EXTRACTION OF RICIN FROM CASTOR

by

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CHAPTER 1

INTRODUCTION

Plants need chemical defenses – natural or synthetic – to survive various stresses, such as pathogen attacks, wounding, application of chemicals including phytohormones and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions. Higher plants protect themselves from these stresses by varying their physiological conditions. Plants also produce toxins as a natural chemical defense against, fungi, insects and other predators. When plants are stressed or attacked by pests, they greatly increase their output of natural pesticides, occasionally to levels that are acutely toxic to humans.

Tens of thousands of these natural pesticides have been discovered, and every species of plant contains its own set of different toxins (Stirpe et al., 1992), usually a few dozen. Ricin from castor beans (*Ricinus Communis*), saporin from soapwort seeds (*Saponaria officinalis*) and abrin from jequirity bean seeds (*Arbrus precatorius*) are some of the best-known examples of these toxins.

Ricin – a broad view

Ricin is obtained from the beans of the castor plant, the same plant from which castor oil is derived. Castor oil pressed from the seeds of castor bean (*Ricinus Communis*) has long been used both as a medicinal purgative and as lubricating oil for machinery (Salunkhe et al., 1992). For just as long, castor bean seed has also been recognized to contain ricin, one of the most potent toxins known. The toxic principle, a

protein called ricin is found in the residue, or pressed cake, which remains following the extraction of the oil. Ricin is a phytotoxin and is one of the most poisonous proteinaceous substances known, like the toxins of tetanus, botulism, diphtheria, etc., and is so powerful that one milligram of it is deadly. Ricin has attained notoriety since World War I both as a potential chemical warfare agent and also as the perfect poison that could kill instantly (Knight, 1979), leaving no telltale signs.

Ricin is a heterodimeric toxin. Ricin molecules are made up of two parts, one part is a ribosome-inactivating protein (RIP), also known as the A-chain and the other part is a carbohydrate binding lectin, also called the B-chain. The B-chain lectin is the aggressor, attaching to cells throughout the body and the A-chain (RIP) is the killer, inactivating the ribosomes of the cells to which it attaches thus destroying the cell. These two chains work in tandem, one chain (lectin) leads the pair to the target cell and the other chain (RIP) kills the target cell.

Objective – Ricin as an Immunotoxin

A major application of ricin, currently being explored, is in the construction of immunotoxins. An immunotoxin is a molecule constituted of a cell-reactive ligand coupled to a toxin or its active sub-unit (Ghetie and Vitetta, 1994). The ligand portion of the immunotoxin is usually an antibody or growth factor that binds with partial or partial selectivity to the target cell. The toxic portion of the immunotoxin then destroys the target cell selectively. Ricin can be used as an immunotoxin by conjugating its A-chain to cell binding ligands such as antibodies or growth factors. If these ligands are tumor cell specific, they preferentially bind the unwanted cells. The toxin then kills the tumor

cells selectively, unlike chemotherapy or radiotherapy, which kills all, rapidly dividing cells, malignant or normal.

The present study revolves around the design and parameterization of an extractor for the extraction of the extremely toxic protein 'ricin' from castor beans. It also addresses the issues involved in the extraction of the ricin, such as the protein chemistry involved and the materials to be used in any such extraction. Due to the hazards involved in the extraction of the toxic protein ricin, it becomes imperative to establish certain guidelines and criteria for the safe and efficient operation of the extractor. Extraction runs were conducted on mechanically delinted cottonseeds to achieve this objective. Similar trials were conducted on autoclaved castor beans with the autoclaving being done to ensure that the ricin is denatured making it safe to work with. This served to simulate similar conditions as would be encountered during the actual extraction of ricin from castor. Effective conditions for the extraction of ricin from castor in a controlled environment were obtained from these studies.

CHAPTER II

LITERATURE REVIEW

Ricin is a protein produced by the castor oil plant, *Ricinus communis*, which is highly toxic. The castor bean plant belongs to the genus *Ricinus* of the Euphorbiace or spurge family (Atsmon, 1985). This stout, robust plant is a shrub-like herb with reddish to purple stems that may reach as much as 12 feet in height and its leaves are 4 to 30 inches across and have 5 to 9 pointed, finger-like lobes. These plants range in color from, bright red stems and leaves rich in anthocyanin, to a uniform dark green. Castor is grown both as an ornamental plant and also as an oilseed crop. Greenish-white or reddish-brown flowers are produced in narrow, upright clusters. The fruit is a three-lobed, green or red capsule with soft, spiny exterior, 1.5 to 2 cm long. Inside each lobe develops one large, mottled, attractive seed. The castor bean seeds are grayish-brown mottled with reddish-brown in color and about 10mm long and 6 to 7 mm wide in size. The seeds are the most dangerous part of the plant because they are the primary source of the toxin ricin.

Ricin is a heterodimeric type 2 RIP, which consists of two smaller proteins held together covalently, by a disulfide bridge. It begins synthesis in the endosperm as a prepropolypeptide (proricin), that contains both A and B chains (Lamb et al., 1985; Butterworth and Lord, 1983). Ricin is not catalytically active until it is proteolytically cleaved by an endopeptidase within the protein bodies. This splits the polypeptide into the A- chain (RTA) and the B- chain (RTB) still linked by a single disulfide bond. Since ricin is inactive until then, the plant avoids poisoning its own ribosomes in case some

proricin accidentally passes into the cytosol during synthesis and transport. When the mature seed germinates, the toxins are destroyed by hydrolysis within a few days. To understand the toxicity of ricin, we need to look at the activities of these two subunits. the A-chain (RTA), a Ribosome Inactivating Protein and the B-chain (RTB), a Lectin.

Ribosome Inactivating Proteins (RIPs)

Many cytotoxic proteins that inhibit protein synthesis by specifically and irreversibly inactivating eukaryotic ribosomes have been identified (Stirpe et al., 1992). These proteins are widely distributed in Angiosperms and also occur in several species of fungi and bacteria. This family of proteins is known as ribosome inactivating proteins or RIPs. The very nature of these proteins, i.e., that they catalytically and irreversibly inactivate the 60S sub-unit of eukaryotic ribosomes, rendering it incapable of binding elongation factor 2 is commonly used as criterion for their inclusion within this group (Stirpe and Barbieri, 1986). This definition was proposed before the nature of the RIP mediated modifications to ribosomes were known. Hartley and Lord indicate that this has been broadened to take into account the recent finding that some RIPs are also active on prokaryotic ribosomes. Protein toxins that act by the catalytic inactivation of protein synthesis accessory factors such as diphtheria toxin and Pseudomonas exotoxin are not included within this group. Due to the extreme cytotoxicity of these RIPs the ribosomes of the organisms producing them adopt two protective strategies:

1. A developed resistance to the toxicity:
2. Sequestration of the RIP thus preventing them from coming into contact with the ribosome.

The most widely adopted classification of RIPs is that attributed to Stirpe and Barbieri (1986). They designated those RIPs existing in nature as single chain proteins or glycoproteins as type I and those consisting of, a catalytically active A-chain, linked to. a cell binding B-chain with lectin properties as type II. The most commonly used nomenclature is to name the RIP after the generic name of its plant of origin. Thus ricin. abrin and dianthin are the RIPs from Ricinus communis, Abrus precatorius and Dianthus caryophyllus, respectively.

Distribution of RIPs

Type I RIPs are widely distributed in Angiosperms and have been seen to be found in some sixty species belonging to fifteen different families according to Barbieri and Stirpe (1982). These RIPs occur in plant organs, like seeds, leaves, lattices, roots and tubers in concentrations ranging from a few micrograms to several hundred milligrams per 100g of tissue (Hartley and Lord, 1990). (See Table 2.1 for details of some of the more common Type I RIPs.)

However, in order to bind to the cell surface galactosides, and to enter the cytosol to reach ribosomes, they require a second monomer, a galactose binding lectin. The monomers are joined by a disulfide bridge thus forming, the toxic heterodimer (Type II RIPs). Some plants, such as wheat and barley, have only Type I RIPs, and are not poisonous, while others, such as the castor bean plant seed, contain the Type II RIPs that are among the most potent cytotoxins in nature. Figure 2.1 illustrates the basic difference between the two types.

The other type of RIPs viz. type II, are less common than their type I counterparts and have been identified in five species from four different families. In all cases the A-chain is joined through a single disulfide bond to a galactose binding B-chain also of around 30 kDa. (Table 2.2 lists some of the known Type II RIPs.)

Lectins

In his dissertation published in 1888, H. Stillmark is said to have first reported the fortuitous observation that aqueous extracts of castor bean caused agglutination of mammalian erythrocytes (Franz, 1988). It was later recognized that the agglutination caused by castor bean extracts was due to the presence of another toxin RCA (Ricinus Communis agglutinin) and not due to ricin. In due course of time, protein extracts from a variety of plants were found to cause agglutination (clumping) of mammalian erythrocytes. These were termed Lectins (L. legere = to choose), as it was learned that their hemagglutinin property resulted from the presence of specific carbohydrate binding sites on the protein. The terms, phytohemagglutinins, phytagglutinins, and lectins are used interchangeably. In a broader sense, lectins are carbohydrate binding proteins capable of recognizing and reversibly binding with specific complex carbohydrates (Sharon, 1989). As compared to other proteins lectins are relatively small with molecular masses ranging from 50 kDa to 120 kDa.

Ricin – The Protein

Ricin is obtained from the beans of the castor plant, the same plant from which castor oil is derived. The castor bean plant belongs to the genus *Ricinus* of the

Euphorbiace or spurge family (Atsmon, 1985). The endosperm tissue of castor seeds contain two proteins which are highly toxic (Lord et al., 1994), Ricinus Communis agglutinin (RCA), a 120 kDa hemagglutinin (coagulates red blood cells), and ricin, a 65 kDa cytotoxic lectin lethal to eukaryotic cells. They were first described in castor seeds in the late nineteenth century by H.Stillmark, an Estonian scientist, after aqueous extracts of the seeds caused agglutination of mammalian erythrocytes (Franz, 1988).

Balint (1974) discusses over 700 cases of human intoxication dating back to the late 1800s. Ricin, through the past century has attained notoriety due to its use as a weapon of destruction, by means of assassination attempts and also by attempts at mass destruction through use as a chemical weapon. Ricin has been used by secret intelligence services as a weapon in many assassination attempts, some successful (Cooper and Johnson, 1984; Griffiths et al., 1987), some botched.

It was evident that ricin could be prepared as an odorless powder capable of being dispersed as a particulate or dust cloud (Blair et al., 1943). With the absence of odor and the complexity of the consequent detection problem, ricin was more insidious than any available chemical warfare agent was, at that time. During World War I, ricin was examined as a candidate chemical warfare agent and its preparation was studied. The investigation of the preparation and properties of ricin pertinent to its use as a chemical warfare agent was renewed in Great Britain early during World War II and in this country during the fall of 1942. Systematic work on the use of ricin as a chemical warfare agent was begun in the United States during the fall of 1942. Its immediate objective was the production on a pilot plant scale of a sufficient quantity of an active product to make possible field trials of methods of dispersal of this novel type of agent.

During the course of these studies, no systematic investigation of the mechanism of the action of ricin was undertaken. The knowledge about the intrinsic nature of the toxic action at that time hence remained fragmentary.

Butterworth and Lord (1983) revealed that ricin has two polypeptide chains, the A-chain and the B-chain, while the RCA protein has four polypeptides, linked by disulfide bonds. Two of the agglutinin chains are similar to the ricin A-chain and two are similar to the B-chain. The A and B chains of ricin, together are highly lethal to mammalian cells, while the Ricinus Communis agglutinin has limited cellular toxicity, but increases agglutination of cells. RCA is a powerful hemagglutinin but a weak cytotoxin whereas ricin is a weak hemagglutinin and a potent cytotoxin. Poisoning by ingestion of the castor bean is due to ricin, not RCA, because RCA does not penetrate the intestinal wall, and does not affect red blood cells unless given intravenously. If RCA is injected into the blood, it will cause the red blood cells to agglutinate and burst by hemolysis. Ricin E is a variant of the ricin toxin, with an A-chain similar to ricin and a B-chain hybrid between the ricin and RCA B chains (Ladin et al., 1987).

The phytotoxin ("plant toxin") ricin in castor bean is a water-soluble protein concentrated in the seed. It is said to be one of the most toxic natural poisons, poisonous to people, animals, and insects. Ricin is a type II RIP (ribosome inactivating protein) that consists of two smaller proteins held together covalently by a disulfide bridge. These two proteins are the A-chain (**RTA**) and the B-chain (**RTB**).

Lord et al. (1994) made it clear that the A-chain of ricin is a ribosome inactivating protein. This 32kDa subunit prevents protein synthesis by irreversibly altering the ribosomal subunits involved in translation. The ricin A-chain cannot enter the cell

without the B-chain. The lectin portion of the ricin is the B-chain. The B-chain attaches to the eukaryotic cell and the intact toxin enters the cell by receptor mediated endocytosis (Bilge et al., 1994)

The RTA portion of the heterodimer specifically targets a sequence in ribosomal RNA and completely inactivates the ribosomes. Ribosomes are the machinery that produces proteins in the cell. Without functional ribosomes, the cell cannot produce the enzymes it needs to operate and hence dies. On its own, RTA cannot enter the cell to get access to the ribosomes.

RTB is a lectin (a protein which binds to sugar), which is specific for sugars containing galactose. Since, complex sugar chains – several of which contain galactose – decorate most proteins on the outsides of cells, the RTB has lots of places to stick to. Besides sticking to the outside, RTB can piggyback on these proteins as they are internalized into the cell (via the TGN) thus being carried into the cell itself.

So by attaching RTA to RTB, castor oil plants have created a ribosome inactivator that can be carried into the cell where it is toxic. A normally benign toxin is linked to a protein, which gives it a way into the cell, where it is deadly.

Ricin – The Chemistry

The proteins we observe in nature have evolved, through selection pressure, to perform specific functions. Proteins are basically built up by amino acids that are linked by peptide bonds into a polypeptide chain. All of the amino acids, identified so far, have in common a central carbon atom to which are attached a hydrogen atom, an amino group (NH 2), and a carboxyl group (COOH). What distinguishes one amino acid from the

other is the side chain attached to its central carbon atom. Amino acids are joined end-to-end during protein synthesis by the formation of peptide bonds. The carboxyl group of the first amino acid condenses with the amino group of the next, eliminating a water molecule in the process, and yield a peptide bond. These peptide bonds are repeated as the chain elongates. The formation of a succession of peptide bonds generates a "main chain" or "backbone" from which the various side chains project (Branden and Tooze, 1991).

The amino acid sequence of a protein's polypeptide chain is called its primary structure. Different regions of the sequence form local regular secondary structure, such as alpha helices or beta sheets. Packing such structural elements into one or several globular units called domains, forms the tertiary structure. The final protein may contain several such polypeptide chains arranged in a quaternary structure. By formation of such ternary and quaternary structures amino acids far apart in the sequence are brought together in three dimensions to form a functional region called the 'active site'. The functional properties of proteins depend upon their three-dimensional structure. The three dimensional structure as explained arises because particular sequences of amino acids in polypeptide chains fold to generate, from linear chains, compact domains with specific three-dimensional structures. The folded domains either serve as modules for building up large assemblies such as muscle fibers or provide specific catalytic or binding sites as found in some enzymes or proteins.

The most remarkable feature of the protein molecule is its complexity and its lack of symmetry. In spite of these, there are several regular features present in protein structures – the most important of which is the secondary structure. There are two main

types of secondary structure, alpha helices and beta sheets. Combinations of these secondary structural elements form the core regions – the interior of the molecule – and loop regions at the surface connect them.

The coding region in the ricin protein is a 24 amino acid N-terminal signal sequence preceding a 267 amino acid chain. The B-chain is 262 amino acids and a 12 amino acid linker joins the two chains. Figure 2.1 (Lord et al., 1991) depicts a three-dimensional ribbon drawing of ricin modeled from X-ray crystallography data. In Figure 2.2, the upper right half, the dotted ribbon, is the A-chain (or RTA), and the lower left half, the solid ribbon, is the B-chain (or RTB).

The RTA is a 267-amino acid globular protein. It has 8 alpha helices and 8 beta sheets. The substrate-binding site is at the cleft, marked by the substrate adenine ring. The carboxyl-terminal end of the A-chain folds into a domain that interacts between the two domains of the B-chain. The A-chain exhibits a substantial amount of secondary structure and 30% of the protein is helical. The A-chain folds into three more or less arbitrary domains. The 'active site' of the A-chain is located at the cleft created at the interface between all three domains (Montford et al., 1987)

The RTB is a 262-amino acid protein that is shaped like a barbell. RTB, the galactose specific lectin, forms two distinct globular domains with identical folding topologies (Montford et al., 1987). Each domain contains two internal disulfide bonds, one glycosylation site, which is usually occupied, and one sugar-binding site which lies in a pocket formed, in part, by a kink in the polypeptide chain (Rutenber et al., 1987). As a result it has a binding site for galactose at each end (depicted by lactose rings). These two sites allow hydrogen bonding to specific membrane sugars (galactose and N-acetyl

galactosamine). A disulfide bridge (-S-S-) joins RTA with RTB (far-right, center). The spheres in the figure are trapped water molecules.

A model for the active site region of RTA that has been proposed by some researchers (Katzin et al., 1991) indicates the presence of the several residues. Substitution of one or several of these residues reduces the ribosome-inactivating activities of the mutant polypeptides by a factor of greater than 100 (Frankel et al., 1990).

Ricin – How It Works

The RTB portion of ricin binds to both glycoproteins and glycolipids at cell surfaces that contain terminal galactose residues. Mammalian cells contain an abundance of such binding sites, ensuring a high concentration of bound toxin. A small portion of the toxin bound at the surface of the target cell is internalized.

Ricin enters cells by endocytosis primarily, but not exclusively (Moya et al., 1985), via coated pits and vesicles (van Deurs et al., 1985). Though a large portion of the ricin taken into cells is either recycled back to the cell surface or is delivered into lysosomes, where it is degraded there is a small portion of ricin avoids recycling or degradation. It is from this small portion that RTA crosses an intercellular membrane to reach its ribosomal substrates in the cytosol. Toxic action occurs when RTA (A chain) penetrates the Trans Golgi Network (TGN) membrane and is liberated into the cytosol, or cell fluid. Once inside the cytosol, the RTA catalyzes the depurination of the ribosomes, halting protein synthesis. An extremely low concentration is enough to inhibit protein synthesis. Just a single ricin molecule can inactivate over 1,500 ribosomes per minute when it enters the cytosol, or cell fluid, killing the cell.

This series of transports facilitates the toxins rapid entry into the cytosol. The enzymatic active site on the A-chain is exposed when the A-chain is released from the B-chain (Olsnes and Pihl, 1982). The A-chain binds and depurinates a specific adenine (at position 4324) found in the 28S ribosomal RNA (rRNA) subunit in the 60S ribosome subunit (Robertus, 1991). The adenine at this specific position is removed, leaving the sugar-phosphate backbone intact. Thus the A-chain acts as a highly specific hydrolase, cleaving a single N-glycosidic bond among approximately 7000 nucleotide residues in rRNA. The removed adenine (A) lies near the center of a 14 nucleotide sequence that is the most strongly conserved structural feature of the large rRNA. As the structural integrity of this sequence is of crucial importance to the functioning of the ribosome, removal of this adenine inactivates the ribosome.

Ricin – Its Toxicity

In very small doses, ricin only causes the human digestive tract to convulse. But it is supposed that less than one milligram of 100% ricin taken orally can kill an adult (Bush et al., 1946). When inhaled as a small particle aerosol, this toxin may produce pathologic changes within 8 hours and severe respiratory symptoms followed by acute respiratory failure and death in 36 to 72 hours. When ingested, ricin causes severe gastrointestinal symptoms followed by vascular collapse and death.

Ricin has a LD_{50} of 3 ppm in mice (Olsnes and Pihl, 1973). The symptoms of ricin poisoning via ingestion include abdominal pain, vomiting, and diarrhea, which can sometimes be bloody (www.ansci.cornell.edu/toxicagents/ricin). Within several days there is severe dehydration, a decrease in urine, and a decrease in blood pressure. Ricin

works as a slow poison, inhibiting protein synthesis and ultimately causing a total body "shut-down" because essential proteins are not being replaced.

Ricin as an Immunotoxin

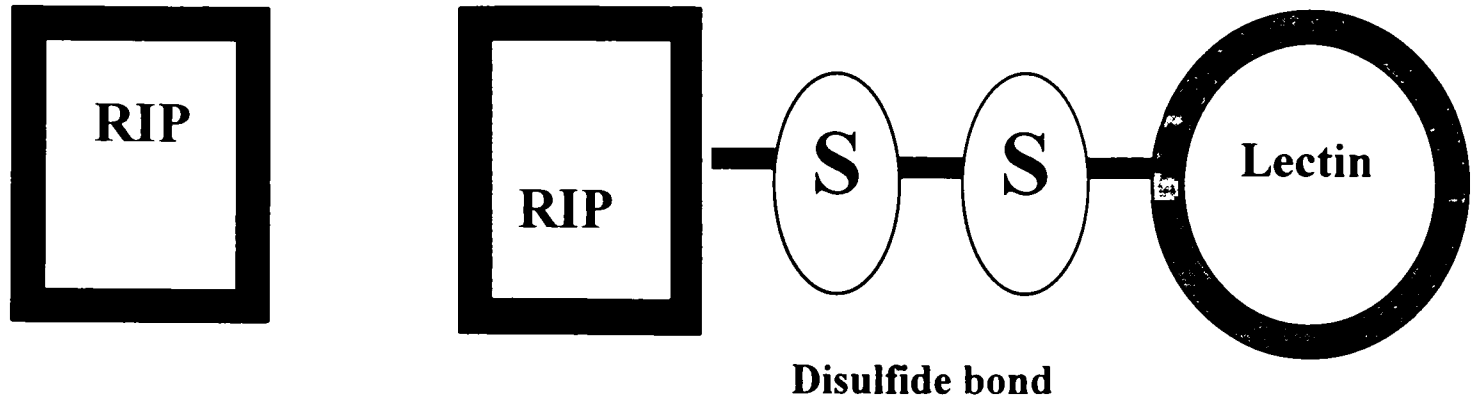
A major application of ricin, currently being explored, is in the construction of immunotoxins. Ricin can be targeted to specific cells such as cancer cells by conjugating its A-chain to cell binding ligands such as antibodies or growth factors. If these ligands are tumor cell specific, they preferentially bind the unwanted cells. The toxin then kills the tumor cells selectively, unlike chemotherapy or radiotherapy, which kills all cells that are dividing rapidly regardless of whether they are malignant or normal.

Table 2.1 (Type I RIP Classification) List of some common Type I RIPs and their salient details (Stirpe et al., 1987; Hartley and Lord, 1990)

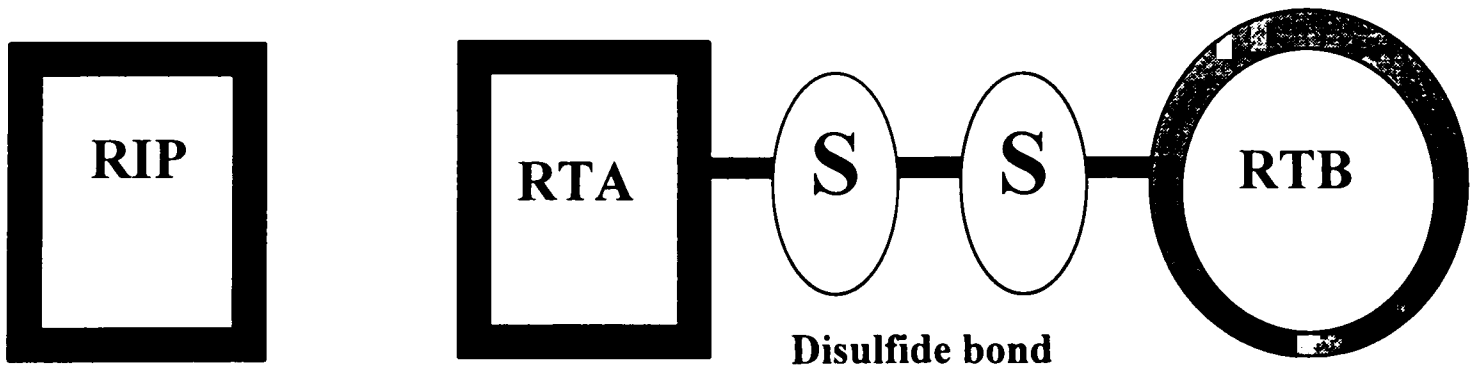
Plant source	**Inhibitor**	**Mol.Wt. (kDa)**	**Glycosylated**
Pokeweed seeds or leaves *Phytolacca americana*	Pokeweed antiviral proteins (PAP)	30	No
Wheat seeds *Triticum aestivrum*	Tritin	30	No
Gelonium multiflorum seeds	Gelonin	30	No
Bittergourd seeds *Momordica charantla*	Momordin	31	No
Soapwort seeds *Saponaria officinalis*	Saporin	29.5	No
Carnation seeds *Dianthus caryophyllus*	Dianthin	30	Yes
Maize seeds *Zea mays*	Maize RIP (Dimeric)	16.5 18.5	

Table 2.2 (Type II RIP Classification) List of some common Type II RIPs and their salient details (Stirpe et al., 1987; Hartley and Lord, 1990)

Plant source	Inhibitor	Approx. Mol.Wt. (kDa)	Glycosylated
Castor bean seeds	Ricin	65	Yes
Ricinus communis	A-chain	32	Yes
	B-chain	34	Yes
Jequirity bean seeds	Abrin	65	Yes
Abrus precatorius	A-chain	30	No
	B-chain	36	Yes
Adenia digitata roots	Modeccin	63	Yes
	A-chain	28	
	B-chain	31	
Adenia volkensil roots	Volkensin	62	Yes
	A-chain	29	
	B-chain	36	
Mistletoe leaves	Viscumin	60	Yes
Viscum album	A-chain	29	Yes
	B-chain	32	Yes



RIP Type I **RIP Type II**



WHEAT
BARLEY

RICIN--- from CASTOR BEAN

Figure 2.1. The basic structural differences between RIPs of Type I, II (shapes of lectin, RIP and disulfide bond are arbitrary), from the website www.ansci.cornell.edu/toxicagents/ricin.

Figure 2.2. A 3-dimensional ribbon drawing of ricin modeled from X-ray crystallography data. (Lord et al. from www.ansci.cornell.edu/toxicagents/ricin)

CHAPTER III

LEACHING

Leaching is a two-phase, mass transfer process in which solutes from a solid, usually in a particulate form, are transferred to a contiguous liquid, the extract. In simpler terms, leaching is a separation technique that removes a solute from a solid mixture with the help of a liquid solvent. Separation, through leaching, usually involves selective dissolution, with or without diffusion. In the extreme case of simple washing it may consist merely of the displacement, accompanied by some mixing, of one interstitial fraction by another with which it is miscible. Though leaching, almost invariably involves diffusion of solutes in the solid it may also involve washing of solutes or extract off the solid's surfaces, displacement from interparticle pores, and solubilization, reaction induced creation of solutes from insoluble precursors.

Components of a Leaching Process

The solid and the liquid in these systems are generally termed as phases even though the solid phase is rarely structurally homogeneous. The solid phase may consist of a matrix of insoluble solids and the occluded solution. Solute particles may exist either in the, or on the inert solid in a variety of ways. The soluble constituent may be a liquid or a solid, and it may be incorporated within, chemically combined with, adsorbed upon or held mechanically in the pore structure of the insoluble material. The solute may exist on the surface of the solid as in the case of simple washing. It may be surrounded by a matrix of inert material, may be chemically combined or may exist inside cells as in

the case of many vegetable bodies (Schweitzer, 1996). In effect, a given solid-liquid extraction system is assumed to consist of the following three components:

- Inert, soluble solids – solute;
- A single solid, which may either, be a single solid/liquid or a mixture of soluble components;
- A solvent, which selectively dissolves the solute but, has little or no effect on the inert solid.

Leaching is known by such other names as decoction (use of solvent at its boiling point), lixiviation, percolation, infusion, and elutriation, based upon its application in the industry (Perry and Green, 1989). All of these solid-liquid extraction methods or leaching methods involve four steps, viz.:

- Pretreatment of the solid;
- Contact of liquid solvent with the solid to effect transfer of solute from the solid to the solvent;
- Separation of resulting solution from the residual solid;
- Recovery of solute from the solvent.

Pretreatment of the Solid

Knowledge of the physical characteristics of the solid matrix is very important to determine whether it needs prior treatment to make the solute more accessible to the solvent. Prior treatment or preparation may involve crushing, grinding, cutting into pieces or reforming into special shapes such as flakes. The solid matrix in some special

cases, generally in the case of toxic proteins, may also be heated to destroy the cellular structure and to detoxify the material being handled. All of the above treatment methods have to be optimized with due consideration being given to the requirements and to the nature of the solid matrix being handled.

Dry solids first imbibe the solvent and the solvent then dissolves the solute content. The solutes then diffuse through occluded solution contained in pores and cells in the solid. The solvent easily leaches solute adhering to the surface. However when the solute exists in pores surrounded by a matrix of inert material, the solvent has to diffuse to the interior of the solid to leach the solid and then diffuse out to the solid. In such cases, subdivision of the solid by prior treatment increases the solute surface exposed to the solvent. The leaching process is favored by increased surface per unit volume of solids to be leached and by decreased radial distances that must be traversed within the solids, both of which are functions of particle size. Fine solids, on the other hand, cause slow percolation rate, difficult solids separation and possible poor quality of solid product. The optimum particle size is established by these characteristics.

Selection or Design of a Leaching Process

The major aspects that need to be approached with care for the leaching operation are the selection of process operating conditions and the sizing of the extraction equipment. The process operating parameters that have to be fixed or identified are:

- The solvent to be used;
- Temperature of operation;
- Terminal stream compositions and quantities;

- Leaching cycle;
- Contact method, and based upon all the above;
- The specific choice of extractor.

Choice of Solvent

The solvent selected will offer the best balance of a number of desirable qualities. The specifics of each leaching process determine the balance and relative significance of each quality. Any one of the below factors can be the determining one under the right combination of process conditions.

The solvent has to have a high saturation limit and selectivity for the solute to be extracted. The solvent should not contaminate the product, i.e., the quality of extracted material should be unimpaired by the solvent. The solvent should remain chemically stable under process conditions and should preferably have low toxicity and flammability. The ease and economy with which the solvent can be recovered from the extract also is one factor to be considered.

Hexane has been extensively used for most vegetable oil extractions. In the recent past other organic solvents like, alcohol and alcohol-water mixtures have been used as solvents.

Temperature

The temperature of the extraction should be chosen with the view of obtaining the best balance between several factors such as; solute solubility, solvent-vapor pressure, solute diffusivity, solvent selectivity, and sensitivity of product to temperature.

Solid-Solvent Compositions and Quantities

These are basically linked to the desired production capacity of the leaching process. The compositions and quantities should be chosen such that they maximize process economy, while confirming to requirements.

Leaching Cycles

The choice between continuous or batch operation is largely a matter of the size and nature of the extraction process. Though continuous flow reactors are likely to be most economic for large-scale production, batch reactors provide very real advantages. especially for smaller scale production. Small batch reactors generally require less auxiliary equipment, such as pumps, and their control systems are less elaborate and less costly than those for continuous reactors are. In some processes, batch reactors are preferred because the interval between batches provides an opportunity to clean the system thoroughly and ensure that no deleterious intermediates build up and contaminate the product. Batch reactors are best suited for cases where in a few batches per year are sufficient to meet the production requirements for an unusual product.

Contact Method

The basic operating methods used in leaching systems depend upon the solid-liquid contacting method that is desired. The basic types of solid liquid contacting methods that may be distinguished are (Schweitzer, 1996):

1. Fixed-bed contacting – In fixed-bed contacting, the solid particles are stationary while the solvent is contacted with the fixed bed of solids in different ways.
2. Dispersed contacting – Dispersed contact involves the motion of the solid particles relative to each other and also relative to the liquid. This sort of a contact is usually affected by agitation.

The choice of the solid-liquid method of contact depends, principally upon the nature of the solid-solvent pair and upon the properties of the solid. The difference in the above types of leaching is in the manner in which the solid and liquid are contacted in different ways.

Reduction of solids to finer particle size increases the surface exposed to the solvent and thus enhances extraction. Hence, it is undesirable to apply the fixed-bed contacting method in situations where very finely divided solid particles are to be treated. In the fixed-bed contacting method, very fine particles may pack the solids during extraction, preventing free flow of solvent through the solid bed. In such cases, the dispersed contacting method has to be used. On the other hand, dispersion of particles in liquid solvent by agitation permits thorough contacting of the solid with the solvent, but has its own limitations. Agitation while giving good extraction may cause suspension of fine particles in the solution, which may subsequently hamper the filtration or clarification step. This may also affect the quality of the solid product.

Type of Reactor

The final choice for the type of reactor to be employed for a particular leaching process is totally based upon, the chosen combination of the preceding parameters. The specific type of reactor that is most compatible with these selected parameters is very rarely perceived, clearly and unequivocally, without difficulty. The ultimate criteria for the design are reliability and profitability.

Leaching Systems

The following features are generally used to distinguish leaching systems designed with the above factors.

1. Operating cycle: Batch, continuous, or multi-batch intermittent;
2. Direction of streams: Co-current, counter current, or hybrid flow;
3. Staging: Single-stage, multistage; and
4. Method of Contacting: Sprayed percolation, immersed percolation, or solids dispersion.

The mechanism of leaching may involve simple physical solution or dissolution made possible by chemical reaction. The rate of transport, of solvent into the mass to be leached, or of soluble fraction into the solvent, or of extract solution out of the insoluble material, or some combination of these rates may be significant. The concept of equilibrium for leaching differs from the one applied in other mass-transfer separations. This is due to the fact that the overflow and the underflow streams are not immiscible phases but streams based on the same solvent. Usually, it is not feasible to establish a

stage or overall efficiency, or a leaching rate index, without testing small-scale models of likely apparatus.

CHAPTER IV

EXTRACTION OF RICIN

Most methods for extracting ricin from castor involve a preliminary delipidification process. In this phase, a substantial portion of the oil present in the castor bean is extracted using organic solvents like heptane, acetone, etc. The castor bean cake thus obtained is then treated with a buffer solution of controlled pH to extract the ricin. Among the solvents that have been used to extract the toxin from castor beans are water, dilute salt solutions, glycerol, ethylene glycol etc.

In one of the commercial processes employed in the past (Craig et al., 1946.), castor beans are ground, heated to 60^0 C and pressed using a Carver press. This cold pressed pomace or cake was recommended as the starting material for any large-scale production of ricin. The toxin is best isolated if the product is further extracted with heptane to remove the remaining castor oil.

Extraction

The extraction of ricin from castor beans involves three major steps each step in turn involving several process operations.

Pretreatment of the Castor Beans

Castor seeds are flattened oval in shape with spiny brittle testa enclosing a white kernel. The seeds vary in size from 4mm to 25mm in length and from 5mm to 16mm in breadth (Salunkhe et al., 1992). The seed coat makes up about 25% of the seed by

weight. The epidermal cells have a thick cuticle, are pigmented and show a characteristic pitting. The germ with cotyledons occupies 75 to 80% of the total seed volume and is made up of soft parenchyma tissue.

The whole seed contains about 45 to 50% oil, 12 to 16% protein (~ 1% of which is ricin), 3 to 7% carbohydrates, 23 to 27% fiber and 2% ash. The pretreatment phase involves the grinding of the castor beans, to dehull them and to ensure an optimally fine particle size, thus facilitating extraction. The grinding was to be carried out very carefully so that the delicate nature and structure of the proteins are not damaged.

Delipidification

The castor bean as explained above contains a high percentage of oil, removal of which is an important step in the extraction of the protein. Pinkerton, 1997, reveals that use of acetone for extraction of castor oil from castor beans gives satisfactory yields of ricin. Hence, the castor beans were to be delipidified by extraction with acetone.

Extraction of Ricin

Acetone Filtration and Removal

The ricin rich, relatively oil-free castor was the starting material for the extraction of ricin. Hence the acetone used in the previous phase, as also the oil extracted, had to be separated from the ground castor bean cake by filtration.

Extraction with Solvent and Recovery of Ricin

The ricin rich castor was then to be extracted with the apt solvent. The solids and the solvent were to be mixed thoroughly to enable effective solid-solvent contact. The next step would be the recovery of the extracted ricin by physical separation, in this case filtration.

Denaturing and Detoxifying Any Remnant Ricin

The filter cake and any remnant ricin resulting as a result of spills and leaks was then to be detoxified by heating to a temperature of around 100-125^0 C for around 3-4 hrs.

Extractor

The nature of the product, the scale of production desired and the need for non-continuous operation of the reactor suggested that a batch reactor would best suit our requirements. The design of the batch extractor attempted to achieve the above mentioned process operations within its simple apparatus. The objective in the design of the extractor was to transform relatively innocuous whole castor seeds into a very toxic aqueous extract of ricin, and a residue in which any remaining ricin had been deactivated by heat inside one custom-designed grinder/extractor container. Grinding of the castor beans, their subsequent delipidification and the extraction of ricin from the crushed castor beans was carried out in the batch extractor.

The extractor was designed after taking into consideration the nature of the solid-solvent pair to be handled. The nature of the solid particles to be handled, their initial

state and the pretreatment desired were some of the other factors that were analyzed. It consisted of the following parts.

Extraction Column: Design Considerations

The column within which the extraction proper was carried out was designed to meet the following requirements.

- Serve as a compact extraction vessel within which the solvent and solids could be brought into contact (Figure 4.1).
- Crush and finely grind the castor beans without degrading the delicate protein structure of the ricin.
- Filter the ground slurry to remove the acetone (after delipidification) and also to obtain the protein.
- Pressurize the column during filtration, if desired.

Extractor Components

Column

A ridged commercial Pyrex glass pipe (Figures 4.1 and 4.2) with a nominal diameter of 101.6mm and length of 305mm was the extraction vessel within which the extraction took place. Glass was chosen as the material for the column because of obvious reasons like, ease of visually monitoring the operation, easier clean up procedures and minimal chance of contamination. The glass column was affixed upon an axis and could be pivoted on its axis. The column could also be fixed at any desired

angle. As shown in Figure 4.1, the extraction column had bottom and top stainless steel flanges held in place by one-bolt couplings. These stainless steel flanges had several features that would perform the desired process operations (Figure 4.3). One flange had auxiliary equipment that would aid in grinding the castor beans and ensuring optimum mixing of the solids and the solvent. The other flange was designed as the filtration/ recovery unit of the extraction column. Several process operations could thus be carried out in the same column without dismantling it. The entire extraction column could be dismantled and cleaned before and after each run with ease.

Grinder

The bottom flange had impeller blades that would crush the beans without having too vigorous an action. This was necessary to prevent degradation of the delicate ricin protein. The blades, referred to as 'Osterizer blade assembly' in Fig. 4.1, were replacement blades for a nominal one-quart Waring blender. The impeller blades were connected to the motor drive through a flexible shaft. The motor had a nominal speed of 10,000 rpm and was to be operated in the range of 3000-6000rpm to achieve the desired grinding. The motor was mounted on a track assembly by which the height and position of the motor could be altered as desired.

The bottom flange had a 100mm X 25mm stainless steel baffle, which could be rotated around its axis and positioned, at different angles with respect to the impeller blades. This would enhance grinding and also aid in efficient mixing thus ensuring optimum contact between the solids and the solvent. A facility for inserting a temperature probe (not shown in Fig. 4.1) that could monitor the temperature of the

column was provided in the bottom flange. The bottom flange also had an appendage by means of which fluids could be let into the system either to pressurize the column (with pressurized nitrogen) or to wash the filter cake (with water).

Filtration Apparatus

The filtration unit would attain the objective of removing the acetone from the slurry after the delipidification process. Filtration of 'ricin' after extraction would also take place through this unit. The filtration unit was held in place by a single-bolt coupling and was the top flange. A simple filter assembly consisting of a stainless steel filter, fixed onto a steel funnel was designed for this purpose. Later, the stainless steel filter was replaced with 325-mesh stainless steel screen since the original filter tended to get plugged. A cylinder of nitrogen, connected through PTFE tubing with nominal diameter of 0.635mm, could be used to pressurize the column during the filtration phase.

Oven

The extraction column and all its components were housed in an oven of insulating material. The oven had a glass front through which all operations could be suitably monitored. The oven had stainless steel strip heaters that when switched on would heat the column up to temperatures of 125-150^0 C. This was a precautionary measure that would ensure that 'ricin' spills and leaks from the column could be denatured after the process and thus would cease to be hazardous.

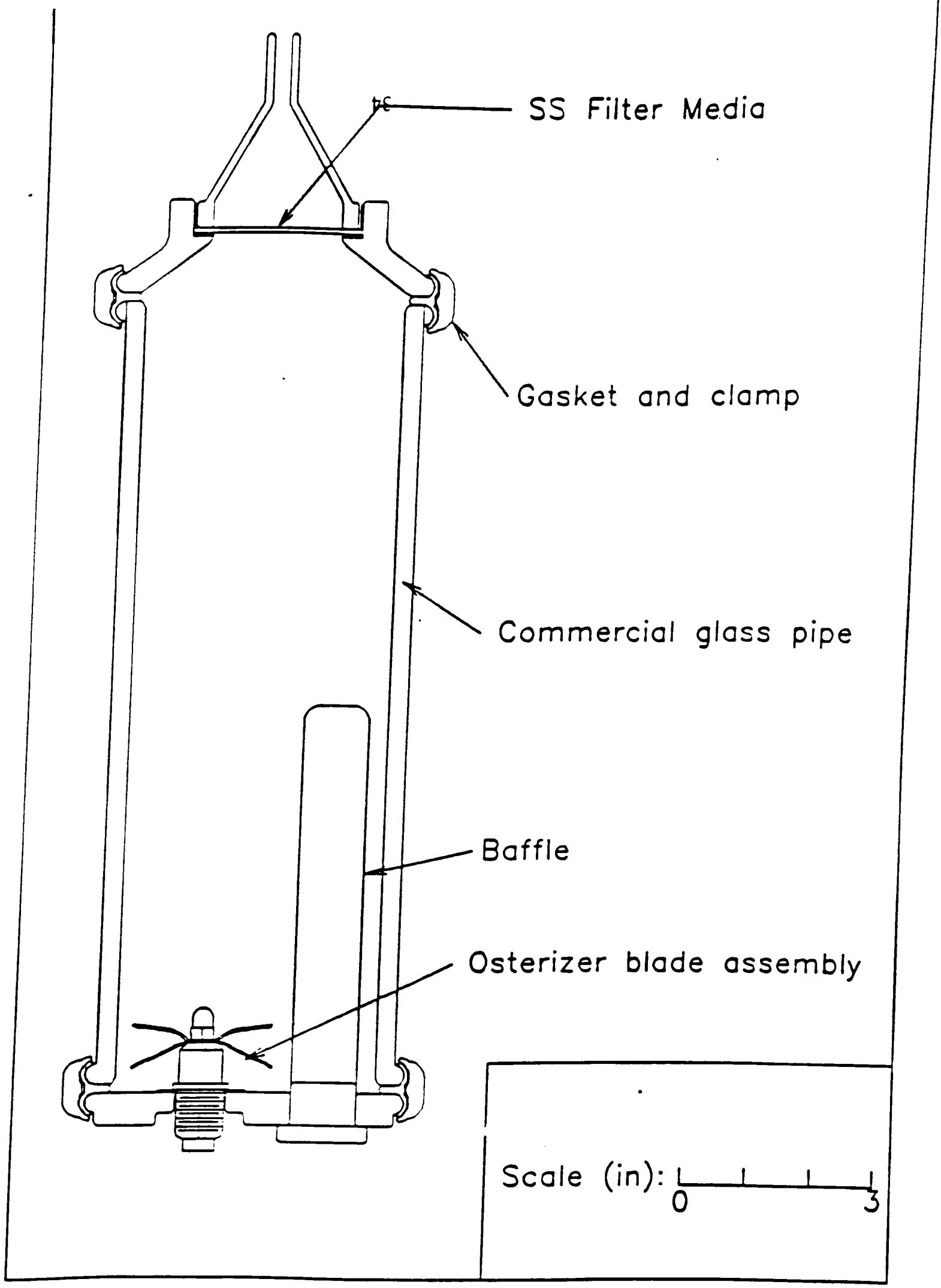


Figure 4.1. Extractor Body (Cross Section of Cylinder)

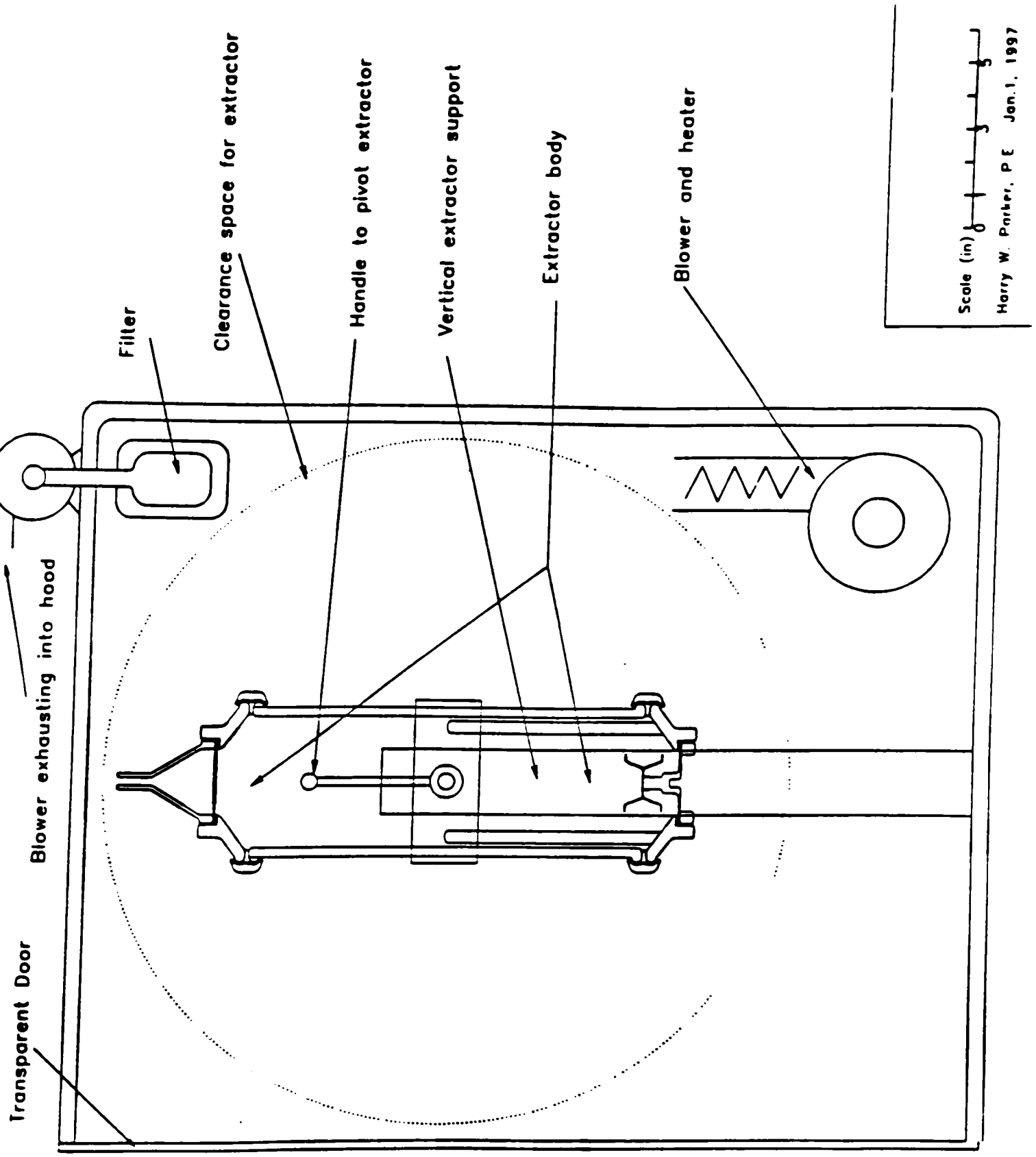


Figure 4.2. Extractor (Side View of Extractor)

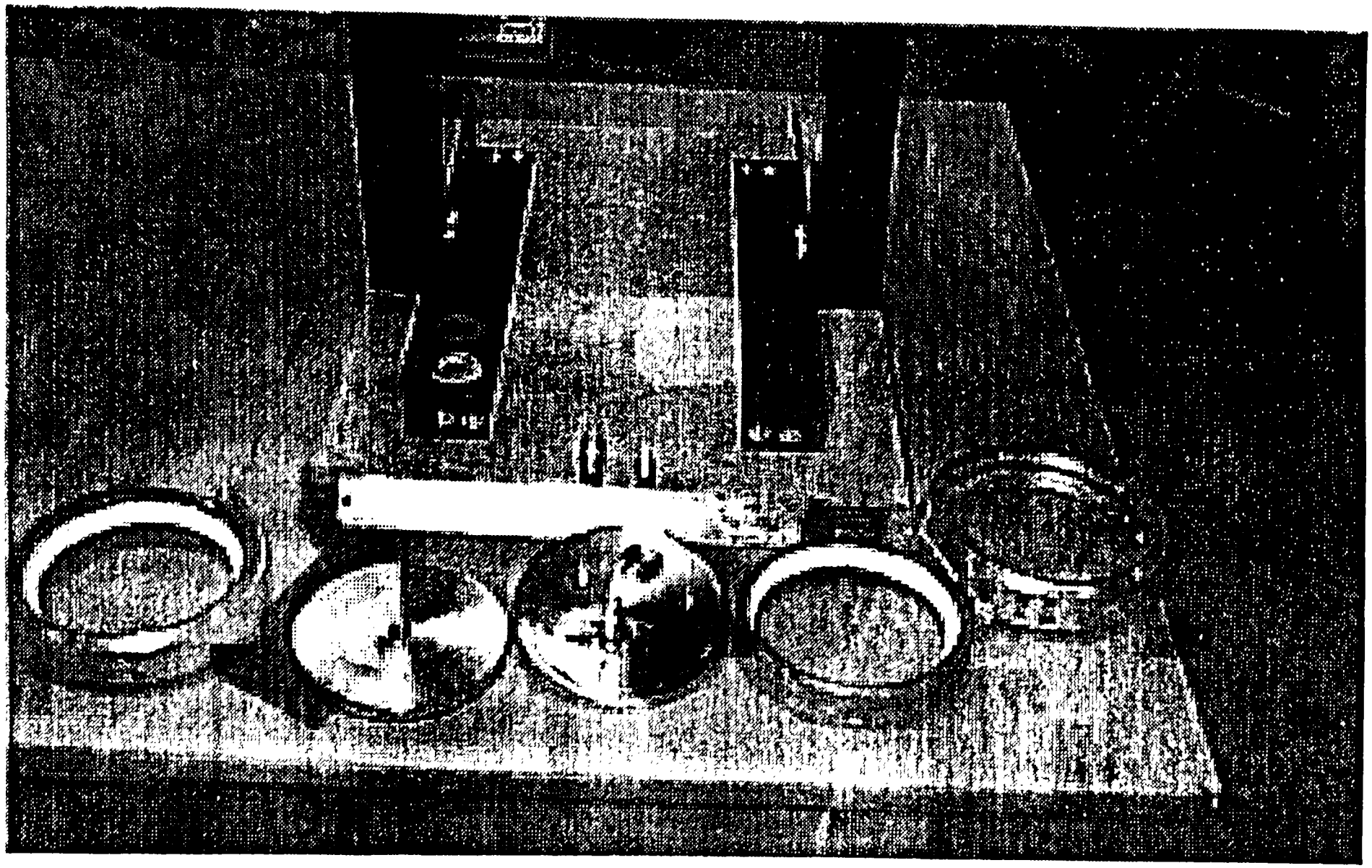


Figure 4.3. Parts of the Extractor

CHAPTER V

EXPERIMENTATION

The extraction of the protein ricin from castor involves many hazards primarily due to the extreme toxicity of the ricin. It thus becomes necessary to establish certain guidelines and criteria for the safe and efficient operation of the extractor. This was achieved by conducting extraction runs on cottonseeds. Similar trials were conducted on castor beans autoclaved for 24 hrs at 120^0 C. This would serve to simulate conditions as bound to be encountered during the extraction of ricin from castor. Autoclaving of the castor beans denatures the ricin thus making it relatively safe to work with in an uncontrolled environment.

Specific Objectives of the Experimentation

As explained above the experiments were carried out in two phases, due to the hazardous nature of the extraction of ricin from castor. The first phase of the experimentation involved the parameterization of the various components of the extractor by running tests on delinted cottonseeds. It was desired to approximately establish the effective operating conditions for the extraction process through the means of these tests. The cottonseeds were delipidified and then extracted with water in this process. The next phase was extraction runs on 'dead' ricin, i.e., tests on castor beans that had been autoclaved to denature the toxin. Several such tests were conducted to establish reasonable extraction parameters such as:

- The quantities of solids and solvent that could be optimally handled;
- Inclination of the column with respect to the vertical axis;
- Optimum time of grinding;
- Baffle position with respect to the impeller blades;
- Type of filter to be used for the best filtration;
- Filtration pressures and time of filtration;
- Number of stainless steel heaters to be turned on and the time of heating the oven.

Phase I – Tests on Cottonseeds

The starting material for the first phase of operations, were mechanically delinted cottonseeds obtained from Plains Co-op Oil Mill, Lubbock, TX. The cotton seed is pointed, ovoid (8mm to 12 mm long) varying in color from brown to nearly black. The cotton seed coat is relatively thin but tough. The mature kernel consists of a major portion of embryo developed at the expense of reserve endosperm (Salunkhe et al., 1992). The cottonseed hence has a thick testa, which covers a thin endosperm. These seeds store oil as the major energy source. Salunkhe et al. (1992) states that researchers have found the range of oil to be 25.6-16.5 % of the total seed. These delinted cottonseeds were delipidified with acetone and then extracted with water.

Extraction with Cottonseeds

The extraction column was assembled with the grinder unit fixed on to the bottom flange and the filtration unit was the top flange (Figure 5.1). The top and bottom flanges were held in place by one-bolt couplings. A torque wrench was used to tighten these couplings to the torque rating of 55 in-lbs as specified by the manufacturer. Around 100-125 g of cottonseeds and 350-400 ml of acetone were placed in the extraction column before putting the top flange in place. The column was positioned at an angle of 45 degree to the horizontal, with the bottom flange facing downwards. The impeller blades were connected to the motor drive through a flexible shaft. The motor had a nominal speed of 10,000 rpm and was operated in the range of 3000-6000rpm to achieve the desired grinding. The grinder was turned on and the cottonseeds ground for about 10 minutes. The baffle was positioned at the 12 o'clock position with respect to the impeller blades during grinding for most effective grinding.

The column was then completely upturned with the filter flange now facing downwards and filtered under nitrogen pressure of 0.7-1.0 atm to remove the acetone. Very good filtration was observed during this preliminary filtration (as described in Tables 5.1-5.5). The residue was then left to dry overnight and then treated with water. The contents were then thoroughly mixed using the grinding unit. The slurry obtained was again filtered under a pressure of 0.7-1.0 atm. It was observed that the filter tended to clog up resulting in very poor filtration. This was because of the very fine nature of the micropore filter used. An alternate filter unit with a 325-mesh stainless steel woven filter cloth as the filter was designed. Good filtration rates were observed while using

this filter assembly. It is evident from comparison of Tables 5.1 and 5.3, that the 325-mesh stainless steel woven filter is best suited for the extraction process.

Phase II – Tests on autoclaved castor beans

As the oil content, hardness and texture of cottonseeds vary from that of castor beans, any analysis of the extractor without conducting tests on castor beans of some sort would be incomplete. Actual tests on 'live' ricin can be carried out only in a highly controlled environment. Hence tests were carried out on 'deactivated' castor beans. i.e., castor beans autoclaved for 24 hr. at 120^0 C.

The starting material for this process was castor beans from the semi-dwarf cultivar Hale, acquired from Mr. Eli Boroda and Dr. Dick Auld of the Plant and Soil Sciences Department, Texas Tech University, Lubbock, Texas. The Hale cultivars were developed by Texas A&M to allow mechanical harvest of castor seed grown for oil production and is well adapted to the Texas High Plains. As this cultivar has high ricin content (14,000 ppm ricin) (Auld et al., 1995), this would be the ideal candidate for the extraction of ricin.

As explained in earlier sections, the toxin is best isolated after a large quantity of the oil is removed during the first extraction itself. Acetone was selected as the solvent for this extraction because of the ease with which acetone could be removed from the castor bean residue.

It is proposed that the physiological buffer solution (PBS) currently being used by the Plant and Soil Sciences Department, Texas Tech University, be used for our extraction. This buffer is a solution of sodium di-hydrogen phosphate in water (pH

around 1.8). This is then made up to a pH of around 7.0 by adding sufficient quantity of 1M solution of sodium hydroxide.

Extraction with Autoclaved Castor Beans

The extraction column was assembled with the top (filter unit) and bottom flanges (grinder unit) held in place by one-bolt couplings. Around 90g of castor beans and 350ml of acetone was placed in the extraction column before putting the top flange in place. The column was positioned at an angle of 45 degree to the horizontal, with the bottom flange facing downwards. The motor was operated in the range of 3000-6000rpm to achieve the desired grinding. The grinder was turned on and the castor beans ground for about 10 minutes. The baffle was positioned at the 12 o'clock position with respect to the impeller blades for grinding. It was observed that better grinding was achieved while working with castor beans than while working with cottonseeds.

The column was then completely upturned with the filter flange now facing downwards and filtered under nitrogen pressure of 0.7-1.0 atm to remove the acetone. The filter unit with the 325-mesh stainless steel woven filter cloth as the filter was used. Very good filtration was observed during this preliminary filtration. The residue was then removed and left to dry overnight. In an actual extraction run involving 'live' ricin the residue would be allowed to dry within the column itself by passing air through it. The residue is then treated with the pH controlled buffer solution. The contents were then thoroughly mixed using the grinding unit. The slurry obtained was again filtered under a pressure of 0.7-1.0 atm. The extract was collected and removed. The hood, glass door were closed, the thermometer inserted inside the extractor and the oven was turned

on. The stainless steel strip heaters were turned on and the total extraction column was then heated inside the oven to a temperature of 100-150^0 C for about 2 hrs. The temperature-time curves were recorded.

Experimental Procedure for Extraction of Ricin

Materials Used:

The designed extractor could handle the following quantities of each material effectively:

- Castor beans 100 g
- Acetone 400 ml
- pH buff. Solution 400 ml

Safety Equipment:

After considering the toxicity of the materials to be handled it is suggested that the following safety equipment be used while conducting the extraction:

- Safety Goggles, rubber gloves, facemask.

Procedure:

The experimental procedure to be followed for the extraction was as follows:

- Assemble the extraction column (minus the top flange) by affixing the bottom flange onto the glass pipe. Make sure that the baffle is at the 12 o'clock position with respect to the impeller blades. Torque the one bolt coupling on the bottom flange to 55 lb. in.

- Install the extractor into the oven with the bottom flange facing downwards, fixing it at the reference height marked out on the walls of the oven. Ensure that the blade assembly is at the correct position. Incline the extractor at 45^{0} to the horizontal and fix it in place by means of the screw on the metal frame.
- Once connected, move the motor assembly to the desired position and insert the motor shaft through the oven into the blade assembly. Connect the nitrogen pressure line to the appropriate connection without turning on the nitrogen supply itself. Insert the thermometer through the slot into the extraction column.
- Weigh 100g of castor beans for extraction and put it into the unit. Add 400 ml of acetone to this.
- Attach the filter assembly, which constitutes the top flange and torque the one bolt coupling on the top flange to 55 lb. in. This completes the total assembly of the extraction column. Once assembled, recheck all connections to ensure no leaks are present.
- Close the hood and start the motor. Maintain the speed of the motor around 3000 – 6000 rpm (5 through 7 on the motor control). Allow the motor to run for about 15-20 minutes. Pulse the motor if necessary for better grinding.
- Disconnect the motor and upturn the extractor so the acetone filters into a beaker. Pressurize the vessel so that the acetone filters out. Do not exceed a pressure of 1atm. Discard the acetone and air-dry the filter cake. Record the dry weight of the filter cake.

- Pour in 400 ml of the pH buffer solution. Reconnect the motor and allow the buffer solution to extract for 30 min., set the motor to run at a speed less than 2500 rpm (speed<4).
- Again, disconnect the motor, and tip the extractor to collect in a clean container. Filter for about 1 hour, pressuring if necessary.
- After draining/filtering, cap the container and set aside in a safe place.
- Close the oven and turn on the thermometer. Turn on the oven and blower. Allow the temperature to rise to ~150^0 C and keep it on for about 30 minutes. Turn off the oven after that time and spray down the extractor with some cleaning agent after the extractor has cooled down.
- Have the ricin solution analyzed.
- Disassemble and clean all parts.

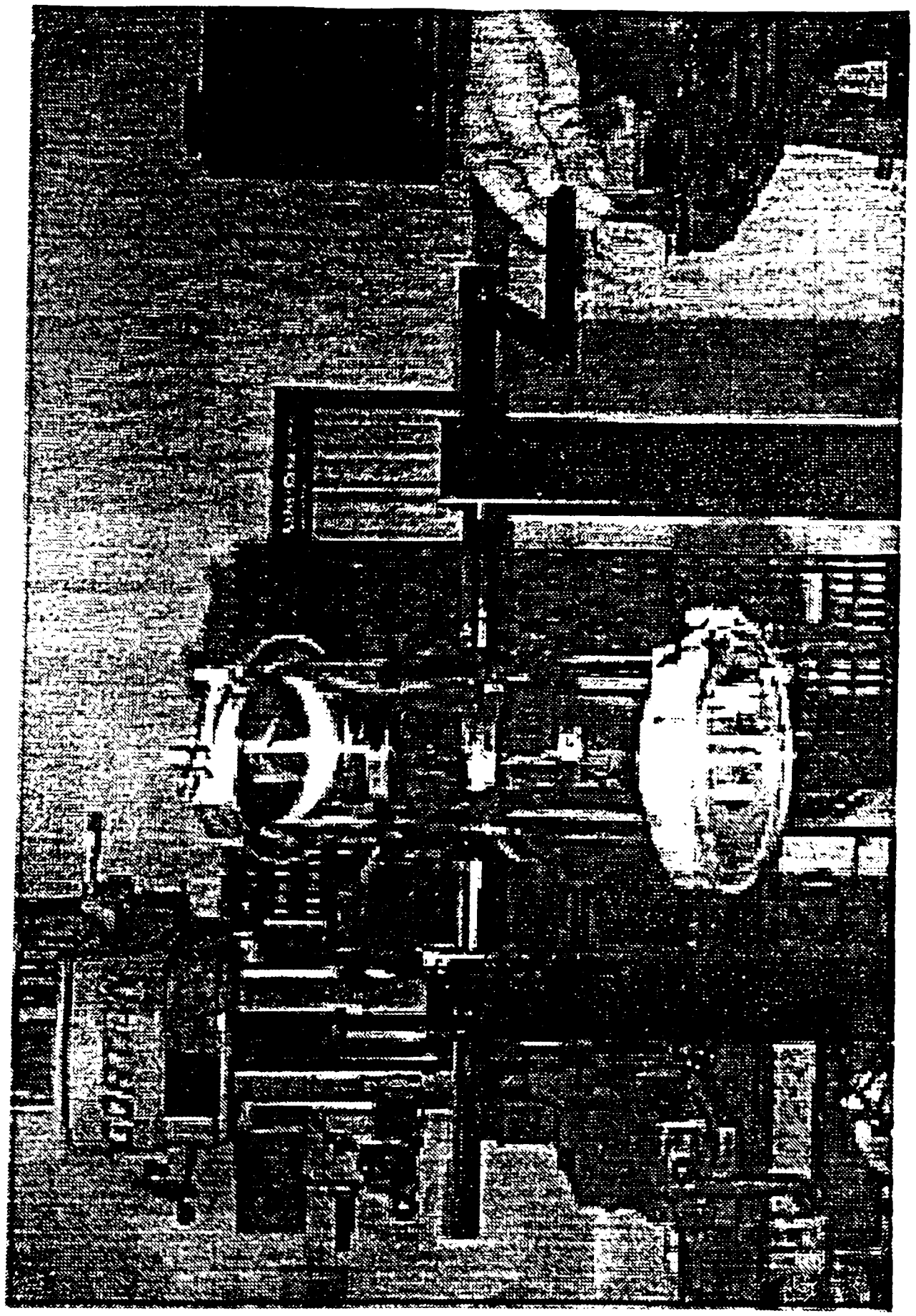


Figure 5.1. Assembled Extractor

Table 5.1: Extraction with cotton seeds

Pretreatment (Delipidification)	
Solid being handled	160 g of cotton seeds
Solvent for extraction	375 ml of acetone
Temperature of Extraction	Room temperature of 22^0 C
Time of Grinding	10 – 20 minutes, baffle at 9 o'clock position to blades
Speed of Grinding	3000 – 6000 rpm (pulsed), column inclined at 45^0
Acetone Filtration	
Filter used	Stainless steel filter
Pressure	0.7 – 0.9 atm (Very good filtration rate, very clear filtrate)
Time of filtration	4 - 5 min under pressure (overnight gravity filtration)
Dry filter cake obtained	143.5 g
Extraction with water	
Solid	Dry filter cake after extraction with acetone
Solvent	Water
Temperature of extraction	Room temperature of 22^0 C
Agitation time	30 - 45 min (not critical)
Speed of agitation	1000 – 2000 rpm
Filter used	Stainless steel filter
Pressure	1 atm (filter clogged, unsatisfactory filtration)
Time of filtration	15 -25 min under pressure (over night gravity filtration)

Table 5.2: Extraction with cotton seeds

Pretreatment (Delipidification)	
Solid being handled	122.7 g of cotton seeds
Solvent for extraction	400 ml of acetone
Temperature of Extraction	Room temperature of 22^0 C
Time of Grinding	10 – 20 minutes, baffle at 5 o'clock position to blades
Speed of Grinding	3000 – 6000 rpm (pulsed), column inclined at 45^0
Acetone Filtration	
Filter used	270 mesh
Pressure	0.7 – 0.9 atm (a little residue passed through)
Time of filtration	4 - 5 min under pressure (overnight gravity filtration)
Dry filter cake obtained	110 g
Extraction with water	
Solid	Dry filter cake after extraction with acetone
Solvent	Water
Temperature of extraction	Room temperature of 22^0 C
Agitation time	30 - 45 min (not critical)
Speed of agitation	1000 – 2000 rpm
Filter used	270 mesh (unsatisfactory filtration)
Pressure	1 atm (some residue passed through)
Time of filtration	15 -25 min under pressure (over night gravity filtration)

Table 5.3: Extraction with cotton seeds

Pretreatment (Delipidification)	
Solid being handled	100 g of cotton seeds
Solvent for extraction	400 ml of acetone
Temperature of Extraction	Room temperature of 22^0 C
Time of Grinding	10 – 20 minutes, baffle at 12 o'clock position to blades
Speed of Grinding	3000 – 6000 rpm (pulsed), column inclined at 45^0
Acetone Filtration	
Filter used	325 mesh
Pressure	0.7 – 0.9 atm (Very good filtration rate, very clear filtrate)
Time of filtration	4 - 5 min under pressure (overnight gravity filtration)
Dry filter cake obtained	83.7 g
Extraction with water	
Solid	Dry filter cake after extraction with acetone
Solvent	Water
Temperature of extraction	Room temperature of 22^0 C
Agitation time	30 - 45 min (not critical)
Speed of agitation	1000 – 2000 rpm
Filter used	325 mesh
Pressure	1 atm (good filtration rate, very clear filtrate)
Time of filtration	15 -25 min under pressure (over night gravity filtration)

Table 5.4: Extraction with cotton seeds

Pretreatment (Delipidification)	
Solid being handled	100 g of cotton seeds
Solvent for extraction	400 ml of acetone
Temperature of Extraction	Room temperature of 22^0 C
Time of Grinding	10 – 20 minutes, baffle at 12 o'clock position to blades
Speed of Grinding	3000 – 6000 rpm (pulsed), column inclined at 45^0
Acetone Filtration	
Filter used	325 mesh
Pressure	0.7 – 0.9 atm (Very good filtration rate, very clear filtrate)
Time of filtration	4 - 5 min under pressure (overnight gravity filtration)
Dry filter cake obtained	78.1 g
Extraction with water	
Solid	Dry filter cake after extraction with acetone
Solvent	Water
Temperature of extraction	Room temperature of 22^0 C
Agitation time	30 - 45 min (not critical)
Speed of agitation	1000 – 2000 rpm
Filter used	325 mesh
Pressure	1 atm (good filtration rate, very clear filtrate)
Time of filtration	15 -25 min under pressure (over night gravity filtration)

Table 5.5: Extraction with autoclaved castor beans

Pretreatment (Delipidification)	
Solid being handled	90 g of castor beans autoclaved at 120^{0}C for 72 hrs
Solvent for extraction	350 ml of acetone
Temperature of Extraction	Room temperature of 22^0 C
Time of Grinding	5 – 10 minutes, baffle at 12 o'clock position to blades
Speed of Grinding	3000 – 6000 rpm (pulsed)
Observation	Grinding much easier and better than with cotton seeds
Acetone Filtration	
Filter used	325 mesh filter on a cone assembly
Pressure	0.9 atm (Very good filtration rate, very clear filtrate)
Time of filtration	4- 5 min under pressure (overnight gravity filtration)
Dry filter cake obtained	59.1 g
Extraction with buffer	
Solid	Dry filter cake after extraction with acetone
Solvent	pH controlled buffer solution (pH = 7.0)
Temperature of extraction	Room temperature of 22^0 C
Agitation time	30 - 45 min (not critical)
Speed of agitation	1000 – 2000 rpm
Filter used	325 mesh filter on a cone assembly
Pressure	0.9 atm (filtration rate a little low, clear filtrate)
Time of filtration	10 -15 min under pressure (over night gravity filtration)

CHAPTER VI

RESULTS AND CONCLUSIONS

The first phase of experimentation yielded some approximate results with regard to the operating parameters as evident from Table 6.1. Phase I of the experimentation established some criteria by which further extraction can proceed. The optimum quantities of solid and solvent that could be handled, the baffle position, inclination of the column, pressure, and time of filtration as well as other operating parameters were more or less established by the end of this phase of experimentation.

It was also observed during the first phase that the filter tended to get clogged, thus bringing to light the inadequacy of the stainless steel filter to handle very fine particle sizes. The percentages of cotton oil extracted in phase I of our experimentation compared well with the literature values of around 16.5-25.6 % of cotton oil (Lawhon et al., 1977).

Phase II of the experimentation was intended as a follow up on phase I and served to corroborate that the criteria established during extraction with cotton seeds would also be valid for castor beans. Effective conditions for the extraction were arrived at and a set of guidelines for the safe operation of the extractor was obtained by the end of the second phase of experimentation. The optimum quantities of solid (100 g) and solvent (400 ml), which the extractor could handle, were ascertained (Table 6.1). It was observed that grinding for 10 minutes with the column inclined at an angle of 45 degree yielded the desired results. The baffle at the 12 o'clock position provided reasonable mixing, enhanced grinding and extraction.

The one-bolt couplings had to be tightened to a torque of 55 in-lbs.. in order to hold the top and bottom flanges in place during pressurization of the column. The column should not be pressurized beyond 1 atm., and pressurizing the column for around 30 min during delipidification and for an hour during the extraction was sufficient. The filter cake must be dried for 24 hrs outside the extractor to attain satisfactory results.

Complete operating procedures have been developed. It is evident from Table 6.1 that the tests on autoclaved castor beans corroborated the observations of the experiments on cottonseeds. The effective extraction parameters that were established are as follows in Table 6.2.

Table 6.2: Effective Extraction Parameters

Operating Parameters	**Delipidification**	**Extraction with buffer**
Solid quantity	100 g	100 g (initial)
Solvent quantity	400 ml	400 ml
Temp of extraction	Room temp (22^0 C)	Room temp (22^0 C)
Grinding/Agitation speed	3000 – 6000 rpm	1000 –2500 rpm
Time of grinding/agitation	10 –15 minutes	30 – 45 minutes
Filtration pressure and time	0.5 – 0.7 atm for 4-5 min	0.7 – 1.0 atm for 10 – 25 min
Baffle position	12 'O Clock position to blades	12 'O Clock w.r.to blades
Column inclination	45^0 to vertical	45^0 to vertical

The temperature – time curves (Figure 6.1) recorded for the oven showed that two stainless steel heaters were sufficient to heat the extraction column to the desired temperature. An effective procedure for extracting ricin was recorded.

Projection for Commercial Production of Ricin

In clinical trials conducted using RTA in an Immunotoxin – XomaZyme –791, patients with cancer, received infusions of XomaZyme –791, doses ranging from 0.02 to 0.2 mg/kg/day (Ghetie and Vitetta, 1994). The total dose infused was between 5 and 61.4 mg/patient (Byers et al., 1989; Byers and Baldwin, 1991). These immunotoxins contained around 4% of free RTA (Ghetie and Vitetta, 1994).

According to the American Cancer Society (www.cancer.org), cancer is the second leading cause of death in the US, exceeded only by heart disease. One out of four deaths in the US is due to cancer. As per the society, this year about 563,100 Americans are expected to die of cancer.

The annual production requirement for ricin is around 1kg (personal correspondence of Dr. Harry W. Parker with Dr. Victor Ghetie). The quantity of ricin in castor beans was found to be around 8.5 to 14 mg/g of seed (Scott Pinkerton, 1997). In trials conducted under National Defense Research Committee (NDRC) Division 9 around 80 % of the extractable ricin was recovered (Bush et al., 1946). So if a particular extraction efficiency of ricin is assumed, then the quantity of castor beans required to produce 1 kg of ricin could be calculated based upon this yield.

Assumptions for Projected Commercial Production of Ricin

The data obtained from the tests, as also the data available presently in literature is insufficient to make accurate estimates of requirements and production. Hence several assumptions were made in order to predict and project production of ricin on a commercial basis:

Annual requirement of ricin = 1 kg;

Quantity of ricin in the castor beans = 10 mg/g of seed;

Extraction efficiency = 70%;

Time required for each extraction using the present extractor = ~3 hrs (not including time taken to heat the oven up to 100-150^0 C);

If Ricin extracted per g of seed = 7 mg.

Initial Estimates of Materials Required

As explained earlier in Table 6.2, around 100 g of castor beans can be subjected to extraction effectively, in one run, using the present extractor. This would require around 400 ml quantities of Acetone and PBS, respectively for each extraction. The hypothetical analysis of material requirements to meet the projected annual production of 1kg of ricin yielded the following results.

Analysis

It is assumed that it would be able to recover 7 mg of ricin from 1 g of seed. Hence, the total quantity of castor beans required to extract 1 kg of ricin is computed to be around 1430 kg. Around 5720 liters of acetone would be required for the preliminary

extraction of 1430 kg of castor beans and 5720 liters of buffer solution would be required for the actual extraction.

Results of Analysis

As indicated in Table 6.2., the present extractor handles 100g per run. This would mean that around 14300 runs would have to be undertaken with present system in order to meet the projected production requirements.

Scaled up Estimates

With the above analysis it is evident that the present extractor needs to be scaled up for it to extract the projected amounts. A scale up of projected production by a factor of 3.4 would imply a scale up of volume by a factor of 3.4. This would enable the extractor to handle 340g of castor beans per run thus allowing for the required amount to be hypothetically extracted in 4205 runs. So it would be desirable to design six such extractors and run four of them at any time with two spares being put into service every other day.

Current extractor handles 100g;

Extractor to be scaled up to handle 340g;

Quantity of acetone and buffer solution required for each extraction = 1360 ml each;

Quantity of castor beans to be extracted to get 1 kg ricin = 1430 kg - castor beans;

Total runs to be conducted / year in scaled up extractor = 4205 runs;

Number of such extractors in service at any time = 4;

The number of extractions each extractor has to handle / year = ~1050;

Total runs to be conducted per day = 3 runs;

Number of hrs per each run = 3hrs.

Requirements

As outlined in the previous section the extractor would have to be scaled up by a factor of 3.4 by volume to meet the projected production requirements. Bisio (1985) defines scale up ratio as the ratio of the commercial production rate to the small-scale unit in which tests were conducted. The scaleup ratio would hence be 3.4.

This would imply that the dimensions of the extraction column would be scaled up by a factor of 1.5. The existing dimensions of the column as indicated in Chapter 4 are 101.6mm i.d., and 305 mm in length. The scaled up extraction column would hence be 152.4mm i.d. and about 458mm long. All other parts of the extractor would be scaled up considering these dimensions and with the desired scaleup ratio in mind.

Conclusions

A facility has been designed and tested for the safe extraction of ricin from castor. Though tests on 'live' ricin have not been carried out, it could be safely presumed that such an extraction would be definitely possible if carried out in a controlled environment. It is suggested that such tests be carried out in the next phase of experimentation. The Department of Environmental health and safety has to be informed before any such experimentation is carried out.

Though the capacity of the extractor is 100 g of seed, it could easily be scaled up to large sizes. It is also suitable for extraction of other products from seeds. The facility allows for grinding of seeds in slurry, extraction of oils with solvents such as acetone or hexane, and the aqueous extraction of proteins such as ricin.

Table 6.1: The summarized results of the experimental trials carried out are shown.

	Preliminary Delipidification (Removal of Oil using Acetone)					**Extraction with water (Protein extraction)**	
Substance	Mass Solid in g	Volume of Acetone in ml	Mass of Res., in g	Extracted Oil as % of mass	Filtr. Press. atm	Filter Material	Filtration Observed
Cottonseeds	160	375	143.5	10.3	1.02	Stainless steel filter	Poor filtration, Filter clogged
Cottonseeds	122.7	400	110	10.4	0.82	270 mesh	Residue passed through
Cottonseeds	100	400	83.7	16.3	1.02	325 mesh	Good filtration
Cottonseeds	100	400	78.1	22.9	0.82	325 mesh	Good filtration
Autoclaved Castor beans	90	350	59.3	34.1	1.02	325 mesh	Good filtration

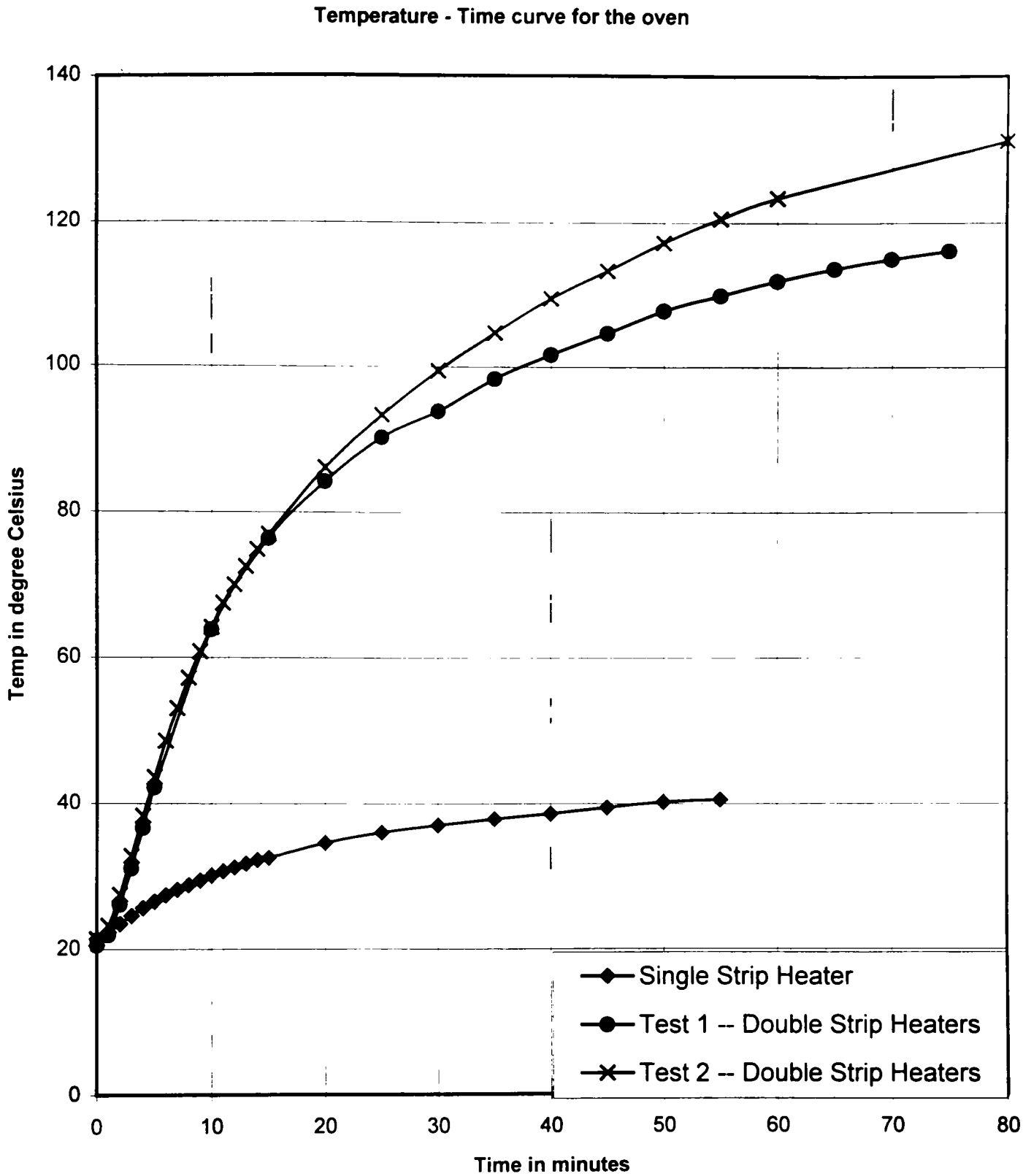


Figure 6.1: The Temp -Time curve in the extractor with different heaters.

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