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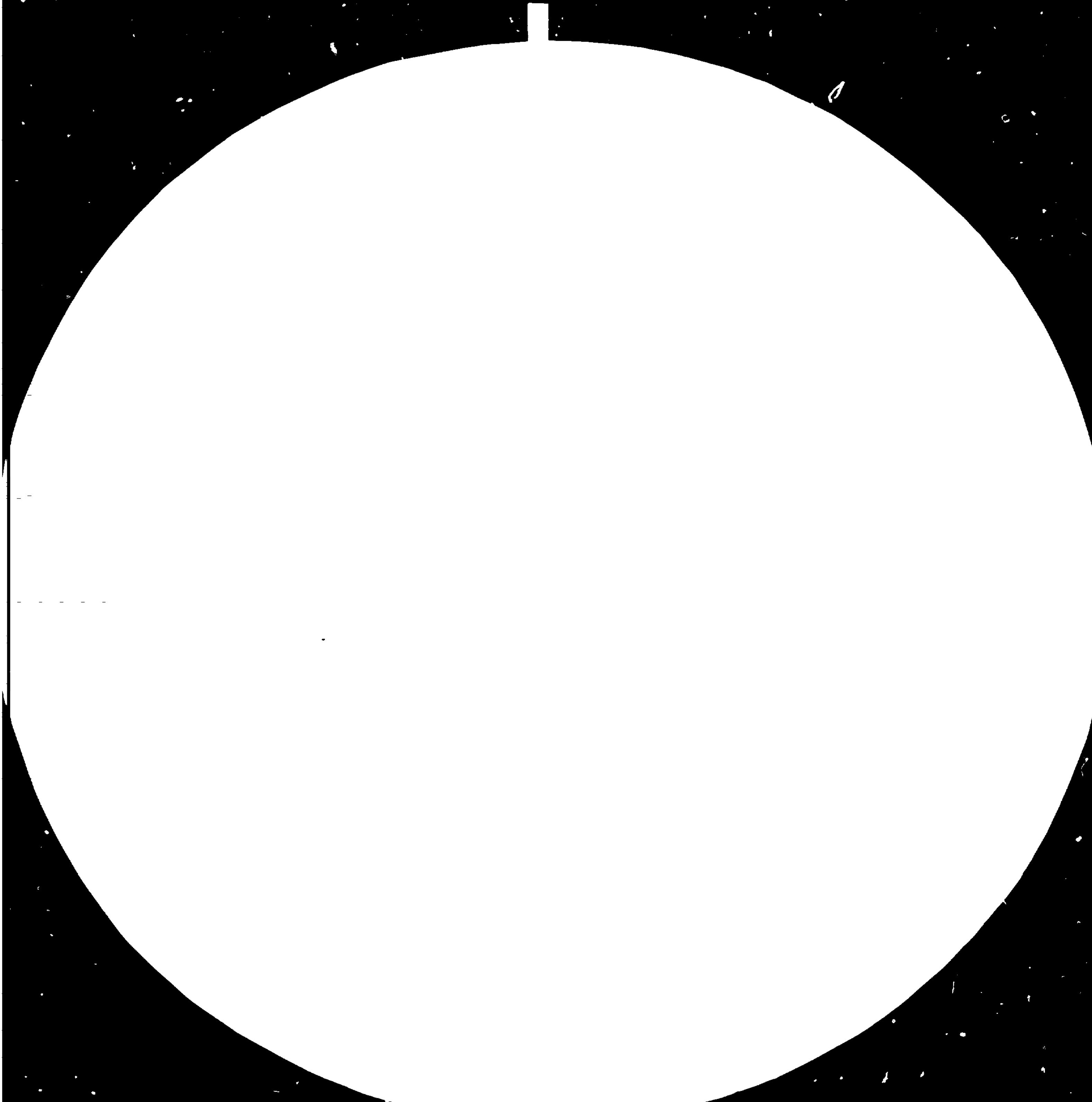

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CASTOR BEAN DETOXIFICATION TECHNOLOGY RESULTING IN THE SETTING-UP OF A DEMONSTRATION PLANT IN A SELECTED DEVELOPING COUNTRY

– RICIN AND RICININE

Final Report

Contract No. 80/70
Project No. US/GLO/77/033

003011

June 1982

FUNDAÇÃO TROPICAL DE PESQUISAS E TECNOLOGIA

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I- INTRODUCTION

The castor plant (Ricinus communis) belongs to the Euphorbiaceae family and its origin is sometimes given as Asiatic, sometimes as African and occasionally even as American. It was known already in ancient times, having been cultivated in China and India some 3000 years ago and, according to same classic authors, also by ancient Egyptians who considered it a miraculous plant.

It is usually an arboreous plant 1-5 m high, but may sometimes reach a height of 10 m or more, when growing wild. The seeds are borne in capsules clustered in spikes and in many varieties the capsules burst when ripe and drop the seeds. Each fruit contains, as a rule, 3 oblong, flat seeds, the size and colour of which depend on the variety. Actually, the castor plant is encountered in practically all hot and temperate regions.

According to FAO Production Yearbook of 1978 the total cultivated area of the castor plant amounted to 1.418.000 ha, to which India contributed with 540.000 ha and Brazil with 344.000 ha. The world seed production in the above year amounted to 921.000 tons, India and Brazil, the two principal producing countries being responsible for 256.000 tons and 370.000 tons, respectively. About 94% of the seeds are used for oil production.

The castor seed contains about 50% of oil, whose main component-ricinoleic acid-may reach up to 93% conferring to the oil some special characteristics such as:

a) Solubility in ethanol

b) Low solubility in light petroleum.

c) Solubility only in hot hexane.

d) High viscosity and chemical reactivity.

Castor seed oil and its chemical transformation products find important applications in modern industry, such as: raw material for plastics manufacture, paints, varnishes, adhesives, high quality lubrificants specially for jet aircraft, hydraulic fluids, toilet articles, leather preservatives, sulphonated oils, electric wire isolation products, nylon 610, pharmaceutics and others. Contemporary industry finds each day new forms of castor seed oil utilization, thus explaining its growing demand.

Each ton of castor seed oil leaves about 1200 kg of pomace, which, despite its high protein content (ca 36%), cannot be used directly in animal feeds owing to the presence of toxic components represented by ricin, ricinine and powerful allergenes. As a result, the pomace is being used mainly as fertilizer.

COMPOSITION OF THE CASTOR SEED

Castor seed weighing 0.1 - 1 g consists of ca 25% of hull and 75% of kernel. The quantity of hull depends on the seed variety.

The percentual composition of Indian (Bombay) Seed variety reported by Jones (1947) is shown in Table 1.

TABLE 1

	Seed composition %	Water	Protein	Oil	Carbohydrates	Fibre	Ash
Whole Seed	100	5.14	17.88	46.65	12.61	14.99	2.73
Kernel	70	3.60	23.43	66.02	4.01	0.70	2.24
Hull	30	8.76	4.76	0.98	32.92	48.69	3.89

Castor seed contains, as already mentioned, toxic compounds which are not extracted with the oil, but remain as components of presscake or meal. The main toxic principle is the protein, ricin. Ricinine, a simple alkaloid of low toxicity, can also be isolated from the extracted residue. Powerful allergenes, the inactivation of which is rather difficult, are also present.

The presscake and meal contain a relatively high phosphorus content. The phytin accounts for 91% of this quantity. Bolley and Holmes (8) stress that the phytin content of castor seed is higher than that of the majority of vegetable seeds.

Castor flour (residue after oil is removed from decorticated castor seeds) contains a powerful lipolytic enzyme. Crude castor lipase exists in castor flour prepared without heat treatment.

Castor oil is obtained industrially by single or double pressing, or alternatively by mechanical pressing followed by solvent extraction.

Pressing can be effected in hydraulic presses or by the "Expeller", yielding a presscake with appr. 10% of residual oil. The presscake subjected to solvent extraction produces a meal with appr. 1% of oil.

According to Swern (96) the American Oil Chemists' Society Standards for castor oil are the following

Specific gravity at $25^{o}/25^{o}C$	-	0.945 - 0.965
Refractive Index at $25^{o}C$	-	1.473 - 1.477
Iodine number (Wijs)	-	81 - 91
Saponification number	-	176 - 187
Unsaponificable matter %	-	not over 1.0
Acetyl value	-	144 - 150

The gas liquid chromatographic analysis gave the following fatty acid composition of the oil.

Acid	%
Ricinoleic	87
Oleic	7
Linoleic	3
Palmitic	2
Stearic	1

It has been suggested that castor meal would be of value as a nutrient for animals, provided that a reliable method of detoxication could be devised.

Castor seed meal contains about 40% protein, which is deficient in essential amino acids, methionine, lysine and tryptophan. These deficiencies preclude the use of castor meal as the main source of protein in rations for pigs and poultry, but the meal would be suitable as a concentrate for cattle.

II- RICIN: PREPARATION, PURIFICATION, STRUCTURE AND PROPERTIES

According to Jaffe (41) the first description of a hemagglutinin (or lectin) was provided by Stillmark in 1889, who studied the toxicity of castor seeds and press cake from the production of castor oil. From his very thorough investigation he concluded that the toxic action was due to a protein which he called "RICIN" and which he showed to be capable of agglutinating the red cells from human and animal blood.

In 1905, Osborne et al (85) purified a ricin extracted from defatted castor seed meal by treatment with a 10% NaCl solution. The extract was dialysed and filtered. From the filtrate there was obtained, by repeated treatment with ammonium sulphate, a precipitate which was highly toxic and exhibited a strong hemagglutinating activity. Elemental analysis of the preparation showed C = 52.01%, H = 7.02%, O = 23.12%, N = 16.56% and S = 1.09%. The preparation coagulated and lost its activity completely when heated and it was concluded that ricin is a protein of high molecular weight. The ricin thus obtained represented 1.5% of the defatted meal.

Ricin was detected in 21 different varieties of Ricinus communis by Agulhon (1) in 1914.

Kabat et al. (5) extracted ricin from the castor seed presscake with water at pH 3.8. A toxic protein was precipitated from the extract by saturation with sodium chloride, redissolved in water, and reprecipitated at pH 8.0 by saturation

with ammonium sulphate. The protein thus obtained was homogeneous electrophoretically, ultracentrifugally and immunochemically and was denoted "Ricin B_1". Its molecular weight was between 77000 and 85000. It showed high toxicity and strong hemagglutinating power.

"Crystalline Ricin" was isolated by Kunitz and Mc Donald (50) in 1948 by precipitation with sodium sulphate from an aqueous castor meal extract. A concentrated solution of the crude product in water, after storage for several weeks at 5° C. yielded crude crystals. A facily homogeneous product was obtained after three or four recrystallizations. Crystalline ricin was identified as a globulin type protein with an isoelectric point at pH 5.4-5.5. The molecular weight was found to be 36000 by sedimentation. The toxicity of the crystalline ricin was superior to that of Ricin B_1, whereas its hemagglutinating activity was about 1/10 of the latter.

Funatsu (30) related the preparation of an "amorphous ricin" denoted as Ricin Tb which was isolated by Le Breton and Moulé (51) using a modified method of Osborne et al (85) from a small grain type of Ricinus communis produced in in Japan. This preparation exhibited the same toxicity as the previously mentioned "Crystalline Ricin". Despite of its amorphous form, Ricin Tb showed homogeneity when ultracentrifuged.

Regarding new attempts of purification of the crystalline and Tb ricin and the probable inhomogeneity of original preparations, Funatsu reported the obtaining of 3 fractions: TB-I, Tb-II and Tb-III in proportion of 36:38:25 respectively, starting from amorphous ricin, and the obtaining of 2 fractions C, and C_2

(85:12) from crystalline ricin, which was achieved by continuous paper electrophoresis. These preparations showed homogeneity by ultracentrifugation and electrophoresis. Physiological properties of the above mentioned fractions are shown in Table 2.

Table 2. Physiological Properties of Proteins contained in Ricin Tb and crystalline ricin.

Activity	Ricin Tb			Crystalline Ricin	
	Tb-1	Tb-II	Tb-III	C_1	C_2
Toxic T.U.µg N(a)	0.3	0.3	-	0.3	3
Proteolytic	+	+	+	-	-
Hemagglutinating, µg N(b)	5	5	0.1	18	18

(a) T.U. = Toxicity unity: minimum quantity of protein nitrogen Sufficient to kill a mouse of 20g in 24 h.

(b) Minimum quantity of protein nitrogen giving definite macroscopic evidence of agglutination of 0.2 ml of a 4% suspension of washed rabbit erythrocytes.

Ricin Tb-II exhibits toxicity and high proteolytic activity. The fraction C_1 shows the same degree of toxicity as Tb-II, but without proteolytic activity. The Tb-III preparation possesses

high hemagglutinating potential and considerable proteolytic acti_
vity without toxic effects. According to Funatsu this last fraction was subsequently resolved into 2 new components by column chromatography using DEAE cellulose. One of the components was a protease, the other being a hemagglutinin. The C_2 component showed hemagglutinating activity, very low toxicity and no proteolytic activity.

According to Funatsu (30) the amino acid composition of ricin C_1 is as follows:

Glycine	2,05
Alanine	5,45
Valine	4,16
Leucine	8,93
Isoleucine	6,65
Proline	5,20
Phenylalanine	4,50
Glutamic acid	12,72
Aspartic acid	12,14
Arginine	7,14
Histidine	3,28
Lysine	3,09
Serine	8,05
Threonine	6,46
Tyrosine	4,56
Tryptophan	3,57

Cystine	2,50
Methionine	2,44
Amide ammonia	3,22
Total	106,11

Funatsu described further the purification procedure and properties of a crystalline material denoted "Ricin D" isolated from the seeds of a large grain, type Ricinus communis L., produced in Thailand. The toxicity of Ricin D was 10 times greater than that of Ricin C_1, but without hemagglutinating and proteolytic activities.

Physico-chemical properties of Ricin D were: molecular weight-60000; sedimentation constant-4.64 S, and the iso-electric point measured by electrophoretic mobility - 5.9.

Ricin D was found to be a glycoprotein containing about 5.6% mannose and about 2% glucosamine. Of its amino acids, totalling a number of 493, 105 were of acidic and 47 of basic character. Since the isoelectric point of this ricin was 5.9, a relatively large number of acidic amino acids must be present as amides. Twelve half cystine residues were shown to exist in disulphide form, so that ricin D contains 6 disulphide linkages.

Ishiguro et al (39) studied the terminal structure of ricine D. The N-terminal aminoacids of this preparation were alanine and isoleucine as determined by di-nitro-phenylation. The C-terminal aminoacids were quantitatively analysed by the hydrazinolysis method and by hydrolysis catalyzed by carboxypeptidase. A phenylalanine and serine were identified. The difficulties

in separating these 2 components led to the supposition that ricin D consisted of 2 polypeptidic chains, which were denoted as Ala and Ile chains linked by disulphide linkage or linkages. It was, in fact, possible, to separate ricin D into 2 different chains by oxidation or reduction of the disulphide linkage or linkages.

Funatsu and Funatsu (23) described the separation of 2 polypeptidic chains existing in ricin D. Such chains were isolated from performic acid oxidized ricin D by DEAE-cellulose column chromatography in a phosphate buffer, pH 7.0, containing 8 M urea or from reduced carboxymethylated ricin D by gel filtration on Sephadex G-75 followed by DEAE-cellulose column chromatography in Tris-HCl buffer, pH 8.5. Aminoacid analyses of the two isolated chains (Table 3) revealed that they were distinct molecules possessing similar molecular weights of about 30000 and linked by one disulphide bond in ricin D.

The amino acid compositions of reduced carboxymethylated ricin D (RCM-chains) were almost identical with those of the respective oxidized chains except with respect to cystine, and approximately one mole of CM-cystine per mole of the chain was found in each chain. From these results, it was concluded that the two constituent polypeptide chains of ricin D are linked by a single disulphide bond which was exposed on the surface of the ricin D molecule.

Table 3. Amino Acid Composition and N-terminal Amino Acids of the Oxidized Subunits of Ricin.

Amino Acid	Number of Residues		
	Fraction P-1	Fraction P-2	Ricin D
Aspartic Acid	24	35	59
Threonine	16	19	35
Serine	17	19	37
Glutamic Acid	27	21	46
Proline	14	13	26
Glycine	16	18	36
Alanine	22	15	37
Cystine/2	0	7	12
Valine	13	12	29
Methionine	3	3	6
Isoleucine	20	16	35
Leucine	21	23	42
Tyrosine	13	8	20
Phenylalanine	12	5	18
Lysine	2	7	9
Histidine	3	3	6
Arginine	18	14	32
Tryptophan	2	6	8
$CySO_3H$	2	7	0
Total	245	(248)	493
N-terminal amino acid	Ile	Ala	Ile Ala

According to Funatsu (30) the subunit structure of ricin D can be drawn schematically as in Fig. 1.

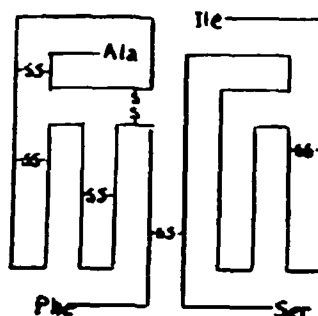


Fig. 1. A schematic representation of the main chain structure of ricin D.

Some physical, chemical and physiological properties of the two subunits isolated by reduction from ricin D and carboxy methylated (S-CM Ile and S-CM Ala chains) were studied by Hara et al. (36). Physical properties of subunits are summarized in Table 4. The S-CM subunits consisted of 244 amino acid residues (S-CM Ile chain) and 254 residues (S Ala chain). By the specific cleavage of the single intermolecular disulphide bond of ricin D, no remarkable change in conformation of the polypeptide chains of ricin D was detected, but the toxicity was markedly decreased.

The relative toxicities of ricin D, S-CM-Ile and S-CM-Ala chains were estimated to be 100, 1.5 and 0.3, respectively. It was also found that the separated subunits could be easily digested by trypsin although ricin D is not easily attacked by this proteolytic enzyme. These observations, decrease in toxicity and increase in

susceptibility toward trypsine, due to the cleavage of the single disulphide-bond, indicate that the toxicity of ricin D can be ascribed to the quaternary structure of the ricin D molecule.

Table 4. Physical Properties of Ricin D and S-CM-Subunits of Ricin D

	Ricin D[2.6]	S-CM-Ile chain	S-CM-Ala chain
UV-absorption spectra			
max. nm	280	280	280
min. nm	251	251	250
E_{max}/E_{min}	2.32	2.09	2.38
Optical rotation			
a_o (deg)	-138	-134	-133
b_o (deg)	-66	-2	-158
Helical content assumed (%)			
(by b_o)	10.5	0.3	25.0
(by λ_o)	16.4	0.7	26.4
Ultracentrifugation			
Sedimentation constant	4.64S	3.45S	3.52S
Molecular weight	60,000	30,000	30,000
Molecular weight by SDS Polyacrylamide gel electrophoresis		30,000	30,000
Ampholine electrophoresis			
Isoelectric point	7.34	7.42	5.17

Investigating the nature of the carbohydrate component of ricin D, Nanno et al. (76) found that this glycoprotein contains 6 moles of glucosamine and 17 moles of mannose per mol of protein. Isolation of two constituent polypeptide chains, namely Ala-chain and Ile-chain, and subsequent proteolytic digestions with Nagarose and Promase revealed two glycopeptides (Asp_1 Thr_1 $glucosamine_2$ $mannose_6$ and Asp_1 Thr_1 $glucosamine_2$ $mannose_7$) from Ala chain and glycopeptide (Asp_1 Ile_1 Phe_1 $glucosamine_2$ $mannose_4$) from Ile chain. These authors consider that the carbohydrate components are covalently linked to the polypeptide chains in the sites to form this glycoprotein.

The structures of these glycopeptides were determined (77) and are given below:

```
               (glucosamine2 mannose)
                     |
Ala-chain:     Asx - Asx - Gly - Thr

               (glucosamine2 mannose7)
               |
               Asx - Asx - Thr - Glu - Pro

Ile-chain:     (glucosamine2 mannose4)
                     |
               Ile - Asx - Phe
```

It was found that sugars combined either with aspartic acid or asparagine as oligosaccharides. In the Ile-chain the sugar moiety consisted of a hexasaccharide containing two glycosamine and four mannose

residues. In the Ala-chain, one o gosaccharide was an octasaccha ride composed of two glucosamine and six mannose residues, and the other was a nonasaccharide containing two glucosamine and seven mannose residues.

Regarding the primary structure of ricin D there were described: Isolation of tryptic peptides from the Ile-chain (29) and amino acids sequences of the tryptic peptides from the Ile-chain and isolation and alignment of cyanogen bromide fragments from two constituent polypeptide chains of ricin D (106). A complete amino acid sequence of Ile-chain consisting of 265 amino acid residues was determined by Yoshitake et al (107) and is shown in Fig. 2.

Fig. 2. Complete Amino Acid Sequence of the Ile-chain from Ricin D.

Distribution of positively and negatively charged amino acid residues in the Ile-chain is shown in Fig. 3 . There are three clusters of charged groups which stand out: two groups of 4 negatively charged residues between Asp 96 and Glu 102, and between Glu 135 and Glu 146, and separately spaced positively charged arginine cluster between Arg 189 and Arg 197. Distribution of aromatic non-polar amino acid residues in the protein is also shown in this Figure.

Prediction of protein conformation from the amino acid sequence data is shown in Fig. 4: Six α-helical regions were detected and accounted for 23% of the amino acid residues in the protein. There are 9 β sheet regions in the molecule accounting for 33% of the amino acid residues in the polypeptide chain.

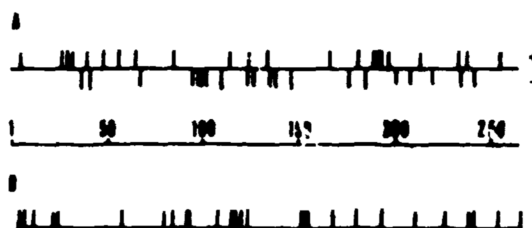


Fig. 3 . Distribution of Amino Acid Residues in the Linear Structure of Ile-chain.

A, positively charged residues, lysine, arginine and histidine (+), and negatively charged residues, glutamic acid and aspartic acid (-). B, aromatic residues, tyrosine, phenylalanine and tryptophan.

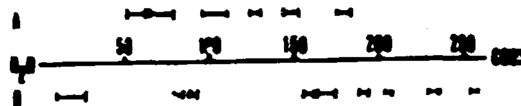


Fig. 4. Prediction of the Secondary Structure of the Ile-chain from Its Amino Acid Sequence.

A, predicted α-helical region. B, predicted β-sheet region.

Funatsu et al (26) have suggested that tyrosine and lysine residues in each chain of ricin D are involved in the toxic action of this protein. In the opinion of Yoshitake et al (107) the relevant lysine residue is Lys 238, suggesting that the carboxyl terminal region of Ile chain may have an important function in promoting toxicity of ricin D.

A complete amino acid sequence of the Ala chain of ricin D was provided by Funatsu et al. (24) and is shown in Fig. 5.

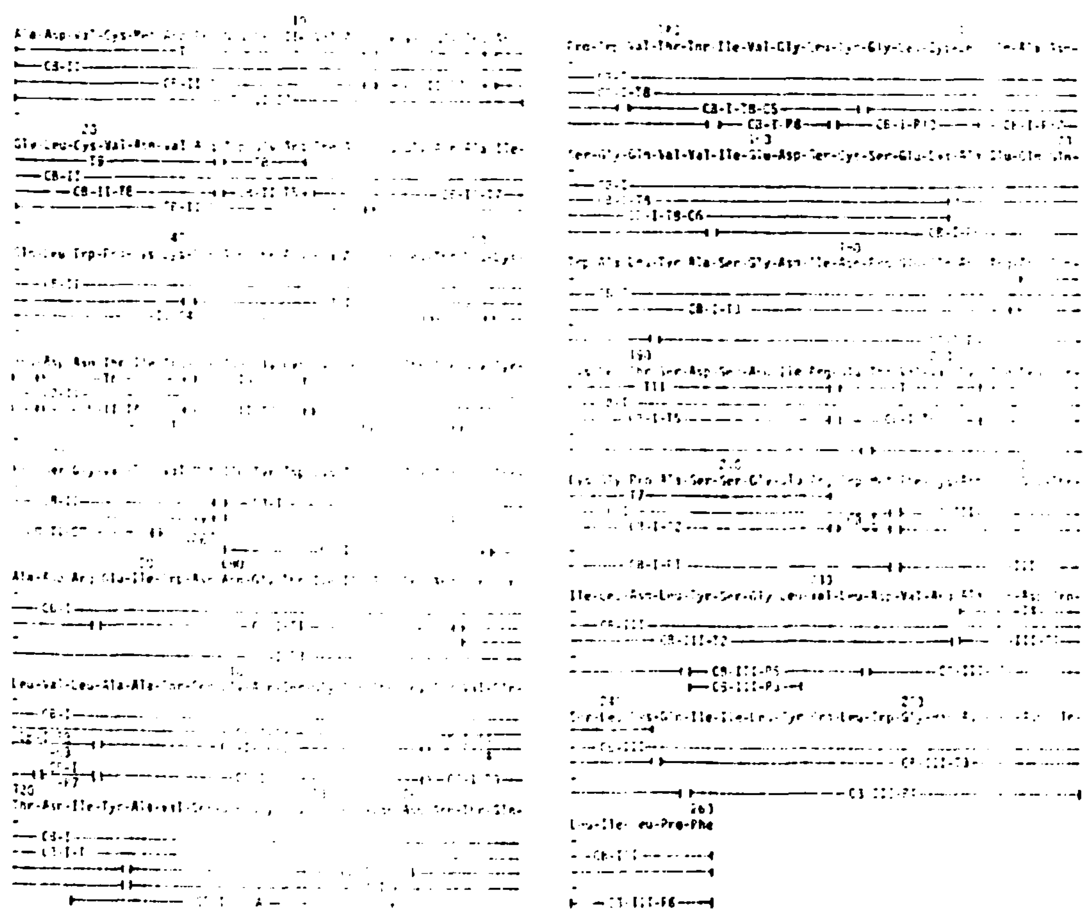


Fig. 5. Primary Structure of Ala Chain of Ricin D

T, C, P, CB and A numbers represent the peptides resulting from tryptic, chymotryptic, peptic digestions, CNBr cleavage and partial acid hydrolysis, respectively.

The molecular weight calculated from the sequencial analysis is 31.432 (including $glucosamine_4$ and $mannose_{13}$). The 22 basic and 21 acidic amino acid residues are distributed in both N- and C-terminal regions and there are no charged residues in the central region between Ser 101 and Ile 159. Many continuous sequences of hydrophobic amino acids occur over the chain; especially the C-terminal region, which is a typical region including the leucine and isoleucine-rich hydrophobic amino acids.

The secondary structure predicted from the amino acid sequence indicated that the Ala chain possesses 4 α-helical regions (27 amino acid residues or 10.4% of total residues), 12 β-sheet regions (82 residues, 32.3%) and thirty one β-turns.

According to Funatsu et al (24) the two types of polypeptide chain Ile and Ala, which constitute ricin D, correspond to A and B chain respectively, described by Olsnes and Pihl (82). The Ile or A-chain inhibits protein synthesis, while the Ala or B-chain induces not only cyto-agglutination, but also the entry of the Ile chain into the cytoplasm by binding to the cell surface receptors carrying galactose.

A toxic protein distinct from ricin D was isolated by Mise et al (66) from castor seeds produced in Japan and referred to as "Ricin E". It behaves differently from ricin D in DEAE - cellulose column chromatography at pH 8.5 and in disc-electrophoresis at pH 8.3, indicating that ricin E is not identical to ricin D. It is not encountered in the castor seeds (large grain) produced in Thailand. Ricin E was found to be a glycoprotein with two N-terminal amino acids Ile and Ala identical

to those of ricin D. The molecular weight of ricin E was found to be 64000 by SDS-polyacrylamide gel electrophoresis. The isoelectric point of ricin E, estimated to be 8.8, was higher than that of ricin D. It showed equal toxicity for mice and equal cytoagglutinating activity for sarcoma 180 ascites tumor cells.

Two constituent polypeptide Ile e Ala chains of ricin E were separated by Funatsu et al (25). The isoelectric point, amino acid composition,and tryptic peptides pointed towards similarity of the Ile chain of ricin E to that of ricin D. The toxicity of the hybrid molecule consisting of the Ile chain of ricin E and Ala chain of ricin D to malignant cultured cells was shown to be similar to that of ricin D. It was concluded that the difference in the toxicity of ricin E and D toward the cultured cells was due to the difference in the function of Ala chain.

Castor bean hemagglutinin - The toxic and hemagglutinating activities are properties of two different protein species of castor seed, which possess similar structures as shown by Takahashi et al (97).

According to Ueno (101) castor seed hemagglutinin (CBH) has been isolated and characterized in various laboratories. Working with seeds produced in Thailand the authors showed that CBH consisted of 4 polypeptide chains of similar size and possessed similar sugar specificity to that of ricin. The molecular weight of CBH was determined as 130,000 by SDS-polyacrylamine gel electrophoresis and its isoelectric point as 7.8 by isoelectric

focusing electrophoresis. CBH possessed the identical N-terminal amino acids (Ile and Ala) and an amino acid composition very similar to that of ricin D whereas its toxicity for mice was about 1% of the latter. The protein was separated into 4 fractions A, B, C and D. Hemagglutinating activity was detected in all four fractions but the major A fraction possessed the highest activity.

Two constituent polypeptide chains of castor seed hemagglutinin (CBH-A) were isolated from the performic acid-oxidized and from reduced carboxy methylated CBH-A, by DEAE-cellulose chromatography or sepharose 4B column (27). Analysis of N-terminal amino acids, the amino acid composition, and the tryptic peptides of each chain, showed that the layer chain with a molecular weight of 34,000 and the smaller chain with a molecular weight of 31,000 were homologous with Ala and Ile chains of ricin D, respectively.

According to Funatsu et al (28) despite the fact that the two lectins-ricin and hemagglutinin (CBH) - present in castor seeds have similar physicochemical properties, they are remarkably different in their toxicity and hemagglutinating activity. The ricin D and CBH-A have subunit structures denoted as $\alpha\beta$ and $(\alpha\beta)_2$, respectively, and the high hemagglutinating activity of CBH is due to its tetramer structure possessing two binding sites for red blood cells. On the other hand, it has been shown that the high toxicity of ricin is exhibited by a cooperative action of the Ile chain inhibiting protein synthesis, whereas the Ala chain binds to galactose - like residues on cell surface.

III- TOXICITY EVIDENCE AND PHYSIOLOGICAL EFFECTS OF RICIN

The toxic characteristics of castor seed which affect human and domestic animals have been known for a long time. In 1888 Edson (19) reported nausea and vomit symptom in the case of 15 children who had consumed up to 4 castor seeds. Meldrum (65) recorded the death of an adult caused by consumption of 2 seeds. The death of Ayrshire cattle resulting from castor seed ingestion with the minimum lethal dose of 0.5 lb was reported by Anderson (4).

Dixson (18) in 1887 was apparently the first one to recognize and isolate the castor seed toxic protein. Stillmark, however (95) as already mentioned previously, seems to be the first one to observe in 1889, that a protein fraction of the castor seed, the "Ricin", was capable of agglutinating red blood cells, a property due to proteins called phytohemagglutinins or lectins.

Ricin, prepared in a highly pure state by Kabat et al (45) has been recognized as pertaining to a small group of phytotoxins which includes highly toxic compounds such as abrin, crotin, circin and rubin. All these compounds are antigenic, thermolabile and possess hemagglutinating properties.

Ricin is a protein of considerable stability and can be maintained during months without deterioration. It agglutinates not only the blood but also cholesterol suspensions and other suspensions of particles with a negative charge (34).

Ricin was the first phytohemagglutinin to attract the attention of research workers, presumably owing to its extreme toxicity. Its MLD is about 0.001 μg/1 g (rat), which makes it 1000 times more toxic than other seed lectins. Its toxicity persists even after oral ingestion. For this reason a detoxication of castor seed press cake and meal is essential to make possible their safe utilization in animal feeds (57).

According to Mosinger (69) the quantity of ricin injected intraperitoneally and capable of killing an experimental animal such as mice, amounts to 10 μg/kg of body weight in less than 96 h. Experiments showed that 7.2 μg/kg of body weight killed a rat in less than 48 h and 1.5 μg/kg of body killed a rabbit in less than 72 h. All parts of the plant, especially the seed, are toxic to human, horses, cattle, sheep, pigs and birds.

The toxicity varies with the animal species, guinea pigs being apparently most susceptible (44) and birds more resistant than mammals to the toxic action of castor seed protein.

Physiological effects of ricin are similar to those of many bacterial toxins and of toxalbumins. It induces, after a few hours, paralysis of the respiratory and vasomotor system. Oral ingestion causes diarrhea, general prostration and intestinal hemorrhage, renal congestion and hypermia of the spinal medulla and brain. It coagulates the red blood corpuscles. Ricin also affects the eyes causing inflamation and severe panophthalmitis.

Regarding the pyrogenic action in rabbits, rats, guinea

pigs and cats, Esperjessy et al (20) showed that it is capable of causing prolonged temperature elevation (40-41°C for 60-72h).

It has also been found (3) that ricin limits the development of the embryo during the hatching of the hen eggs. The development of the heart, liver and stomach, but not of the goiter, was markedly limited.

Koga (49) studied the tissue affinity of ricin D labelled with I^{131}. The toxoid ^{131}I-ricin D, injected intra-peritoneally to rats, was traced in the liver, pancreas, digestive organs, and in blood. The I^{131} content in the rat tissues was expressed as percentage of the injected total dose. ^{131}I-ricin D was encountered initially in the liver and reached the maximum value of 15% of the injected dose 2 hours after injection. Finally it passed to the pancreas reaching a level of 30% of the injected dose after 5 hours as shown in Fig. 6, taken from the publication of Funatsu (30).

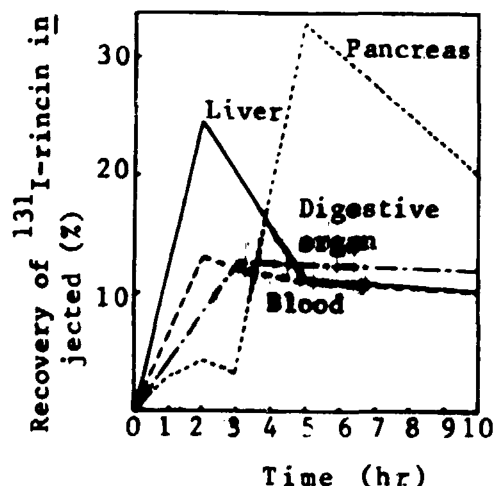


Fig. 6. Time course of ^{131}I-ricin D uptake by the liver, pancreas, digestive organs and the blood.

Waller et al (105) carried out pathological studies on the action of ricin. The necropsy of rats killed by subcuta neous injection of crude ricin revealed as greatest hystopatholo gical changes: hyperemia, exudation, and necrose near the injection surroundings; hyperemia and multifocal necrose in the liver; and hyperemia, hemorrhagic edema and necrose in lymphatic modules.

Olsnes and coworkers (81, 82, 83 and 84) / carried out a detailed investigation of the structure of ricin and of the mechanism of its toxic action. They established that ricin is composed of 2 polypeptide chains with a molecular weight of 32.000 and 34.000, respectively, unified by disulphide linkages (see Fig. 7).

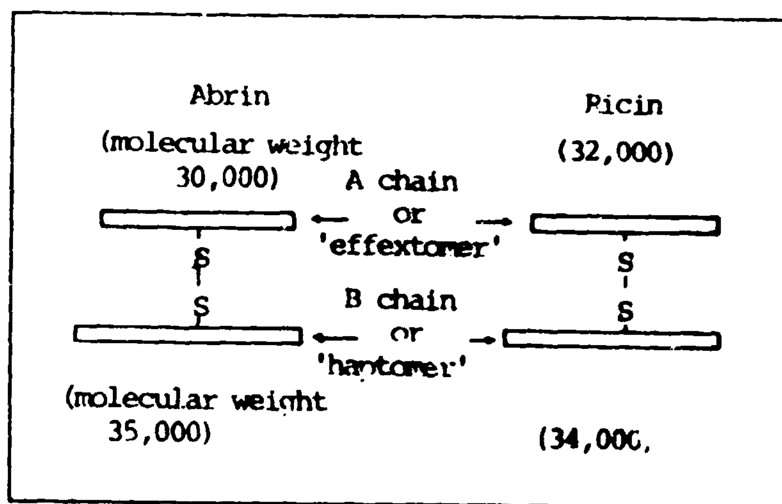


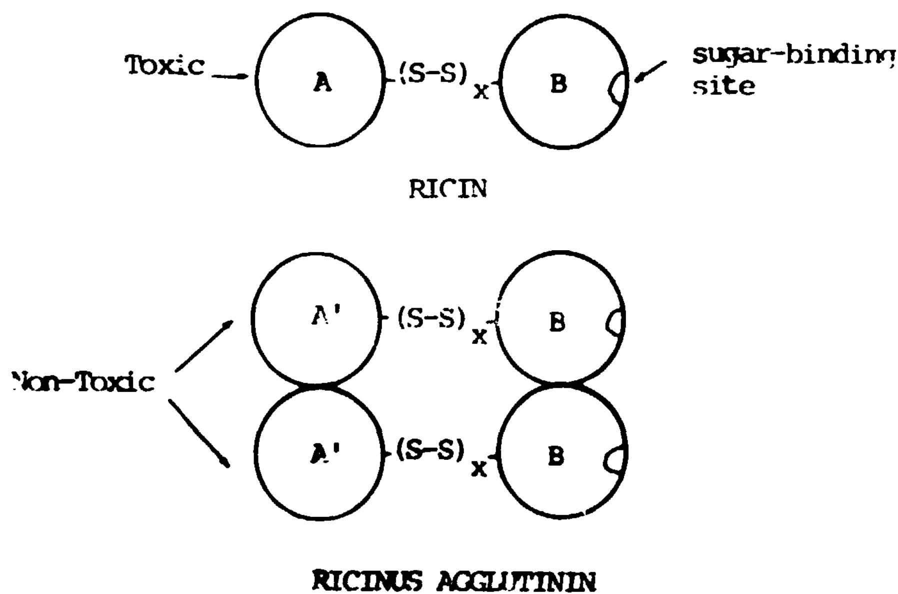
Fig. 7. Schematic structure of abrin and ricin.

When these two chains were separated by reduction with mercaptoethanol, the lesser of the two, the A chain or "Effectomer" inhibited protein synthesis in a cell-free system of rabbit reticulocytes. On the other hand the B chain, the "haptomer", did not affect the protein synthesis, but similarly to the A chain was capable of binding to sepharose. Thus it seems that the B chain functions as a carrier of the portion which serves to anchor the toxin to the cell surface. The binding of the toxin produces an increase in membrane fluidity and the A chain (or possibly the whole toxin) is transported into the cytoplasm, where it exerts its toxic effect.

That the toxic effect of ricin is the result of protein synthesis inhibition has been well established by various investigators and is explained by the growth inhibiting action of ricin on tumors (59, 60, 61 and 99) and on virus-transformed cells (78). Lugnier et al (63) reported that tryptic ricin fragments with a molecular weight of approximately 12,500 retained its hability of inhibiting protein synthesis. The specific target of ricin is the 60S subunit of ribosome (14, 33, 68 and 93) where it interferes in some way with the elongation of the peptidic chain (14, 67 and 80). It has been suggested that ricin action could be of enzymatic nature and that its specific reaction site may be guanosin triphosphatase.

Studies on the ricin structure in relation to that of agglutinin has revealed interesting similarities. The native agglutinin with a molecular weight of 120,000, is a tetramer composed 2

different subunits: one with a molecular weight of 33000-37000, and the other with 27,500-31,000 . Chemical and immunological evidence indicated that the heavier chain is probably identical to the ricin B chain, whereas the lighter one is homologous but not identical to the A chain of ricin. (see Fig. 8)



ig. 8. Schematic structure of ricin and the Ricinus agglutinin. The A and B chains shown here are equivalent to those shown in Fig.7 for ricin. The B chain carries a sugar binding site. The A chain is toxic and differs somewhat from the nontoxic A' chain of the agglutinin. The number of disulphide bonds connecting chain A or A' with chain B is not known.

As the B chain of ricin is known to contain a linking site for sugar one can foresee that agglutinin has 2 linkage sites. This

has been verified experimentally (83 and 102). Whatever be the difference between the A chain of ricin and the lighter chain of agglutinin, it would be responsible for the extreme toxicity of the former (58).

IV- DETECTION OF RICIN

As all hemagglutinins, ricin is a plant protein associated with another protein material, which difficultates its detection by chemical analysis. It is usually determined by its biochemical and biological activities, such as hemagglutination and toxicity.

The hemagglutination test may be very useful for comparative studies of the ricin content as it is simple and does not require special facilities.

Gardner et al (32) described the test for agglutination of blood red corpuscles recommended by the Baker Castor Oil Company as follows:

The red blood corpuscle solution was prepared by removing the plasma from citrated whole human blood (citrated guinea pig blood may be substituted) by centrifugation and pipetting, washing the corpuscles three times with a physiological saline solution (0.85% sodium chloride in demineralized water), and then diluting them to 10 times their volume with saline solution. Pomace to be tested is placed in solution by mixing a ground 0.5 g sample in 49.5 g of physiological saline solution, allowing it to stand 1 h and filtering. It is then stored under refrigeration until used.

The test procedure is as follows: Test tubes are placed in a rack, and 0.9 ml of saline solution is pipetted into the first tube and 0.5 ml into each of the remaining tubes. To the first tube 0.1 ml of the pomace solution is added and mixed, using a small glass rod. One half or 0.5 ml of this solution is transferred

to the second tube and mixed; a like amount is transferred to the third tube, etc, down to tube nº 10. Then, to each tube, 0.5 ml of red blood corpuscle solution is added and thoroughly mixed. Thus the dilutions of the pomace solutions in the 10 tubes are: 1:10, 1:20, 1:40, etc. and 1:5120, respectively. After standing for 5 minutes, the tubes are centrifuged exactly 2 min. Observations are then made relative to the appearance and characteristics of the red blood agglutination. The tubes are then shaken gently to redisperse the corpuscles, and the following ratings are made:

4+ complete agglutination with no dispersion upon shaking.

3+ complete agglutination with some breaking up of blood upon shaking.

2+ agglutination, but complete breaking up of blood in the agglutinated particles.

1+ agglutination, easily dispersed to visible agglutinated particles.

± upon redispersion, a magnifyng glass is required to see the agglutinated particles.

The highest final dilution showing a 1+ is called titre. The titre values are in direct proportion to the amounts of ricin contained in the pomace sample.

In 1973 Bukhatchenko (11) suggested the following test: a 5 g of ground castor seed was extracted with saline solution, which was filtered and diluted in proportions 1:10,

1:100, and 1:1000. 1 ml of a 2% critrocite suspension was added to the diluted solutions. The mixtures were left for 24 h at room temperature and the quantity of ricin present in the original sample was judged by visual comparison with the non-agglutinated control sample.

Numerous modifications have been proposed to turn the method more sensitive and quantitative (46). The use of Na_2EDTA as an anticoagulant, elimination of eritrocite washing, maintenance of pH = 7.0, and temperature of 20°C are conditions recommended by Rigas et al (41). Short-time centrifugation of the tubes increases sedimentation according to these authors.

Most workers in biological fields still seem to be content to measure agglutinating activity towards various kinds of cells by a serial dilution technique with visual estimate of the end-point. Some of these techniques have been critically evaluated by Burger (58). Attempts have been made to improve the precision of such assays by spectophotometric techniques which measure the decrease in the turbidity of a suspension of cells induced by their agglutination (47 and 62) or by the direct measurement of the rate of formation of aggregated cells (58).

For ricin the hemagglutination test is not as sensitive and specific as the toxicological assay since there apparently exist interfering factors in mixed feeds (41). It may serve for distinguishing heated from raw castor seed press cake or pomace and for detecting grossly underheated material. For the assay of residual toxicity it should be supplemented by animal tests.

For parentereal toxicity tests mice or rats are mostly used. Feeding experiments have been performed with a wide variety of animals, but rats and chicks are often preferred because of the ease of handling and for economical reasons.

In the feeding test, growth retardation and mortality as compared with appropriate controls are taken as a measure of toxicity. When the test substance is injected, the percentage of animals which die and the time between injection and death are useful parameters for the estimation of the toxicity. Several different doses should be used and expressed as µg/kg of body weight. As susceptibility may vary considerably with weight and age, the animals must be selected accordingly.

Clarke (16) proposed the following test for ricin: 10g of the finely ground material is twice extracted with 50 ml of 0.02 N HCl for several hours with mechanical stirring. The extract is centrifuged and 300 ml of acetone is added. The precipitate is separated by filtering through sintered glass, dissolved in 5 ml of physiological saline solution, and filtered. Two series of identical dilutions are prepared: 0.1 ml of normal serum is added to each tube of one dilution series;and 0.1 ml of serum from a rabbit immunized with ricin to each of the other series. A suitable number of mice is injected with an equal amount of the mixtures of each tube. The mice injected with the extract of the first series will have higher mortality than those of the second. Usually 1 ppm of ricin can be detected, but in a mixture with linseed meal about 0.1% has been found to be the lower limit of detection.

V- DETOXICATION

One of the most serious problems facing the large scale production of castor seed oil is the toxicity of the resulting press cake or meal. This toxicity is due mainly to the properties of the protein ricin, which is so toxic that the defatted residue is used as a rule as fertilizer. The inactivation of the toxic principle would permit the use of the residue as proteinous supplement in animal feeds in view of its protein content of approx. 40% (86, 88, 44, 22, and 5).

Various methods of detoxication have been suggested aiming at a complete destruction of the toxic action of ricin without affecting the nutritional value of the residual protein.

Already in 1889 Stillmark discovered that the toxalbumin ricin was destroyed by heat (41).

The characteristic property of ricin, similarly to other toxalbumins, is to agglutinate the red blood corpuscles of the mammals. This property disappears when a solution of the toxic protein is heated to the boiling point or coagulated by heating to 60 –70°C. Other investigations have shown that dry heat does not destroy completely the toxic property of the protein (44).

According to Carmichael (12 and 13) ricin can be detoxicated by treatment with sodium ricinoleate, potassium permanganate, hydrogen peroxide or halogens.

Some authors state that the desolventization of solvent extracted meals promotes a total detoxication of ricin (57, 70 and 73) whereas others maintain that such meals display,

without exceptions,a high degree of toxicity, a more intensive treatment being necessary to render them innocuous (48).

Boiling for 1-2 hours was used by Petrosyan and Ponomorov (87). The press cake treated in this manner was used with sucess in feading pigs. In the experiments of Tangl (98) the castor seed press cake was heated to 140°C for 60-90 minutes. The press cake thus treated was used in sheep feeding without noticing toxic effects.

Various patents have been obtained for the detoxication of castor seed meal. In Germany a patent was granted for " the extraction of toxic principles of castor seed press cake". The process consisted in boiling repeatedly for short periods of time, a mixture of ground press cake and water, changing the water after each boiling. The resultant material was filtered, washed with water and finally dried (86).

Rudolph (89 and 90) obtained two patents for treating the press cake with hot water and chloroform. In Belgium, Massart (64) proposed a method consisting primarily in the elimination of ricin by extracting the press cake with halogens and alkalis followed by autoclaving. Another patent advocates the use of vapour at 150-300 psi, followed by abrupt decompression (15).

The extraction of castor seed residues with water, acids, and solvents has been suggested by various investigators.

Repeated extractions with hot 95% ethanol were necessary for achieving complete detoxication of castor seed meal,according to Borchers (9). In another study,ricin was hydrolysed

with 1.5 N HCl for 2 h at 110°-$115^{o}C$, the extract neutralized and the amino acids were removed by filtration. Toxic compounds were converted into non-toxic and nutritionally valuable ones (53).

In 1958, Ambekar and Dole (2) found that extraction with solvents such as $CHCl_3$, C_6H_6 or n-C_6H_{14} did not remove ricin but aquous extraction produced a non-toxic meal which could be used for feeding rats. They stated also that extraction with ethanol, autoclaving, steaming and boiling with water produced a detoxicated meal which could replace up to 50% of groundnuts in the diet of newly whined rats without causing significant changes in weight gain.

Waller and Negi (104) obtained ricin, ricinin and allergenes by extracting castor seed meal with water at pH 3.8. The extracted meal did not cause a loss of weight during 8 weeks in rats fed a diet containing 1/3 to 2/3 of the above product.

A patented process treats the castor seed press cake with a physiological serum or with formaldehyde (88).

The use of ultra-violet rays for the detoxication of ricin has been advocated by Balint (6 and 7). According to this author,irradiation can produce a complete detoxication of ricin or only uncouple the 2 chains of this protein effecting a significant decrease of its toxicity.

Various publications report detoxication of the castor seed press cake by microbial methods. It has been afirmed that the press cake could serve as human or animal food after treatment with proteolytic enzymes, autolysed yeast,and autolysed acetobacter (52).

Le Brenton and Moulē (54) treated ricin with papain, pepsine, triptase and pancreatic polypeptidase observing a reduction of toxicity.

Another author reported that Clostridium obtained from sewage water was used for fermentation and autodigestion of castor seed press cake. The digested mass was autoclaved for 1 h at 100-120°C and dried. The final product contained about 32% of half-digested and non-toxic proteins (17).

According to Motolla et al (71) toxic effects, hemagglutinating activity,and proteolytic properties are all destroyed by the combination of heat and humidity. It is of great importance to know the exact heat treatment condition which would assure complete destruction of toxicity, any practical application being limited to thermal treatment (41 and 56).

Steaming and autoclaving have been tested by various investigators. Jaki (42) treated castor seed press cake with steam and removed the excess of humidity <u>in vacuo</u>.

Kodras and co-workers (48) cited various types of thermal treatment as being capable to promote the detoxication of castor seed meal: a) steaming for 30'; b) incubation of the meal in the presence of a mild alkali or acid followed by neutralization or mild oxidation with hydrogen peroxide ; c) "dry" autoclaving for 15 minutes at 125°C. The authors reached the conclusion that the treatment which led to a most complete destruction of ricin with minimum changes in physical characteristics of the meal was autoclaving without water addition for 15 minutes at 125°C.

Hot steam as used in the recuperation of solvents which serve for extraction of castor oil, produced a reduction to 1/1000 of the original toxicity, yielding a meal inofensive to sheep, rabbits and rats, when employed in a proportion not higher than 10% of the diet (41).

Jenkins (43) used autoclave treatment for 1 h at 15 lb/in^2 and obtained a reduction to 1/2000 of the original toxicity. Rats maintained on a diet containing 23.2% of the press cake thus treated remained healthy at the end of 4 weeks, but the growth and food conversion were lesser than in control diets.

In 1960 Gardner and co-workers (32) carried out extensive investigations aimed at detoxication and deallergenization of castor seed meals. Various treatments were applied during the conditioning of the seeds before the solvent extraction of the oil or after the extraction. The authors cited the following treatments as the most effective ones: a) dry heat at 205°C; b) moist cooking with 0.9% of HCl and 3% of HCHO; c) moist cooking with 2% of NaOH and 10% of HCHO; d) moist cooking with 2% of NaOH at a pressure of 20 psig; e) moist cooking with 1% of NaOH. In order to detoxicate completely only ricin a mild moist cooking of the flaked meats, either with or without added alkali, was sufficient.

Calcium hydroxide has been also employed as an additive before applying heat treatment up to complete destruction of the ricin and the allergenes (94).

Residues of castor seed have been detoxicated by autoclaving at 125°C and 2 atm pressure for 5 minutes in the presence

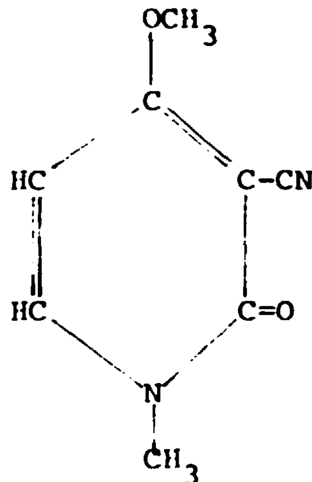
of excess of water. The quantity of albumin soluble in the water remaining after the thermal treatment can be determined turbidometrically and is approximately proportional to the detoxication (31).

As hemagglutination normally accompanies toxicity, the inactivation of ricin can be measured using guinea pigs' red blood cells in vitro (32) , or it can be easily estimated by a mouse essay (40).

In publications which appeared in the period from 1968-1972 Motoila et al (70, 72, 73 and 74) reported a series of investigations on the pilot scale aiming at the detoxication and particularly at the deallergenization of castor seed meal. The most effective treatments were: a) treatment of a 2:1 mixture of meal and water with steam at 10 psig during 60 minutes; b) treatment at 80°C during 45 minutes of a 4:1 weight/volume mixture of meal and 6 Normal NH_4OH; c) treatment at 120°C during 15 minutes of a 1:2 w/v mixture of meal and 4% $Ca(OH)_2$. The authors analysed the effects of the various treatments on essential amino acids and found that all of them affected to a greater or lesser degreee cystine, serine, threonine, methionine and lysine.

VI- RICININE

In the castor seed there has been encountered the alkaloid ricinine with the following structural formula (8):



A review of the literature on its struture was published by Henry (37) in 1949.

Tuson (100) was apparenty the first to isolate ricinine from the castor seed. He obtained the alkaloid by boiling the seed with water, filtration, evaporation to a thick syrup consistency, and extraction with boiling ethanol. He states that ricinine is soluble in water and alcohol, very little soluble in ether and benzene,and when heated with solid potassium hydroxide evolves ammonia.

Schulze (8) obtained a nitrogenous substance from the germinated seed of Ricinus communis, by extracting with alcohol, distilling off the solvent, taking up the residue with water , treating it with tannic acid and lead acetate, filtering ,

removing lead with hydrogen sulphide and evaporating the filtrate. The impure product was purified by boiling the aqueous solution with animal charcoal and finally by crystallizing the substance from absolute alcohol from which it separated in small colourless prisms meeting at 193°C.

In 1900, Evans (21) studied the chemical properties of ricinine, using separation methods described by previous authors. He concluded that Schulze's method was the better one and introduced some modifications: he used boiling toluene instead of alcohol to extract the residue remaining after the evaporation of the aqueous solution. Upon treatment of an alkaline solution of ricinine with potassium permanganate, the latter was almost immediately reduced, the reduction being accompanied by a faint odour of hydrocyanic acid. After filtering off the manganese oxide a colourless solution was obtained, which became red with a yellow fluorescence on acidification with hydrochloric acid; when heated on a water bath the solution became more intensely colored, and after evaporation and subsequent cooling, long, wavy, salmon-pink needles separated out. These proved to be free from inorganic matter and were completely decolorized when their aqueous solution was boiled with animal charcoal.

Ricinine has some characteristics of alkaloids but does not form salts with acids. Although it is not markedly toxic to human beings, it shows toxicity comparable with that of lead arsenate against codling moth larvae (92).

Investigators at Kyushu University (22) have indicated that ricinine has adverse effects on the growth of young chicken.

At the University of Davis, California (22) pure synthetic ricinine was fed to broiler chicks starting at one day of age and continuing until they were four weeks old. Ten chicks were fed, ad lib a control corn-soy diet and the same diet containing different concentrations of synthetic ricinine, prepared according to the method of Schroeter et al (91), was administered to four other lots of chicks.

Results (Table 5) show that ricinine does retard chick growth at higher levels. Examination of the chick livers showed no abnormalities, but a fluorescent material, probably a metabolite, behaving similarly to ricinine, was present in the livers of chicks on diets highest in ricinine.

Table 5. Effect of Synthetic Ricinine on Broiler Chicks

Ration % ricinine	Average weight gain at four weeks, g	Average liver weight, g
0,000	474	11,43
0,010	444	11,03
0,025	456	11,25
0,050	424	10,46
0,075	331	9,07

A more extensive experiment starting with three-day-old chicks is shown in Table 6 . Four pens of seven chicks each were fed the diets shown, for a four-week period. Ricinine was added at four levels to a basal corn-soy ration containing 24.2% protein and 1450 kcal metabolizable energy per pound, supplemented with methionine. In the last four diets, lisine and methionine supplemented ammonia-treated castor pomace replaced an equal weight of the soybean meal,maintaining a constant ration protein content of 24.2% In these isonitrogenous castor rations,the total metabolizable energy was decreased due to the difference in energy between castor and soybean meal. The soybean meal contained 47,5% protein and 1050 kcal metabolizable energy per pound. Ammonia-treated castor meal was partially decorticated, so that it contained 47,5% protein; its ricinine content was 0,29%. Chick weight gain or feed efficiency values, are significantly different at the 95% confidence level. In the equicaloric rations more than 0,4% ricinine was needed to depress weight gain, but in any of the levels fed the feed efficiency was not affected . The metabolizable energy of castor meal seems to be appreciably lower than that of soybean meal since the weight gain per unit of feed is decreased in all the castor diets. However, the adverse effect of ricinine in castor occurs only at high castor levels. Castor meal should thus be limited in diets of chickens to about 10-15% of the total ration. Probably its best use is in ration for laying hens.

Table 6. Weight gain and Feed Efficiency for Chicks Fed Ammonia-Treated Castor Meal and Ricinine.

Ration Nº	Supplement	Percent lysine added	Chick Weight gain, g	Feed efficiency gain/feed
1	Corn-soy Control	-	687	0,679
2	0,01% ricinine	-	666	0,681
3	0,02% ricinine	-	673	0,680
4	0,04% ricinine	-	674	0,665
5	0,08% ricinine	-	626	0,683
6	5% castor meal	0,09	662	0,654
7	10% castor meal	0,18	678	0,651
8	20% castor meal	0,35	657	0,624
9	41,85% castor meal	0,74	675	0,524

Fuller et al (22) developed also a method of ricinine detection, based on its fluorescence when excited at 363 m . According to the intensity of this fluorescence, ricinine may be determined quantitatively in the absence of interfering substances

The quantity of ricinine present in the seed, which is small in the first place, diminishes further during the processing. A meal obtained in the laboratory by extraction with ether contains 0.1% of ricinine, whereas a commercial meal contains 0.02% (104).

Massart (64) extracted ricinine from the castor seed press cake with a solution containing alkali halides and alkali hydroxides.

Okamoto et al (79) detoxified castor seed meal washing it with water followed by extraction with ethanol or further washing with diluted hydrochloric acid. Although no detrimental effects were found in laying hens fed castor seed meal containing diets, retardation in growth of 2 to 7 weeks old chicks was observed even when as little as 5 or 10% of the meal was incorporated into their diet.

Vilhjalmsdottir and Fisher (103) carried out a series of experiments with commercial castor seed meals to determine their use as a protein source for growing chicks. They found that extraction with hot water was the best means of removing the major part, if not all, of the castor meal components responsible for depressing chicken growth. It was concluded that hot water extracted castor meal properly supplemented with the limiting amino acids lysine and tryptophan, is an acceptable protein source for chick diets.

Hinkison et al (38) verified that when castor seed meal is treated with ammonia, in order to destroy ricin and/or allergenes, the quantity of ricinine is also reduced by ca 25%. It was shown that the majority of ricinine loss can be accounted for by the production of 4-amino-3 cyano-N-methyl-2 pyridone, a substance formed by displacement of the original methoxyl group of ricinine with ammonia. In vitro enzymatic studies have demonstrated that neither this compound nor ricinine significantly effects lactate dehydrogenase.

Murase et al (75) report that although the toxic substance (I), remained in the castor meal after heating it at 123°C for 30 min, it was easily and completely extracted from the meal

with H_2O, EtOH, or acetone. The toxic substance was dialyzable and was not adsorbed on a Dowex 8 column. It was separated and crystallized from an EtOH solution of a freeze-dried water extract of the autoclaved meal, after evaporating EtOH *in vacuo*, dissolving the residue in acetone, and storing at -20°C. Sucrose was contained in the EtOH extract as a by product. The toxic substance was identified as ricinine by elemental analysis, melting point (201°C) and its u.v. absorption spectra. The content of ricinine in the autoclaved meal was approximately 0.13% after refluxing the meal with acetone, evaporating acetone to dryness, refluxing the residue with n-hexane, dissolving the residue in acetone, decolorizing the solution with activated C, and concentrating.

The minimal lethal dose of ricinine tested by intraperitoneal injection into mice and by oral administration to chickens was 0.016 and 0.1 mg/g body weight, respectively. The body weight of the chickens increased normally on feed containing 0.035% ricinine, for two weeks and on feed containing 0.028% ricinine for 4 weeks.

Ricinine is used as an insecticide,and according to Haller and Mc Indoo (35), is highly toxic to young larvae of codling moth (Carpocapsa pomonella), but was of slight toxicity to adult houseflies (Musca domestica). Applied as a contact poison, it was non-toxic to grasshoppers.

VII- Experimental Results

1. Composition of castorbean meal and castorbean cake.

Table 1 - Analysis of Samples

SAMPLES	HUMIDITY (%)	TOTAL PROTEIN (%)	OIL CONTENT (%)	TOTAL SUGARS (%)	ASh (%)	FIBRE (%)
Cake (C_1)	6.50	40.26	9.67	4.35	7.86	9.68
Cake (C_2)	7.81	31.94	7.94	4.66	14.16	16.38
Meal (M_1)	8.00	51.56	1.54	5.65	10.01	9.31
Meal (M_2)	10.22	39.44	1.33	5.07	9.05	14.57
Meal (M_3)	8.06	35.59	2.77	5.07	20.60	13.55
Meal (M_4)	8.72	43.30	1.60	6.79	13.55	17.07

2. Toxicity of cartorbean meal and cake on guinea pigs.

Table 2 - Results of the testes with guinea pigs using untreated samples.

SAMPLE	ANIMAL WEIGHT (g)	INJECTED TOTAL PROTEIN g/g weight	DEATH TIME
Meal			
M_1	404	10.3	39:00
	484	15.0	24:10
M_2	361	10.0	72:00
	-	-	-
M_3	284	10.3	14:00
	351	15.0	17:00
M_4	470	10.3	59:00
	-	-	-
C_1	340	10.3	33:00
	416	15.0	51:10
C_2	295	10.3	41:30
	448	15.0	42:00

3. Changes in the Properties of castorbean oil with heat treatment

Castor meal and cake were subjected to steam treatment at 121°C for 3 hours and the following results were obtained.

a. Optical rotation of castor oil

Wave length (nm)	Oil (untreated)	Oil (treated 121°C/3 h)
365	5.930	5.310
405	9.225	8.900
436	8.030	7.775
578	4.520	4.390

b. Refractive index

Original oil and treated oil = 1.4785

c. Acidity index and fatty acid content.

	Original sample	Treated oil (121°C/3 h)
Acidity (g ricinoleic/100g)	0.86	0.97
Acid value (mg KOH/g)	1.62	1.83

4. Changes in the sugar content with heat treatment

Samples	sugar (% glucose/l)
meal, untreated	5,65
meal, treated (121°C/3h)	3,60
cake, untreated	4,35
cake, treated (121°C/3h)	3,20

5. Changes in the extractable allergenic protein with heat treatment

Extraction according to Waller, G. and Negi, S. JAOCS: 35: 409-12:1958.

Sample	Extracted (%)	CB-1A crude (%)
Meal, untreated	6.22	4.83
Meal, treated (121°C/3h)	4.30	2.60
Defatted meal, untreated	5.24	3.24
Defatted meal, treated (121°C/3h)	3.51	1.38

6. Change in the extractable protein with saline solution (0.85% NaCl)

Sample	% (g protein/100 g sample)
seed, dehulled	5.91
cake, untreated	3.38
cake, treated (121°C/3h)	1.69

7. Choice of Analytical Control Methods of Heat Treatment

Among various analytical methods, the change in the extractable protein with saline solution seemmed to correlate with the intensity of the heat treatment. The following results are obtained:

Table 1 - Comparison of different methods of protein determination.

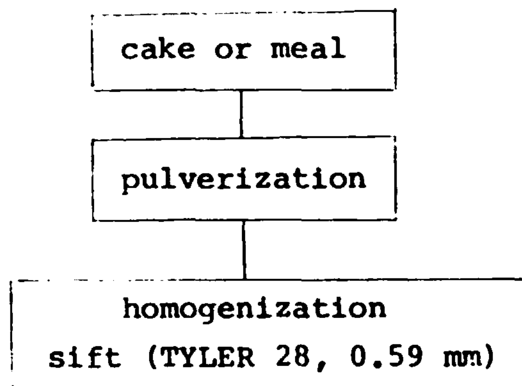
Sample	T (°C)	Time (min)	Extract 1 Biuret * (mg Prot/ml)	Extract 1 Sakaguchi (mg Prot/ml)	Extract 1 S/B	Extract 2 Biuret (mg Prot/ml)	Extract 2 Sakaguchi (mg Prot/ml)	Extract 2 S/B	Δ Protein	Δ S/B	Dest. Ninhydrin ** (μg $(NH_4)_2SO_4$/ml)	Toxicity
		0	8.46	16.27	1.92	7.23	16.17	2.24	1.23	0.32	114.8	+
		4	7.11	15.76	2.22	5.03	13.39	2.66	2.08	0.44	110.2	+
		6	6.01	14.25	2.37	5.35	10.96	2.05	0.66	0.32	101.1	-
		8	3.68	10.71	2.91	3.65	10.61	2.91	0.03	0.00	105.7	-
M_1	120	10	4.54	12.22	2.69	3.83	10.96	2.86	0.71	0.17	125.4	-
		20	2.40	8.69	3.62	1.67	7.02	4.20	0.73	0.58	145.0	-
		30	2.34	8.18	3.50	1.70	7.93	4.66	0.64	1.16	-	-
		45	2.35	8.18	3.48	1.70	7.32	4.30	0.65	0.82	158.7	-
		0	6.00	12.83	2.14	5.11	11.87	2.32	0.89	0.18	104.2	+
		4	5.52	10.96	1.99	4.40	10.35	2.35	1.12	0.36	93.6	-
		6	3.74	9.19	2.46	2.80	7.02	2.51	0.94	0.05	140.5	-
		8	3.07	9.70	3.16	2.12	7.93	3.74	0.95	0.58	134.4	-
C_1	120	10	4.79	12.83	2.68	3.17	9.19	2.90	1.62	0.22	117.8	-
		20	1.97	8.38	4.25	1.43	6.09	4.26	0.54	0.01	105.7	-
		30	2.34	7.93	3.39	1.90	6.16	3.24	0.44	0.15	-	-
		45	2.03	8.08	3.98	2.00	5.95	2.97	0.03	1.01	158.7	-

(*) Average of 3 repetitions
Average Standard Duration = 0,12

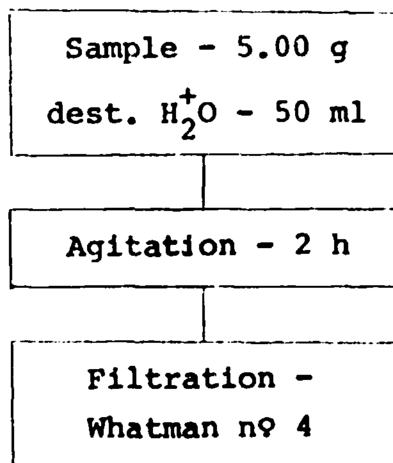
(**) In order to establish parameters for the test of quantification of volatile substances, a thermostat was connected to the heating system and the heating time was strictly measured.

A rapid test which can be performed with relatively simple equipments was developed for the measurement of the heat treatment

A. Preparation of the sample, in accordance with the following scheme:



B. To obtain the extract:



C. Biuret Reaction

Reagents - Dissolve 1.50 g of $CuSO_4 \cdot 5\ H_2O$ and 6.0 g of $NaKC_4O_6 \cdot 4H_2O$ in 500 ml of H_2O agitating constantly, then add 300 ml of a 10% NaOH solution and dilute up to 1000 ml

with H_2O. This reagent can be kept indefinately in a poliethylene bottle but it should be thrown away if a black reddish precipitate appears.

Procedure - Add 4 ml of the biuret reagent to 1 ml of the sample (1:2 dillution). For the blank add 1 ml of water instead of the sample. Read after 30 minutes comparing with the standards.

D. Comparison of the standards

(i) Preparation of the standards

Standard 1 - 1 ppm Violet Crystal solution

Standard 2 - 1 ml of Bacto Methylene Blue
2 ppm solution + 4 ml of violet crystal 1 ppm solution

Standard 3 - 2.5. ml of Bacto Methylene Blue 1 ppm solution + 2.5 ml of violet crystal 1 ppm solution.

Violet Crystal 1 ppm Solution

Dilute 10.00 mg of Violet Crystal in 100 ml of distilled water. Take one measure of 1 ml and complete the volume up to 100ml with distilled water.

Bacto Methylene Blue - 1 ppm

Dilute 10.00 mg of Bacto Methylene Blue in 100 ml of water. Take one measure of 1 ml and complete the volume up to 100ml with distilled water.

(ii) <u>Results</u>

The sample with colour between standard 1 and 2 is toxic and between standard 2 and 3 is nontoxic. Reading is taken in a Pyrex test tube, glass 7740, 16 x 150 mm.

8. Changes in the nutritive components

Amino acid patterns of castor bean cake and meal showing that either lysine, methionine or cysteine losses during common moist heat treatment are negligible. Values given in g of amino acid per 16 g Kjeldahl nitrogen.

Amino Acid	Cake (C_1)		Meal (M_1)	
	No Treatment	120°C 30 min	No Treatment	120°C 30 min
Aspartic Acid	8.99	8.53	8.33	8.51
Threonine	3.66	3.36	3.49	3.61
Serine	4.90	4.58	4.48	4.96
Glutamic Acid	18.00	16.47	17.44	19.69
Proline	3.03	2.82	3.14	3.48
Glycine	4.06	3.87	3.74	4.06
Alanine	4.42	3.90	4.04	4.13
Cysteine	1.40	1.09	1.66	1.59
Valine	5.18	4.93	4.97	5.13
Methionine	1.33	1.22	1.25	1.39
Isoleucine	4.67	4.24	4.16	4.27
Leucine	6.21	5.79	5.61	6.28
Tyrosine	2.74	2.52	2.56	2.70
Phenylalanine	4.22	3.71	3.75	3.88
Histidine	2.07	2.07	2.09	2.13
Lysine	2.95	2.37	2.93	3.06
NH_4	2.09	1.89	1.76	1.70
Arginine	9.57	9.59	11.00	10.18

VIII- CONCLUSIONS

1. Among various treatments for detoxification of castor meal and cake, the steam treatment under pressure seems to be more effective and economically feasible. A treatment equivalent to 10 to 15 minutes at 121°C was sufficient for the purpose.

2. A rapid and semiquantitative method for the measurement of the intensity of the heat treatment could be established. This method is suitable for a modest industrial laboratory.

3. A pilot plant installation is suggested to test technical and commercial feasibility of the heat treatment of castor meal and cake for detoxification.

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