all samples gave a reproducibility SD 0.41 ppm; for 3 sets of blind duplicates, a repeatability SD of 0.26 ppm and a reproducibility SD of 0.32 ppm
2,4-Dichlorophenoxyacetic acid	6	A.E. Smith, *J. AOAC*, 1984, **67**, 794. (Table only)	2,4-D was extracted from dried green wheat material with dilute NaOH, then acidified and solvent extracted, and the residue methylated. The 2,5-D methyl ester cleaned up on Florisil for GC analysis. The procedure has potential for use with wheat grain
NPYR	11	W. Fiddler, J.W. Pensabene, R.A. Gates and J.G. Phillips, *J. AOAC*, 1984, **67**, 521. (Table only)	The DC method followed by the TEA method for NPYR in bacon was tested. 2 outliers were excluded. A recovery of 85.2% average was obtained using *N*-nitrosoazetidine as internal standard. Average recovery was 85.2% for the IS, and the method was seen as an alternative for the MOD-TEA method

APPENDIX 3 *Continued*

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
Dietary fibre	9	L. Prosky, N.G. Asp, I. Furda, J.W. De Vries, T.F. Schweizer and B.F. Harland *J. AOAC*, 1985, **68**, 677. (Chapter 2)	Soy isolate, white wheat flour, rye bread, potatoes, rice, wheat bran, oats, corn bran, and whole wheat flour were tested for TDF using the Prosky method. (*J. AOAC*, 1984, **67**, 1044) for TDF (AOAC, 985.29)
Sulphites	8	Holak and Patel, *J. AOAC*, 1987, **70**, 572. (Chapter 5)	Differential pulsed polarography was used to determine free and total sulphite in shrimps, orange juice, peas, dried apricots and dehydrated potatoes. Recoveries were compared to the official Monier-Williams method
Dietary fibre	13	L. Prosky, N.G. Asp, T.F. Schweizer, J.W. De Vries and I. Furda, *J. AOAC*, 1988, **71**, 1017. (Chapter 2)	TDF, IDF and SDF measured using phosphoric acid in place of HCl, otherwise, the method was as reported earlier for TDF.
Sulphites	21	Hillery *et al. J. AOAC*, 1989, **72**, 470. (Chapter 5)	The optimised-FDA Monier-Williams method was evaluated for sulphites in foods. After familiarisation, 3 food matrixes (hominy, fruit juice, and protein (seafood)) at 3 sulphite levels and a blank were analysed as blind duplicates. Recoveries with reproducibilities giving CVs of 15.5–26.6% for sulphite as SO_2 by weight at the 10 ppm level were recorded
Sulphites	9	H.J. Kim, *J. AOAC*, 1990, **73**, 216. (Chapter 5)	The IEC-EC method was tested with blind duplicates of starch, diluted lemon juice, wine cooler, dehydrated seafood, and instant mashed potato, with and without 2 levels of sulphite spike. Initial sulphite levels varied from 0 to 384 ppm SO_2, and the levels added varied from 10 to 400 ppm. Good agreement was obtained between the IEC-EC method and the Monier-Williams method for initial sulphite levels but spiked levels were higher by the IEC-EC method. The method was adopted official first action

APPENDIX 3 *Continued*

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
p-TSA	11	P.R. Beljaars, R. Van Dijk and A. Brands, *J. AOAC Int.*, 1993, **76**, 570. (Chapter 7)	A very well executed trial by the FIS, The Netherlands, used a CF dialysis extraction of p-TSA from an aqueous sample of ice cream for direct injection on to a LC column *via* a loop
Dietary fibre	13	L. Prosky, N.G. Asp, T.F. Schweizer, J.W. De Vries and I. Furda and S.C. Lee, *J. AOAC Int.*, 1994, **77**, 690. (Chapter 2)	A second collaborative study was made of SDF in which 13 laboratories used the enzymatic–gravimetric method on apricots, carrots, chickpeas, onions, raisins, and sugar beet fibre (Fibrex)
Dietary fibre	9	O. Theander, P. Aman, E. Westerlund, R. Andersson and D. Pettersson, *J. AOAC Int.*, 1995, **78**, 1030. (Chapter 2)	A joint AOAC/AACC collaborative study determined the total dietary fibre content and composition using the Uppsala method. 8 unknown dried products were analysed: 4 cereal products, green peas, potato fibre, carrots, and apples, determined as neutral sugar residues (rhamnose, arabinose, xylose, mannose, galactose, and glucose), uronic acid residues and Klason lignin
Patulin	22	A.R. Brause, M.W. Trucksess, F.S. Thomas and S.W. Page, *J. AOAC Int.*, 1996, **79**, 451. (Table only)	An AOAC-IUPAC-IFJU study extracted apple juice with ethyl acetate, treated with Na_2CO_3 and used RP-LC and UV detection to measure spiked patulin down to 20 $\mu g\ l^{-1}$
Vitamins B-1, B-2, and B-6	16	H. van den Berg, F. van Schaik, P. M. Finglas and I. de Froidmont-Görtz, *Food Chem.*, 1996, **57**, 101. (Table only)	Lyophilised pig's liver, mixed vegetables, and wholemeal flour were analysed by HPLC, using "in-house" and optimal extraction protocol (OEP) preparation (using a common batch of takadiastase enzyme). For B-1 and B-2 the in-house and OEP, gave good and similar results. With B-6, in-house use of AcHyd without EnzHyd showed discrepancies, vitamer interconversion and peak interference needed further study

APPENDIX 3 *Continued*

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
Folates	3	L. Vahteristo, P.M. Finglas, C. Witthöft, K. Wigertz, R. Seale and I. de Froidmont-Görtz, *Food Chem.*, 1996, **57**, 109. (Table only)	Milk powder, lyophilised pig's liver, and wholemeal flour samples, 5-MTHF calibrant, and 2 enzymes, (purified hog kidney and human plasma) were used to measure the folate vitamers. 5-MTHF was successfully measured by all laboratories
Total lipids	10	J.M. Lynch, D.M. Barbano and J.R. Fleming, *J. AOAC*, 1996, **79**, 907. (Chapter 1)	A modified Mojonnier ether extraction and a modified cream Babcock method for determining the fat content of cream were compared using 9 pairs of blind duplicate heat-treated cream samples. Both methods were adopted by AOAC International, the new Babcock method replacing AOAC OM 920.111B-C. Further studies have been reported in 2001 and 2003
Protein	11	P.G. Wiles, I.K. Gray and R.C. Kissling, *J. AOAC*, 1998, **81**, 620. (Chapter 1 and Appendix 1)	The Kjeldahl and Dumas methods compared for routine analysis of proteins in dairy products (milk, skim milk powder, whole milk powder, whey protein concentrate, infant formula, casein, caseinate, 2 reference compounds (glycine and EDTA), and a secondary reference skim milk powder). The two methods gave similar values. The Dumas relative repeatability and reproducibility SDs were consistently about 0.35 and 0.75%, while Kjeldahl values declined generally with N content and were significantly larger. Conclusion: The Dumas method needs Codex Alimentarius status as a recognised test method
Fructans and dietary fibre	9	L. Prosky and H. Hoebregs, *J. Nutr.*, 1999, **129**, 1418S. (Chapter 2)	Inulin and oligofructose are not precipitated in 78% EtOH and, therefore, not included in the standard Prosky method for dietary fibre. This trial of a two-enzyme release of fructans was accepted as an official first action by the AOAC

APPENDIX 3 *Continued*

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
Patulin in apple juices	14	S. MacDonald, M. Long, J. Gilbert and I. Felgueiras, *J. AOAC Int.*, 2000, **83**, 1387. (Table only)	Cloudy apple juice and apple puree were first treated with pectinase and then, along with clear apple juice, were extracted with ethyl acetate, and back-extracted with sodium carbonate solution to remove acidic interferents. 75 ng g^{-1} spiked samples produced RSDs for repeatability and reproducibility of 8–35% and 11–36% respectively
Total lipids	11 and 10	D.H. Kleyn, J.M. Lynch, D.M. Barbano, M.J. Bloom and M.W. Mitchell, *J. AOAC Int.*, 2001, **84**, 1499. (Chapter 1)	The Gerber method for total lipid content of milk gave different results depending on the way the sample aliquot was dispensed. 11 laboratories tested the weighed aliquot and 10 laboratories the pipetted volume. The Mojonnier ether extraction (Method 989.05) was used as the reference method. [The Associate Referee recommended that the Gerber method using a weighed test portion be adopted as First Action with applicability limited to whole milk]
Fumonisins	4	A. De Girolamo, M. Solfrizzo, C. von Holst and A. Visconti, *Food Addit. Contam.*, 2001, **18**, 59. (Chapter 4)	Comparison of extraction solvent, extraction mode, volume of solvent, sample size, and the clean up led to the choice of CH_3CN–water (1:1, v/v) as extraction solvent and immunoaffinity SPE as the clean up, and, based on the general findings of the collaborators, a new method was proposed for the analysis of maize flour, corn flakes, extruded maize, muffins, and infant formula
Mycotoxins	5+5	S. De Saeger, L. Sibanda, A. Desmet and C. Van Peteghem, *Int. J. Food Microbiol.*, 2002, **75**, 135. (Table only)	Field immunoassay kits for the detection of ochratoxin A and T-2 Toxin were used after a simple MeOH-based extraction, filtration and dilution. LODs of 4 and 50 μg kg^{-1} respectively, were reported. The trials were conducted on wheat, rye, maize and barley. Five laboratories (first stage) were joined by another five for the second stage

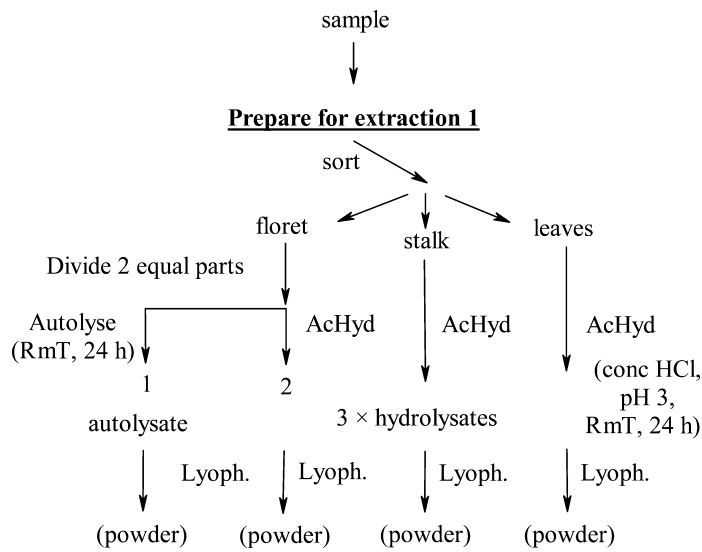
APPENDIX 3 *Continued*

Compound or class	*Size of Trial*	*Reference (chapter location)*	*Comment*
Milk fat	8–15	J.M. Lynch, D.M. Barbano, P.A. Healy and J.R. Fleming, *J. AOAC Int.*, 2003, **86**, 768. (Chapter 1)	8–15 laboratories were involved in a proficiency testing program. Unmodified and modified Babcock methods were compared to the ether extraction method. The unmodified method gave consistently higher (0.022% average) while the modified method gave –0.003% lower values with the ether extraction not significantly different from zero. This trial brought the Babcock data into agreement with the ether extraction data
Acrylamide	62	T. Wenzl, B. De La Calle, R. Gatermann, K. Hoenicke, F. Ulberth and E. Anklam, *Anal. Bioanal. Chem.* 2004, **379**, 449. (Chapter 8)	The recent discovery of the potential toxicity of acrylamide and its formation in carbohydrate-rich foods at elevated temperatures has focused analysts on the development of suitable methods, and this trial on butter cookies and crispbread exemplifies the effort being made to standardise procedures. Many different analytical protocols have already been designed, but this has lead to low reproducibility scores, and more work is required to find a universally acceptable method

APPENDIX 4

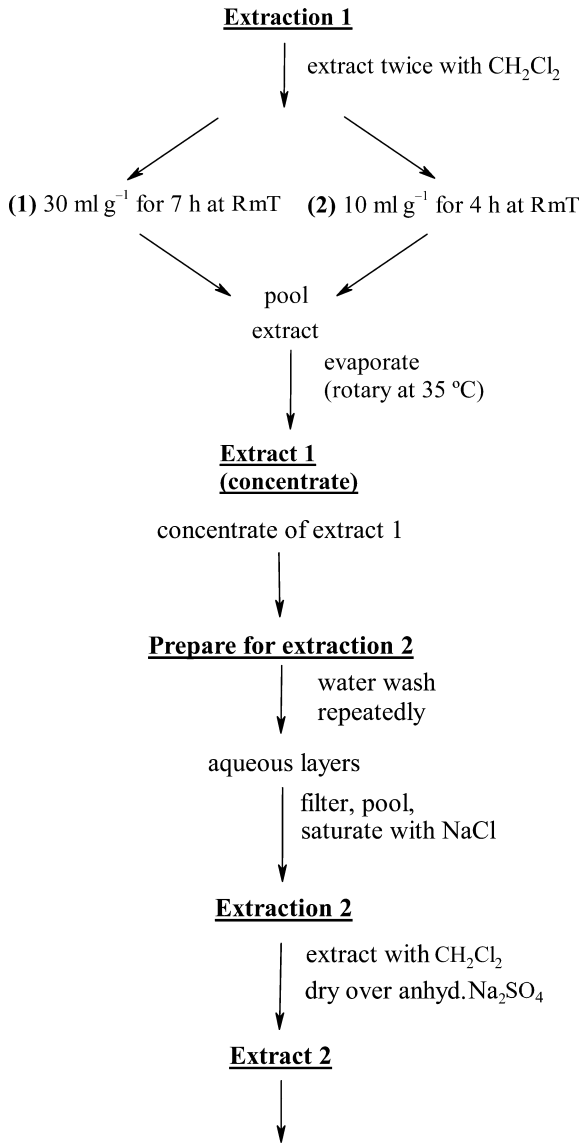
Examples of Preparation and Extraction Schemes

Some interesting examples have been chosen from the literature to illustrate the construction of a "flow diagram" for the preparation for extraction, the extraction and the treatment of the extract of analytes from food sources.

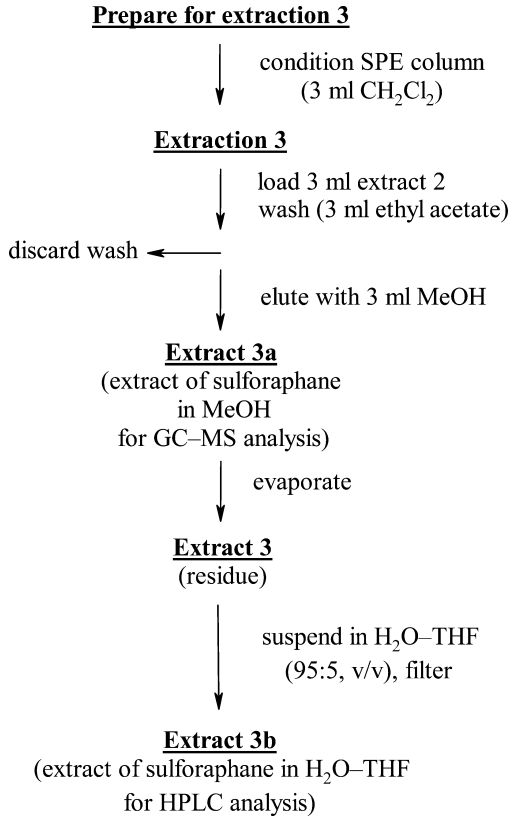


(The lyophilised powders were bulked for Extraction 1)

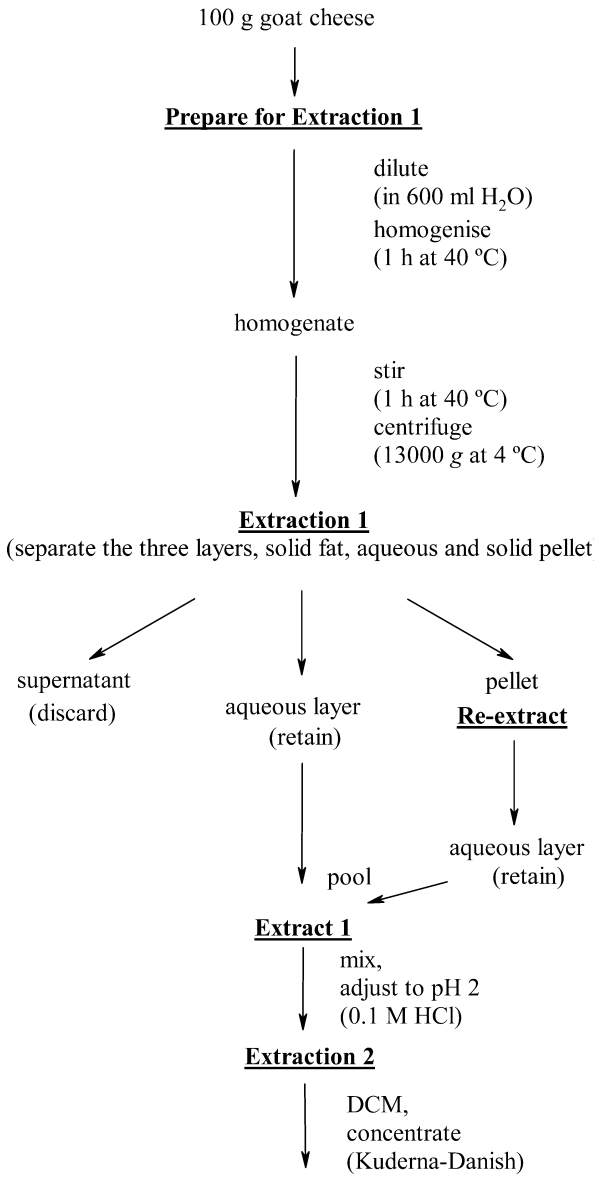
Scheme A4.1 *Extraction of sulforaphane from broccoli. Three extractions (2 × SE + SPE) and their preparations for extraction amounted to 19 analytical procedures*
(Drawn up from data reprinted from Food Chemistry, volume 63, D. Bertelli, M. Plessi, D. Braghiroli and A. Monzani, "Separation by Solid Phase Extraction and Quantification by Reverse Phase HPLC of Sulforaphane in Broccoli", pages 417–421, © 1998, with permission from Elsevier)



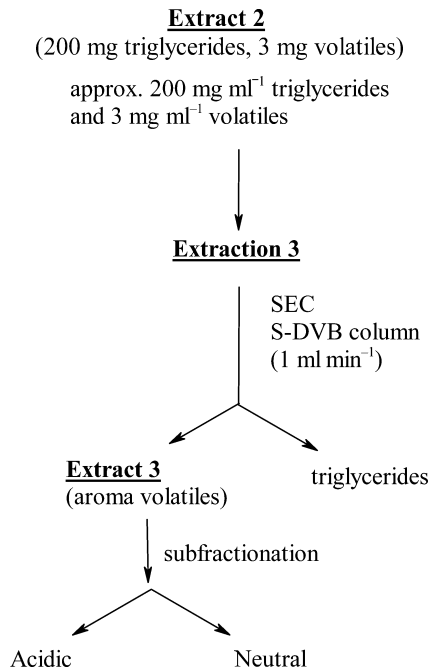
Scheme A4.1 *Continued*



Scheme A4.1 *Continued*



Scheme A4.2 *Prefractionation of aroma volatiles and triglycerides from fatty foods involved three extractions:* 1. *Phase separation after centrifugation,* 2. *DCM solvent extraction,* 3. *SEC fractionation*
(Drawn up from data reprinted from the Journal of Chromatography A, volume 729, M. Lübke, J-L Le Quéré and D. Barron, "Prefractionation of Aroma Extracts from Fat-containing Food by High-Performance Size-Exclusion Chromatography", pages 371–379, © 1996, with permission from Elsevier)

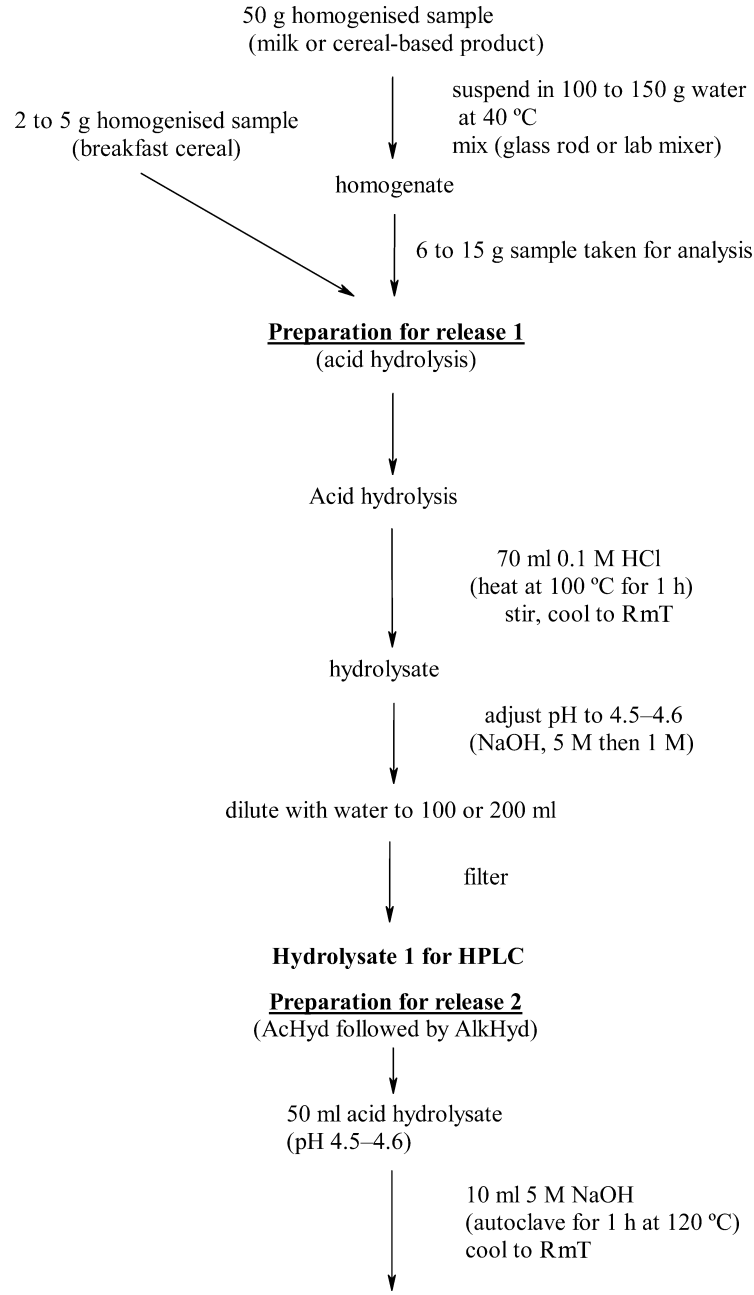


Scheme A4.2 *Continued*

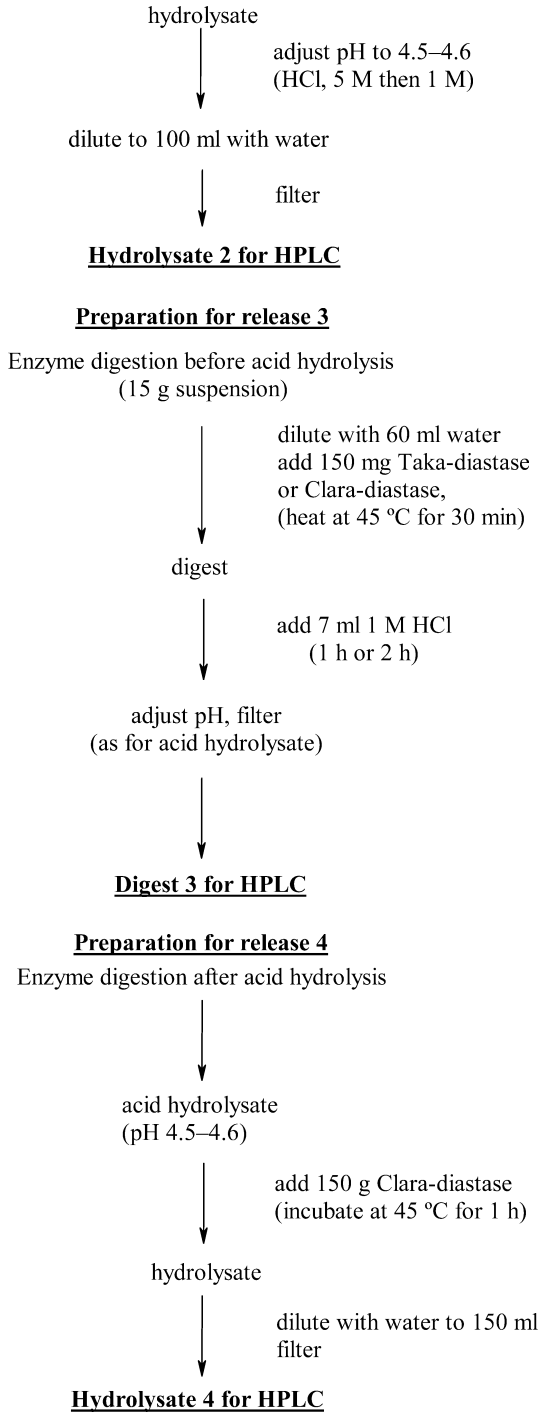
Several experiments were conducted to optimise the biological release of niacin from cereal-based food products:

1. Acid hydrolysis
2. Alkaline hydrolysis
3. Enzyme hydrolysis.

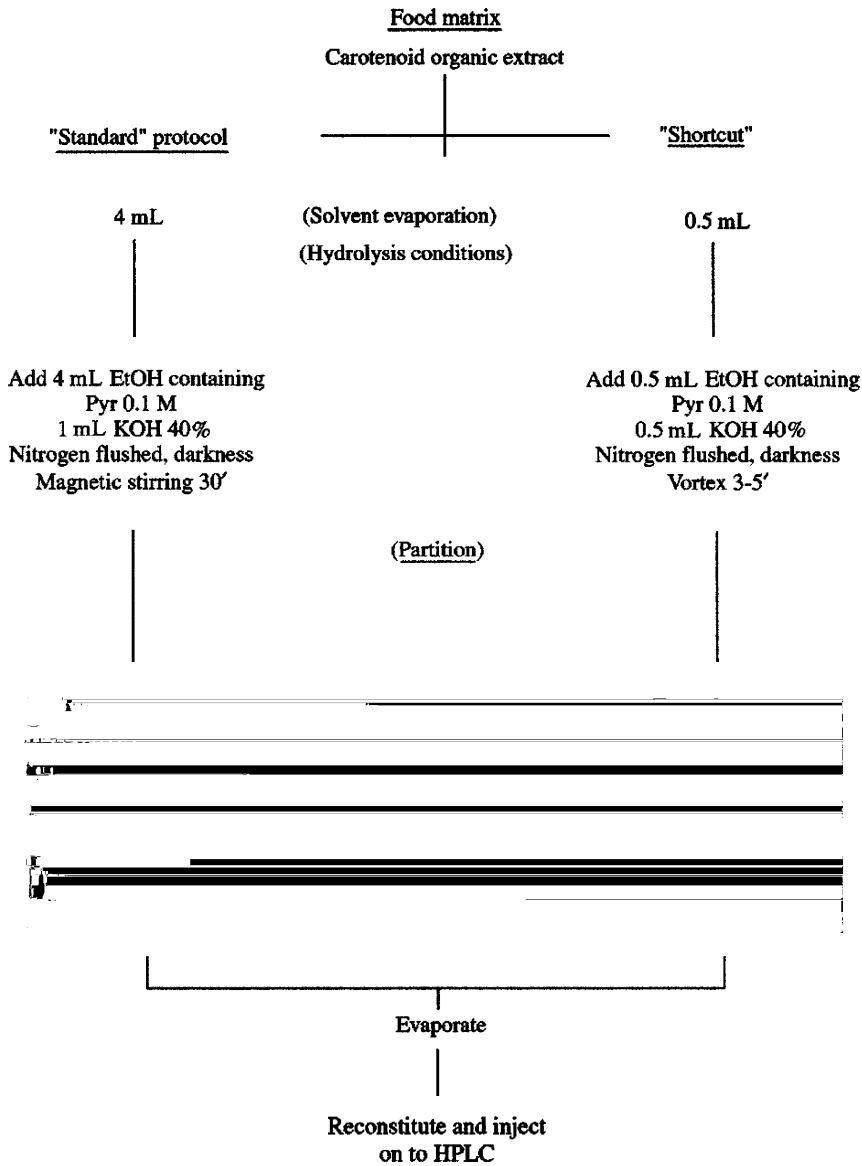
A large laboratory sample was prepared first and then test samples for analysis were drawn from this source. The result of the various combinations of methods and the order of application is reported in the text. In this example, no extraction as such was necessary, the unextracted hydrolysate being clean enough for direct injection into the HPLC.



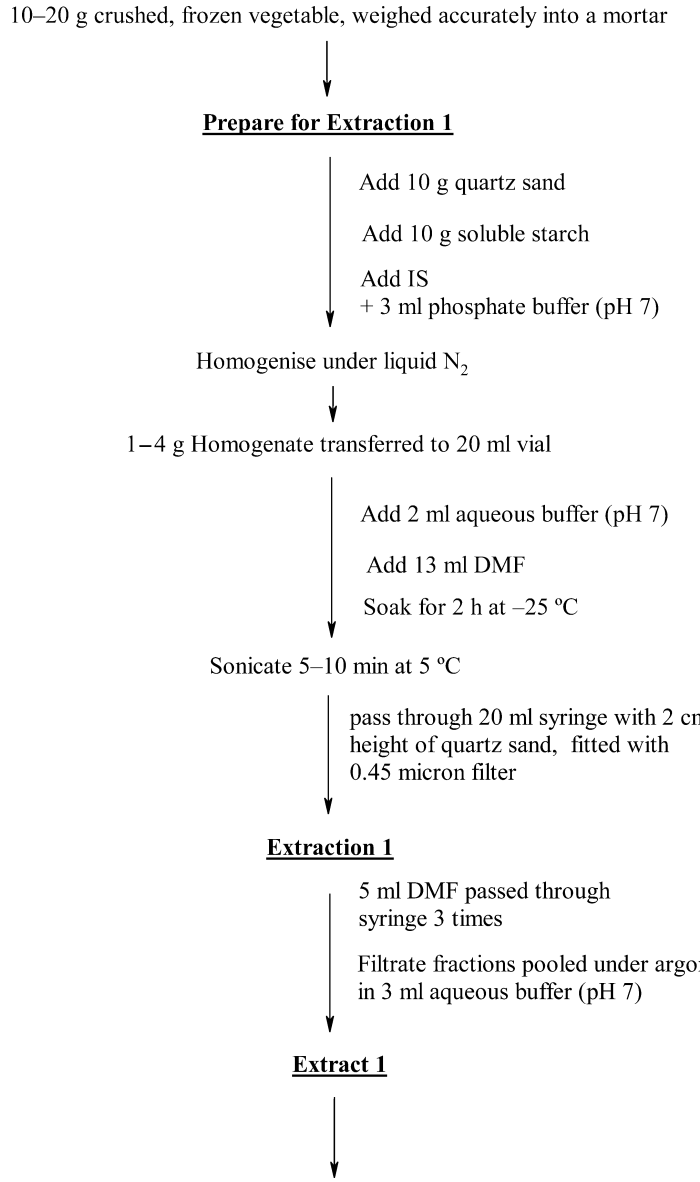
Scheme A4.3 *Release of niacin from cereal-based food products for HPLC separation* (Drawn up from data reprinted from Food Chemistry, volume 73, C. Rose-Sallin, C.J. Blake, D. Genoud and E.G. Tagliaferri, "Comparison of Microbiological and HPLC-Fluorescence Detection Methods for Determination of Niacin in Fortified Food Products", pages 473–480, © 2001, with permission from Elsevier)



Scheme A4.3 *Continued*



Scheme A4.4 *Rapid "shortcut", low-cost method illustrated alongside the standard method for the release of carotenoids from fruit and vegetables*
(Reprinted from the Journal of Food Composition and Analysis, volume 14, F. Granado, B. Olmedilla, E. Gil-Martinez and I. Blanco, "A Fast, Reliable and Low-cost Saponification Protocol for Analysis of Carotenoids in Vegetables", pages 479–489, © 2001, 2001, with permission from Elsevier)



Scheme A4.5 *Extraction of chlorophyll from vegetables. Solvent extraction with* N, N*-dimethylformamide followed by SPE prepared the sample for* C_{18} *RP-HPLC. The novel use of zinc-phthalocyanine as an internal standard was the chief subject. Use an ice bath where possible and work in dim light* (Drawn up from data reprinted from the Journal of Chromatography A, volume 1024, T. Bohn and T. Walczyk, "Determination of Chlorophyll in Plant Samples by Liquid Chromatography using Zinc-Phthalocyanine as an Internal Standard", pages 123–128, © 2004, with permission from Elsevier)

Prepare for Extraction 2

LC-18 SPE column conditioned
with 2 ml MeOH then 5 ml aqueous
phosphate buffer (pH 7)

1–10 ml extract diluted with same
vol of phosphate buffer (pH 7)

washed with 5 ml phos buffer and
6 ml MeOH–Phos Buffer (94:6, v/v)

Extraction 2

eluted with 6 ml 100% MeOH,
followed by 4 ml DMF (1 ml min^{-1})
allow to dry out. Combine the
two eluates

Extract 2
(for HPLC)

Scheme A4.5 *Continued*

APPENDIX 5

Literature Examples of Soxhlet Extractions

(The superscript literature references are included only for those sources not referred to in the body of the text.)

Solvent(s)	*Commodity*	*Analyte*	*Reference No.*	*Comments*
2-propanol	Red peppers	Capsaicin	Polesello and Pizzocaro, 1976[1].	Further clean up by TLC on silica gel (40:45:15, 40–60 pet ether, $CHCl_3$, CH_3CN).
$CHCl_3$	Instant coffee	Dimethylpolysiloxane	Kacprzak, 1977[2].	LOD 5 μg
Acidified acetone	Potatoes, carrots	PCP and TCP	Bruns and Currie, 1980. (Chapter 5)	Optimum yield with 44 h extraction, but 20 h extraction for carrots and a 5 min blending for potatoes were within experimental errors.
N-propanol-water	Bread	Ethoxylated mono- and di-glycerides	Lawrence et al., 1981 (Chapter 5)	22 h extraction followed by further solvent extraction procedures.
?	Infant formulae	Linoleic acid	Bellomonte et al., 1991.[3]	Acid hydrolysis preceded Soxhlet extraction.
MeOH	Wheat	Radiolabelled diflubenzuron (bound residues)	Aly and Dauterman, 1992.[4]	Measure of non-extractable radioactivity.
?	Apples	Ethiofencarb	Clavijo et al., 1996.	Peel compared to interior.

[1] A. Polesello and F. Pizzocaro, *S & TA & NU. Rev. Sci. Tech., Food Human Nut.,* 1976, **6**, 305.
[2] J.L. Kacprzak, *J.AOAC,* 1977, **60**, 1142.
[3] G. Bellomonte, S. Giammarioli, and R. Terilli, *Int. J. Vit. Nut. Res.* 1991, **61**, 91.
[4] M.A. Aly and W.C. Dauterman, *J. Env. Sci. Health, B, Pesticides, Food Contaminants & Agricultural Wastes*, 1992, **27**, 113.

APPENDIX 5 *Continued*

Solvent(s)	*Commodity*	*Analyte*	*Reference No.*	*Comments*
60–80 Petroleum ether	Nigerian foods	Secondary amines	Uheggu, 1997.[5]	Diethylamine, dimethylamine, morpholine, ethylaniline, piperidine, n-propylamine, proline
DCM-hexane (1:1, v/v)	Txakoli wine	Volatiles	Escobal et al., 1997.[6]	Combined with GC/MS
Hexane, $CHCl_3$, acetone	Citrus reticulata peel	Polymethoxylated flavones	Jayaprakasha et al., 2000[7]	Silica gel fractionation of most effective EtOH-soluble extract contained desmethylnobiletin, nobiletin, and tangeretin. Antibacterials.
N-hexane	Sesame seeds	Pesticides	Gebreegzi, et al., 2000.[8]	Method allowed the simultaneous determination of carbaryl, malathion, fenitrothion, and diazinon residues.
Each EtOAc, acetone, MeOH, H_2O	Dried pomegranate peel powder	Antioxidants and antimutagens	Negri et al., 2003[9]	Extracts may have use as biopreservatives in foods
MeOH	Potato chips	Acrylamide	Pedersen and Olsson, 2003 (Chapter 5)	Amount extracted affected by: solvent properties and volume, extraction time, temperature, particle size, and microstructure.
Petroleum ether	Fish and fish feeds	Lipids	Schlechtriem et al., 2003 (Chapter 5)	Hot SOX compared to B&D and Smedes for effect on δ^{13}C-isotope signature.

[5] F.O. Uhegbu, *Plant Foods for Human Nutrition*, 1997, **51**, 257.
[6] A. Escobal, C. Iriondo, and C. Laborra, *J. Chromatogr. A,* 1997, **778**, 225.
[7] G.K. Jayaprakasha, P.S. Negri, S. Sikder, L.J. Roa, and K.K. Sakariah, *Zeit. Naturforsch. C, J. Biosci.*, 2000, **55**, 1030
[8] Y.T. Gebreegzi, G.D. Foster, and S.U. Khan, *J. Agric. Food Chem.*, 2000, **48**, 5165.
[9] P.S. Negri, G.K. Jayaprakasha, and B.S. Jena, *Food Chem.,* 2003, **80**, 393.

1 General Index

2 Commodity/Extraction Method/Analyte, Chemical Class or Utility Index

3 Extraction Method/Analyte, Chemical Class or Utility/ Commodity Index

4 Analyte|Extraction Method| Commodity

5 Chemical Class/Extraction Method/Commodity

6 Utility/Extraction Method/ Commodity