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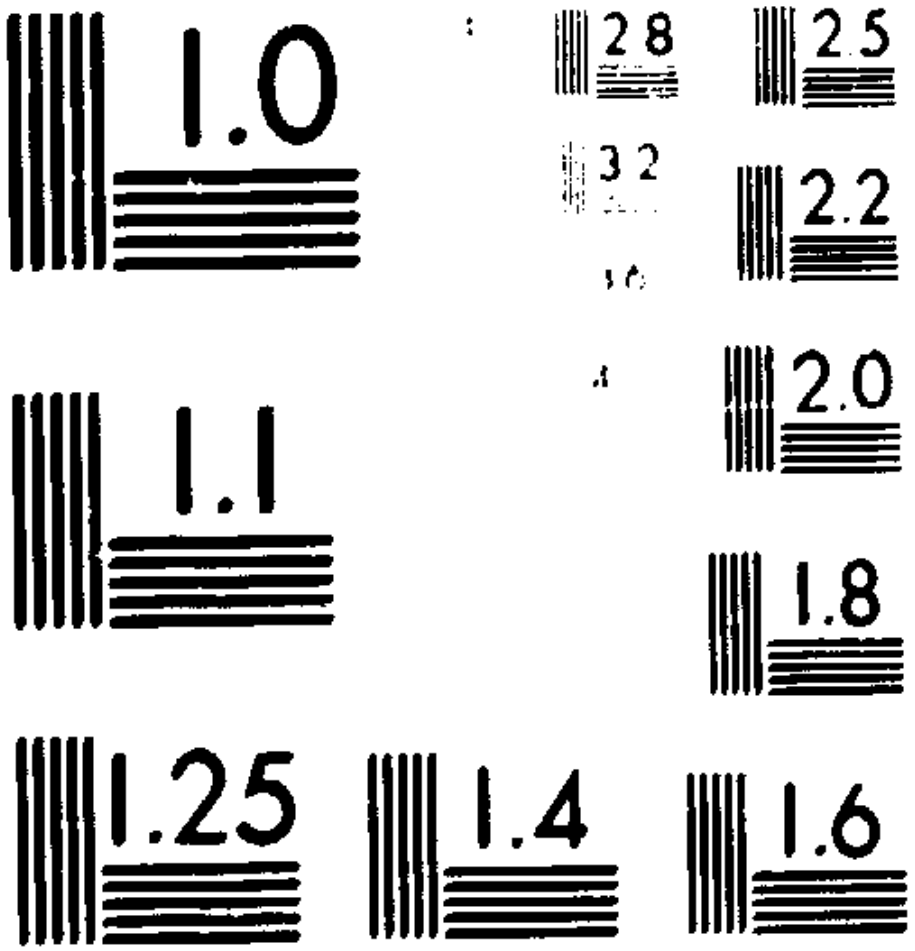

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Title

DEVELOPMENT OF A CASTORMEAL DEALLERGENATION TECHNOLOGY IN A SELECTED DEVELOPING COUNTRY

Project. US/GLO/77/033
Contract no. T81/57

Final Report

December 1982

FUNDAÇÃO TROPICAL DE PESQUISAS E TECNOLOGIA
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1. INTRODUCTION

According to Spies and Coulson (1), the allergic sensitivity of man towards castorseed was described for the first time in 1914, believing that the allergen was identical to the toxic albumine, ricin. In 1929, however Ratner and Gruehl (in Spies and Coulson)(1) showed that castorseed had a distinct anaphylactic agent along with ricin. Later on, classical studies of Spies, Coulson, Bernton and Stevens (1, 2, 3) showed conclusively that the main allergenic agent called CB-1A of the castorseed was different from ricin.

Figley and Elrod (4) in 1928 showed a high incidence of respiratory allergy in certain regions of the United States. Out of 32 cases of asthma studied, 30 were of people who lived near a castor oil factory in Toledo, Ohio. This suggested a close relationship between this sensitivity and this potent allergen. Later, Figley and Rawling (5) reported that in 1932 the Common Pleas Court prohibited permanently castorseed processing in this region. Soon after, the installation was disassembled and asthma caused by castorseed became rare.

In 1952, Small (6) reported on 17 patients that showed a positive cutaneous anaphylaxis for raw castorseed extract 1:10 using the puncture method (skin-prick test). Fourteen of these patients had a background of clinical sensitivity for castorseed residue and eleven had hay fever or asthma due to the inhalation of castormeal commercially used as fertilizer. An occurence of 2.5% of clinical

sensitivity to castorseed was found in a small group of workers of the "California Flaxseed Products Co".

Figley and Rawling (5) mentioned that green coffee in sacks could be contaminated by the allergens of the castorseed. Two coffee roasting installations from Toledo, Ohio, used to import coffee from Brazil or Colombia placed in new fiber bags (caroa fiber) or burlap bags. But, during World War II, when new bags were not available, coffee was occasionally placed in bags that had been used for castorseed. These contaminating allergens caused such symptoms as acute allergic rhinities and/or asthma due to inhalation, on workers of these installations. Eventually, these sacks were resold and even went to homes.

According to Panzani (15), in 1957 epidemics of asthma had been mainly described among citizens in towns where castor oilmills existed, e.g., Toledo, Ohio, in the USA; Baurú, in Brazil; Johannesburg, in South Africa; and in Italy and Germany. This author reported 102 cases of allergy to castorseed in residents of the south of France especially in Marseilles, having described the following etiological cases:

1. Laboratory people, working with castorseeds or castor oil cakes	1 case
2. Dockers, workers on the wharves and drivers	5 cases
3. People growing and gathering castorseed	3 cases
4. Workers and oil-mill employees	1 case
5. Fertilizer retailers, bag merchants, people living in the vicinity	7 cases

6. Country people handling castor oil cakes and inhabitants of the country — 20 cases

7. Citizens of Marseilles living in the vicinity of oil-mills where the dust has been scattered by the wind — 65 cases

When chemical processes are used in the extraction of oil, the meal produced is dryer and more powderly, and therefore, more dangerous from the allergenic point of view, when compared with that obtained from mechanical processes which is granular and still has 6-7% of oil.

Bernton (7) in 1973 reported a case of sensitivity to castorseed of a coffee industry worker. The patient showed intermittent asthmatic symptons which were particularly severe when he worked in the mill. Results of the physical exams showed nothing extraordinary except for engorgement of the tissues of the throat, occlusion of the turbinates, and the presence of loud musical scales throughout the lungs. A chest roentgenogram showed that the heart, great vessels, lungs and pleurae appeared essentially normal.

Karr et al. (28) in 1978 indicated that coffee workers with occupational allergic diseases show serum IgE antibodies, specific for etiologic water-soluble green coffee beans and castorseed antigens, and that these antigens are distinct, unrelated to chlorogenic acid, present in certain industrial dust and sack samples and capable of producing asthma in sensitive subjects.

Clinical symptoms on people who are allergic to castor

seed, generally occur in the nasal, pharyngeal, and respiratory system, mucous, as well as in the ocular conjunctive. On the skin, after a few hours of exposure, cutaneous phenomenon occur like urticaria, in particular on the exposed parts. In the respiratory system it can appear as an allergic type of rhinopharyngitis, probably also presenting general symptoms such as chronic headache, fever, etc. In the eye it produces conjunctivitis similar to that produced by a strange body (8).

Patients sensitive to the allergenic fraction had cutaneous reactions, according to Spies and Coulson (1), when received the allergen in a $1:10^6$ dillution. In guinea pigs it produced sensitivity and anaphylactic shock. The minimum dose used in order to produce sensitivity was 8.4 µg of allergen and to produce shock was of 0.33 µg.

According to Spies et al. (2) the median sensitizing dose (SD-50) in previously sensitized guinea pigs were 63.6 $\pm$ 13 µg and 746 $\pm$ 240 µg (on an ash and water free basis) to CB-1A and CB-65A, respectively. From the dosage response curve the median lethal dose (LD-50) of CB-1A was found to be 2.38 $\pm$ 0.36 µg.

In 1971, Spuzic (29) reported that in the serum of persons with positive intradermal reactions to ricinus communis the existence of corresponding specific antibodies could not be proved with the precipitin reaction in gel, reaction of immune hemolysis and reaction of passive cutaneous anaphylaxis.

In the same year, Wilson, Marchand, and Coombs (30) demonstrated citologically the presence of IgE reagenic antibodies

on the cell membrane of human basophils by a rosette-forming reaction with allergen-coupled erythrocytes. The system involved the in vitro passive sensitization of basophils with the sera of patients exhibiting allergic asthma to castorseed dust. The strength of the cytophilic reaction showed a correlation with the serum titer of specific IgE antibodies measured by the red-cell--linked antigen antibody reaction. Two sera containing IgG, but no demonstrable IgE castor antibodies, failed to passively sensitize basophils for specific rosette formation.

Layton et al.(31) reported in 1970 a study for cross - reactivity to 60 species from 22 other genera of the spurge family (Euphorbiaceae) in fifty patients suffering from primary respiratory allergy to ricinus seed. Sera from these patients were tested for cross-reactive reagents by passive transfer to the skin of monkeys. Passive allergic reactions in the monkeys indicated that the human sera contained reagents with allergen-binding sites directed against several generically and ordinally specific sites in the castorseed antigen. Results of the laboratory and clinical tests conducted by these authors indicated that allergic cross-reactivity to related species, is the rule in primary allergy to castorseed antigens.

The allergens when given to poultry through the feed containing castorseed can be transmitted to man. In 1977, Layton (9) studied the allergenicity of fish fed with allergenic castor meal. The author concluded that catfish can be fed with ration with up to 50% of castormeal without harming the fish, though allergenic substances reach the muscles in sufficient quantity

to produce allergy to those who are sensitive to castorseed.

This author also suggested that, as far as public health is concerned. it would be recommendable to have the fish, which had been fed with castormeal, several days without this feed just befcre harvest. This recommendation is based on tests which the results are shown on Table 1.

Table 1

Reagenic allergic serum transfer test in monkeys[a]

Source of muscle tissue (catfish)	Passive transfer test reading serum source (patient)						
	C.M.	P.B.	V.S.	Lai.	Gaz.	V.M.	Sif.
Catfish on castor	+ 4	+ 4	+ 4	+ 4	+ 5	+ 4	+ 4
Market fish	0	0	0	0	0	0	0
Catfish on castor (7 days)	0	0	0	0	0	0	0

0 = no reacton; + 1 = 1 - 5 mm diameter; + 2 = 5 - 10 mm diameter; + 3 = 10 - 15 mm diameter; + 4 = 15 - 20 mm diameter; + 5 = 20 - 25 mm diameter

[a] Monkeys passively sensitised to castorseed allergens by intra dermal injection of reagenic sera from castorseed - allergic French patients.

The AST (Allergic Serum Transfer) test with monkeys showed intense reactivity for muscle extracts from catfish fed with feed containing castorseed. The catfish that was fed during 7 days with normal feed without castormeal did not show any allergen in the muscle tissue which was indicated by the absence of reaction on the sensitized regions on monkeys (Table 1).

Table 2

Allergic skin-reactions in passively sensitized human volunteers[a]

Source of muscle tissue	Passive transfer test readings serum source (patient)			
	Beau.	Blanc.	Melin.	V.Sec.
Catfish on castor	+ 2	+ 2	+ 3	+ 3
Market-fish	0	0	0	0
Catfish on castor	0	0	0	0

0 = no reaction; + 1 = 1 - 5 mm diameter; + 2 = 5 - 10 mm diameter; + 3 = 10 - 15 mm diameter; + 4 = 15 - 20 mm diameter; + 5 = 20 - 25 mm diameter.

[a] Nonallergic human volunteer passively sensitized to castorseed by intradermal injection of reagenic sera from castorseed allergic patients.

On table 2 we have the AST test with human volunteers which confirm the results obtained with monkeys.

Intravenous injection of muscle tissue extracts from catfish fed castorbean meal did not elicit PCA reactions in guinea pigs. On the other hand, strong PCA reactions were provoked by injecting extracts from the skin, contents of the gastrointestinal tract and feces. These results showed that the castorbean meal was only partially digested by the fish and that antigenically active proteins can be found in every portion of the gastrointestinal tract, as well as the feces. It follows that the pond water containing unconsumed feed and undecomposed feces will contain castorbean antigens.

Although castorseed oil has been considered virtually free of allergens, castorwax or hydrogenated castorseed oil has been reported to contain an allergenic principle. In 1980, Lehrer et al. (18) reported the presence of castorseed allergens in castor wax products determinated by in vivo and in vitro analysis of castor wax extracts. Allergens were detected in one extract of castor wax by the PCA (passive cutaneous anaphylaxis) reaction in mice, the RAST (Radioallergosorbent test) inhibition reaction and skin-prick test in castorseed sensitive individuals. These allergens in the wax were of much lower potency than those in the seed and were not detectable in a deodorant product utilizing castor wax.

2. CHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF ALLERGENS

Both the increasing world production of castorseed oil and the insidious nature of the allergens present in defatted meals prompted researchers to isolate and characterize the compounds responsible for such allergenicity. However we feel that there is still much room for research on the identification and chemical characterization of these allergenic compounds.

During the process of isolation of the allergens it was soon established that most, if not all allergens could be restricted to a proteinacious fraction of the castorseed aqueous extract called CB-1A. This fraction is a mixture of low molecular-weight proteins and glycoproteins (1, 2, 12) which contains the most powerful but not all of the allergens in the seed, as was stated by Spies et al. after examining the meal (12), the pollen, and the flowers (13).

Panzani and Layton (27) observed in 1962 that allergy to commercial castorseed pomace (oil-cakes) was, in many cases, a complex of independent allergies to one or more of several components of the pomace (castorseed, pollen, or femal blossoms) and/or to other contaminants of the pomace (molds, weeds, seeds, pollens, insect bodies, and other miscellaneous biological materials).

The physical-chemical characteristics of the castorseed allergenic principle have been studied by a number of workers

led by Spies (1, 2, 3, 11, 12, 14). In 1943 they (1) isolated a nontoxic and highly allergenic glycoprotein fraction, corresponding to 1.8% of defatted castormeal. This fraction was characterized as being insoluble in water, soluble in alcohol 25%, and insoluble in alcohol 75%, does not precipitate in boiling water and is parcially dialysable. Amino acid determination of the allergenic fraction showed relatively high quantities of arginine and cystine as well as the absence of tryptophan.

Thirty five years after the isolation of the allergenic fraction CB-1A, Youle and Huang (10) identified these allergens as low molecular-weight albumins (storage proteins) present in the protein bodies of the castorseed endosperm. The physical-chemical properties of the CB-1A fraction compared well with those of the purified 2S storage proteins of the seed. SDS-polyacrylamide gel electrophoresis of proteins isolated from protein bodies of castorseed were resolved in two major groups. A group of high molecular weight proteins representing the 11S globulins, 4-6S albumins and its dissociated subunits and another group representing the albumins having a molecular weight of about 11,000 daltons. The sedimentation value, 2S, found by these authors was similar to that found by Spies and Coulson (11) for (CB-1A)E fraction which remained in the interior of the membrane after dialysis and was precipitated with 4 volumes of ethanol. Furthermore, Youle and Huang (10) showed by immunodiffusion tests that no proteins in the total seed proteins other than the 2S albumins had an immunological relationship with the CB-1A. Testing the thermal sta-

bility and solubility of the 2S albuminic fraction, the same authors were able to retain 35% of these albumins using the CB-1A preparation method of Spies and Coulson. The authors also reported a great similarity between the amino acid composition of the albuminous 2S fraction and that of the CB-1A obtained through microbiological experiments 30 years earlier; even if the values used in this comparison did not correspond exactly with one another.

The data reported on the quantity of castorseed allergens varies, depending on the method of analysis used. While Waller and Negi (16) reported a yield of 0,08% of total weight (dry basis) for commercial castormeal and 0,22% for laboratory obtained meal, Coulson et al. (17), using another method, reported that defatted and decorticated meal has from 6,1% to 9% allergens depending on the variety of the castorseed, whereas commercial meal has from 0,09% to 4,2%. Perrone et al. (19) reported values of 8 to 16% allergens in nontreated laboratory meal and nontreated commercial meal. These values found by the immunochemical semi-quantitive process in based on the published analytical data, the authors (17) agreed that the variability found does not encourage a study for the breeding of non-allergenic varieties.

The allergenic proteins from castorseed CB-1A fraction, are among the most heatstable proteins known, heating at 100°C for 1 hour does not destroy or diminish the activity of the native antigenic structures (3). According to Spies (14), heating

the (CB-1A)E fraction for 1 hr at 110°C at pH 5.9 had no effect on its immunoprecipitating capacity. At pH 5.9, this fraction required heating for 1 hour at 150°C and 140°C to destroy completely the precipitating and reagin-neutralizing capacities, respectively. The (CB-1A)E fraction also showed remarkable stability to heating in alkaline solution. Thus, at 100°C and 120°C at pH 12 or over, the reagin-neutralizing capacity was lost in 32 and 8 min, respectively, using sample tests after two-fold time increments, starting at 1 min. There was a difference in stability between the reagin-neutralizing and the precipitating capacity which depended on the pH. At pH values of 5.9 to 8.7, the precipitating property was more stable to heat than the reagin-neutralizing property. But, at pH 10.8 to 11.9 the reverse was true.

It was recognized that the CB-1A proteins contained the principal allergenic specificities and were immunologically distinct from other allergens and antigens present in their respective source materials. Although the 1A fractions as well as the other antigens from the various oilseeds were immunologically distinct from each other, the principal components of each 1A mixture were regarded as having a common antigenic and allergenic specificity (11).

Coulson et al. in 1946 (26) reported that fraction CB-1A from castorseed was capable of precipitating when given to rabbits, although it was uniformly less effective then egg albumin in stimulating the formation of precipitate. When the

allergen obtained from the process of Spies et al. (1) was submitted to DEAE-celulose chromatography and electrophoresis it was fractionated into 6 components. The antigenic differences between the fractions were interpreted as indicating the possible existence of various specific antigenic components (13).

In 1944, Spies et al. (2) obtained a subfraction of the CB-1A called CB-65A which had allergenic properties and was essentially carbohydrate free. Positive passive transfer reactions were produced by 1×10^{-10} g of CB-65A. The principal allergenic and anaphylactogenic specificities of CB-1A were inherent to the essentially carbohydrate-free protein fraction.

Layton et al. (20) described a fractionation by paper electrophoresis of CB-1A and reported that at pH 7.4 with ionic strength of 0.05, six distinct and possibly another two faintly colored electrophoretic bands were obtained. Positive reactions were obtained for PCA tests in all bands using Philippine monkeys and at least five distinct specificities were affecting allergic humans.

In 1967, Spies (23) showed a minimum of eight antigens by gel diffusion analysis of the electrophoretic bands of CB-1A and ten subfractions of CB-1A.

Spies and Coulson (11) obtained a total of ten subfractions from the CB-1A material by using a combination of dialysis and ion exchange chromatography, while the two most important subfractions were further characterized by electrophoresis in celulose acetate. The CB-1A was initially dialysed,

whereas the fraction that remained in the interior of the membrane, designated (CB-1A)E, was separated through precipitation with ethanol, the diffusate, designated as (CB-1A)D was concentrated. These two fractions were chromatographed in an ion-exchange column each one giving 5 fractions. The fractions E_1 and D_5 which represent the separation extremes, were further studied by electrophoresis on cellulose-acetate strips. Both the (CB-1A)D fraction and the (CB-1A)E fraction presented sub-fractions that migrated towards the anode showed by the electrophoresis. The Schultz-Dale test indicated that the E_1 and E_5 fractions contained antigens with identical specificity or closely similar to each other, and that the E_5 fraction had at least one antigen that was not found in E_1. A major common or identical specificity was found in all of the subfractions of fraction 1A even though they were completely separated from each other, and thus chemically distinct. The electrophoretogram of the carbohydrate-free allergenic protein, CB-65A, exhibited four bands, whereas the gel double-diffusion immunoelectrophoretogram pattern showed three bands or groups of compounds. In an attempt to determine if there were any specificity links among the components of the CB-65A antigens, Morris, Spies and Coulson (21) resolved this fraction into four bands by polyacrylamide-gel (7.5%) electrophoresis. It was subsequently shown by gel diffusion that the immunological specificity was closely similar if not identical for the four resolvable components, concluding that any existing structural differences were too slight to impart immunological

distinctiveness among the proteins.

In 1960, Coulson, Spies, and Stevens (17) prepared a fraction (CB-13)E which included a further step in the purification process of the allergenic protein CB-1A. Such purified fraction possessed a potent allergenic activity, causing positive reactions on the skin of castorseed sensitive patients in dilutions greater than $1:10^6$.

Spies and Barron (22) described an electrophoretic fractionation in celulose acetate of CB-1A, a spectrophotometric analysis method of the complex CB-1A - Ponceau S obtained through the precipitation of CB-1A in aqueous solution with a 1% solution Ponceau S in 5% acetic acid and an analysis of the fractions by gel diffusion. Four antigens were demonstrated in the CB-1A - Ponceau S complex by gel diffusion analysis of the electrophoretic fractions. The principal antigen and the principal minor antigen were almost completely separated and both were allergenic.

In 1976, Daussant, Ory and Layton (24) studied physical chemical changes occurring in the proteins of castorseed, particularly the CB-1A antigen and the major storage globulins during germination of the seed. Germination caused an anodic shift in the CB-1A major antigen and a decrease in the most anodic and cathodic portions of the major storage protein. After germination some proteins were no longer detectable by immunoelectrophoretic analysis, but even after 10 days, proteins extracted from the seedlings still contained a significant amount of the

major CB-1A antigen.

There exists a claim that agmatine, arginine and two other unknown guanido derivatives are low-molecular-weight compounds present in the castorseed allergen fraction (32).

Other authors, however, have attempted to confirm such results on the basis of chromatographic, and electrophoretic data, as well as color-producing reactions for amino acid determination, but have concluded that the only guanido compounds present in the Cb-1A allergenic fractions are the low molecular-weight proteins (33).

3. EXPERIMENTS ON CHARACTERIZATION OF ALLERGENS

3.1. The origin of allergens

In our laboratory the following experiments were carried out to see the amount of CB-1A fractions obtainable from different types of castor bean products:

3.11 Industrialized Castormeal

A castormeal sample (500 gr) was placed in a round bottom glass flask (capacity of 3 litres) with 2 litres of distilled water and kept under mechanical agitation for 16 hours. This mixture was then filtered under vacuum through glass wool and washed with 1ℓ of distilled water. The suspension thus obtained (total volume - 3 litres) was centrifuged at 2000 rpm for 2 hours at room temperature (centrifuge IEC Model K).

The supernatant, obtained after centrifugation was heated in a water bath (temperature 96°C), for 5 minutes and centrifuged at 2000 rpm during 10 minutes (Centrifuge IEC Model K). The supernatant thus obtained was concentrated in a rotary evaporator under reduced pressure (bath temperature = 75°C) up to 1/5 of its initial volume. Distilled ethanol was added to the concentrate, in an ice bath until a 75% solution in ethanol was obtained and this suspension was centrifuged at 2000 rpm for 10

minutes at 5°C. The supernatant was discarded and the precipitate was dissolved in 500 ml of distilled water. Distilled ethanol was added until a 25% solution was reached and 100 ml of a 10% solution of basic lead acetate was added. The insoluble material was separated by centrifugation at 2000 rpm for 10 minutes, at 5°C, and ethanol was added to the supernatant up to a concentration of 75%, in an ice bath. The precipitate thus obtained was separated by centrifugation at 2000 rpm for 10 minutes at 5°C, 22.000 rpm for 30 minutes, at 4°C (ultra centrifuge Beckman model L5-65, rotor Ty30). The precipitate was discarded and to the supernatant a 10% solution of Na_2CO_3 was added until a pH = 9,6 was reached. The suspension obtained was centrifuged at 22.000 rpm for 30 min at 4°C (Ultra centrifuge Beckman, model L5-65, rotor Ty 30) and the supernatant was acidified with a 20% solution of acetic acid (pH 6,5) and treated with ethanol under an ice bath until a 75% solution was obtained. The precipitate obtained (CB-1A) was separated through centrifugation at 2.000 rpm for 10 minutes, at room temperature, washed with cold ethanol and dried under vacuun with P_2O_5 during 3 days, resulting in a light brown solid (Table 3).

The result is shown on Table 3 under specification - FL-IND.

3.12 Castormeal from whole seeds obtained in laboratory by extracting the oil with ethyl ether.

The amount of 1 kg of ground whole castorseed was treated

with cold distilled ethyl ether (2 x 1,5 ℓ) during 72 hours. The ethereal extract was separated through filtration and the insoluble material was partially air dried (in a hood) during 24 hours and reextracted with hot ethyl ether (Sohxlet extractor, with 2ℓ capacity) during 32 hours. The insoluble material was left to dry (in a hood) and 5. of laboratory castormeal from seed with skin (CB-FL ether W) obtained.

The extraction of CB-1A fraction from CB-FL ether was carried out according to the procedure described on paragraph 3.11 and the result is shown in Table 3 under the specification FL-ETHER W.

3.13 Castormeal from decorticated seed, obtained in laboratory by extracting the oil with ethyl ether.

The amount of 1 kg of ground decorticated castorseed was subjected to a cold extraction (2 x 1,5ℓ) and to a hot extraction (Sohxlet extrator with 2ℓ capacity), with ethyl ether as described in paragraph 3.12. 350 g of castor meal from decorticated seed was obtained by extracting the oil with ethyl ether (CB-FL ether D) which was worked up according to the procedure discribed on paragraph 3.11 and the result is shown in Table 3 under the specification FL-ETHER D.

3.14 Castormeal from whole castorseed obtained in laboratory by oil extraction with ethanol

The amount of 1 kg of whole castorseed (cut lengthwise in ethanol) was treated with cold ethanol, during 7 days. After filtration, the cut seeds were ground with ethanol and reextracted with ethanol (1,5 ℓ) for 12 days, at room temperature. By filtration and air drying, 610 g of castor meal were obtained, in laboratory, by oil extraction with ethanol (CB-FL EtOH W). The extraction of allergens from this meal was carried out according to the procedure described on paragraph 3.11 and the result is shown in Table 3, under specification FL-EtOH W.

3.15 Castormeal from decorticated castorseed obtained in laboratory by oil extraction with ethanol.

The amount of 1 kg of decorticated castorseed was subjected to a treatment similar to that described on paragraph 3.14, giving CB-1A by the extraction of allergens from the laboratory obtained meal. The extraction of the oil was carried out with ethanol from the decorticated seed (CB-FL EtOH D). The result is shown on Table 3 under specification FL-EtOH D.

Table 3 - Yield and response to the double immunodiffusion test[1] in agar for the isolated CB-1A fractions.

Product specification	Quantity (w/w)	CB-1A[a] Yield %	Minimum Conc. (ppm)[b]
FL-IND	3,2/500	0,6	64
FL-ETHER W	2,95/500	0,6	32
FL-ETHER D	6,2/350	1,8	32
FL-EtOH W	5,9/500	1,2	32
FL-EtOH D	9,6/500	1,9	32

1 The method used in the double immunodiffusion test in agar is described in annex 1.

a Based on the quantity of meal used

b Minimum concentration (ppm) of CB-1A that, still permits a positive answer in tne double immunodiffusion test.

3.16 Results and Discussion

By using the Spies' and Coulson's method (1) for the isolation of the allergenic fraction CB-1A from meals of different origins, allergenic fractions immunologically related were obtained. The yield of CB-1A from meal obtained by the conventional extraction methods (hot extraction with ethyl ether), was higher when decorticated seed was used (Table 3, specification FL-ETHER W and FL-ETHER D). Apparently there seems to be no dependency on

the CB-1A allergenic activity when decorticated seeds are or are not used since no difference was detected in the minimum concentration of the sample necessary for a possitive answer in the double immunodiffusion test in agar (Table 3, specification FL-ETHER W and FL-ETHER D; FL-EtOH W and FL-EtOH D).

Similarly, a previous treatment with ethanol in the preparation of the meal (which could be responsable for a reduction of the allergenic activity of the meal, as suggested by some workers in oil extraction industry), did not lead to any change in the allergenic activity - estimated by the double immunodiffusion method - of the isolated CB-1A. A considerable high yield of the CB-1A fraction from the meal prepared from whole seeds was, obtained as compared to that observed from the meal obtained through hot extraction with ethyl ether from whole seeds (Table 3, specifications FL-ETHER D and FL-EtOH D).

Higher allergenic activity of the sample of CB-1A isolated from industrialized meal (Table 3, specification FL-IND) was observed when compared to the fractions isolated from meals prepared in the laboratory. However, one can see that the presence of allergenic material (fraction CB-1A) is shown in all fractions obtained in laboratory, being characterized as a natural product, existing in the seed itself and not as a product formed during processing.

3.2. The non volatile nature of the allergens

Some workers claimed that some castor seed allergens are volatile and can be spread in air. The following

experiments were carried out in our laboratory.

500g of industrialized meal were placed in a round bottom flask (5 ℓ) containing 1 ℓ of distilled water. It was then connected to a vapor reservoir, in accordance with scheme on Figure 1, with continous heating of the vapour reservoir and intermittent heating the flask containing the meal. Samples were then taken of the condensed vapors (7 x 250 ml) with a flow of a 1 ml/minute.

Results and Discussion:

None of the fractions collected during the distillation indicated a positive answer for the double immunodiffusion test in agar (i.e., no precipitation line corresponding to the antigen-antibody reaction), which allowed us to conclude that the allergenic fraction does not have a volatile nature.

However, whenever particles of the sample were carried over to the collector flask, the fractions gave a positive answer to the double immunodiffusion test in agar, indicating that the local allergenic problems are due to the presence of castormeal or seed particles suspended in the air.

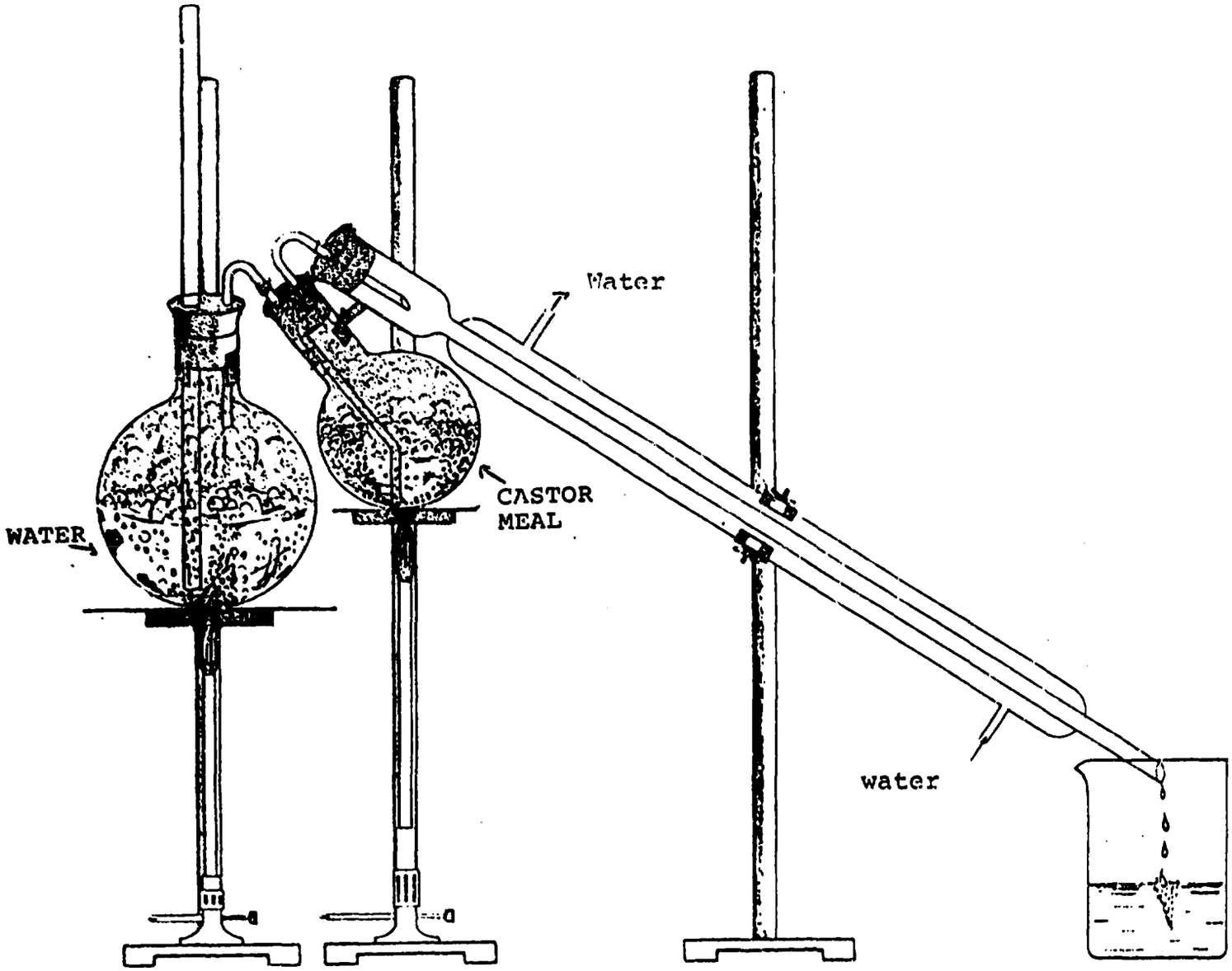


Figure 1: Scheme of apparatus for detection of volatile compounds.

3.3. Fractionation and characterization of allergens

3.31 Ultraviolet spectrum of allergens

The ultraviolet spectrum of the samples in distilled water showed maximum absorption : λ_{max} 273 ($A^{1\%}_{1cm}$ 2.12), 277 ($A^{1\%}_{1cm}$ 2.17), 289 ($A^{1\%}_{1cm}$ 1.14).

The infrared spectrum (samples in KBr pellets) showed intense absorption in the 3500-3100 region (ν_{NH} and/or ν_{OH}) and 1670 cm^{-1} ($\nu_{C=O}$ of amides) and medium intensity at 3060 (ν_{CH} aromatic), 2940 (ν_{CH} alifatic) and 1560 cm^{-1} ($\nu_{C=C}$ aromatic and δ_{NH}).

3.32 Amino acid content of allergens

Amino acid analysis of the FL-ETHER D concentrate, and of the samples obtained from the chromatographic separation of CB-1A is shown in Table 4.

a. Procedure

The sample was hydrolysed with 9.0 ml of 6N HCl in sealed tubes at 110°C during 22 hours. The acid was eliminated through evaporation at a reduced pressure (temperature of 45°C).

The hydrolysate was dissolved in 4,0 ml of sodium citrate buffer (pH 2.2) and it was filtrated through membrane filter Millipore GS-0.22μ). The filtrated solution was injected (100 μl) in an automatic Beckman analyser 119-CL.

The percentage, w/w, of the aminoacids in each sample is listed in table 4, which also shows the estimated percentage of proteins of each sample , assuming that the percentage of the aminoacids in a protein is 118%.

Table 4 - Percentage w/w, of the aminoacids present in the FL-ETHER D and in the fraction obtained from the chromatographic separation (Sephadex - SP-LH 50).

Aminoacids	F33	F25	F19	FL-ETHER D CB-1A	F44
Aspartic acid	3.088	4.184	4.840	4.690	2.120
Threonine	1.268	1.548	0.824	1.149	0.844
Serine	4.208	6.086	9.792	7.552	2.832
Glutamic acid	26.644	42.864	50.056	44.684	15.820
Proline	2.092	2.636	0.818	1.456	1.048
Glycine	3.620	4.530	3.955	3.841	2.244
Alanine	1.860	2.546	2.957	2.405	1.108
Cysteine	N.D.	N.D.	4.362	4.727	N.D.
Valine	2.052	2.484	4.484	3.379	1.232
Methionine	0.252	0.284	1.150	1.045	0.126
Isoleucine	1.920	2.688	3.415	2.970	1.152
Leucine	4.368	5.383	4.414	4.244	2.576
Tyrosine	0.524	0.748	1.970	1.560	N.D.
Phenylalanine	2.184	2.356	0.402	1.120	1.228
Histidine	0.676	0.938	1.086	1.208	0.428
Lysine	1.428	2.144	3.754	3.297	1.020
NH_4^+	2.440	3.426	2.713	2.483	3.932
Arginine	8.132	10.652	14.533	12.556	4.952
TOTAL	66.756	95.496	115.524	104.366	42.662
%PROT.*	56.57	80.93	97.90	88.45	36.15

* Estimated protein content for each sample assuming a total of 118% for a protein.

b. Results and conclusions

The analysed samples showed a high content of glutamic acid (about 40%) and arginine (about 12%), confirming that the 1A fraction of oil seeds is rich in those aminoacids (47).

The aminoacid composition of FL-ETHER D and of the F19 fraction was similar. They were the only ones that had sulfur containing aminoacids cistine (4.5 and 3.8% by weight of the total aminoacids of the samples) besides methionine, when compared with other fractions, F25, F33 and F44.

Fractions F25 and F33 showed two non identified aminoacids, one of which was also detected in the other fractions which were analysed.

Through the estimated percentage of proteins one can see the predominant protein nature of the FL-ETHER D fraction and the F19 and F25 fractions.

3.33 Chromatographic separation of CB-1A in Sephadex SP-LH50.

a. Packing of the column

In a glass column with a sinterized bottom (height = 50 cm, and internal diameter = 4 cm), 10g of previously swollen Sephadex - SP-LH 50 was packed for two days at room temperature, in a buffer solution of sodium phosphate 0,02M, pH 5,0 (height of the absorbent in the column = 30 cm).

b. Elution

The amount of 416 mg of CB-1A, isolated from the extract with ethyl ether of decorticated castorseed, was dissolved in 5 ml of phosphate buffer, pH 5.0 and it was placed at the top of the column. The whole chromatographic process was carried out at 20°C and all aqueous solutions used were stabilized with a 10 ppm thimerosal solution. The flow of the column was controlled to give 2 ml/minute and 300 fractions of 30 ml each were collected while increasing the pH of 0.1 pH unit in the elution mixture (phosphate buffer 0.02 M), from an initial pH of 5.0 and a final pH of 7.0 (figure 2).

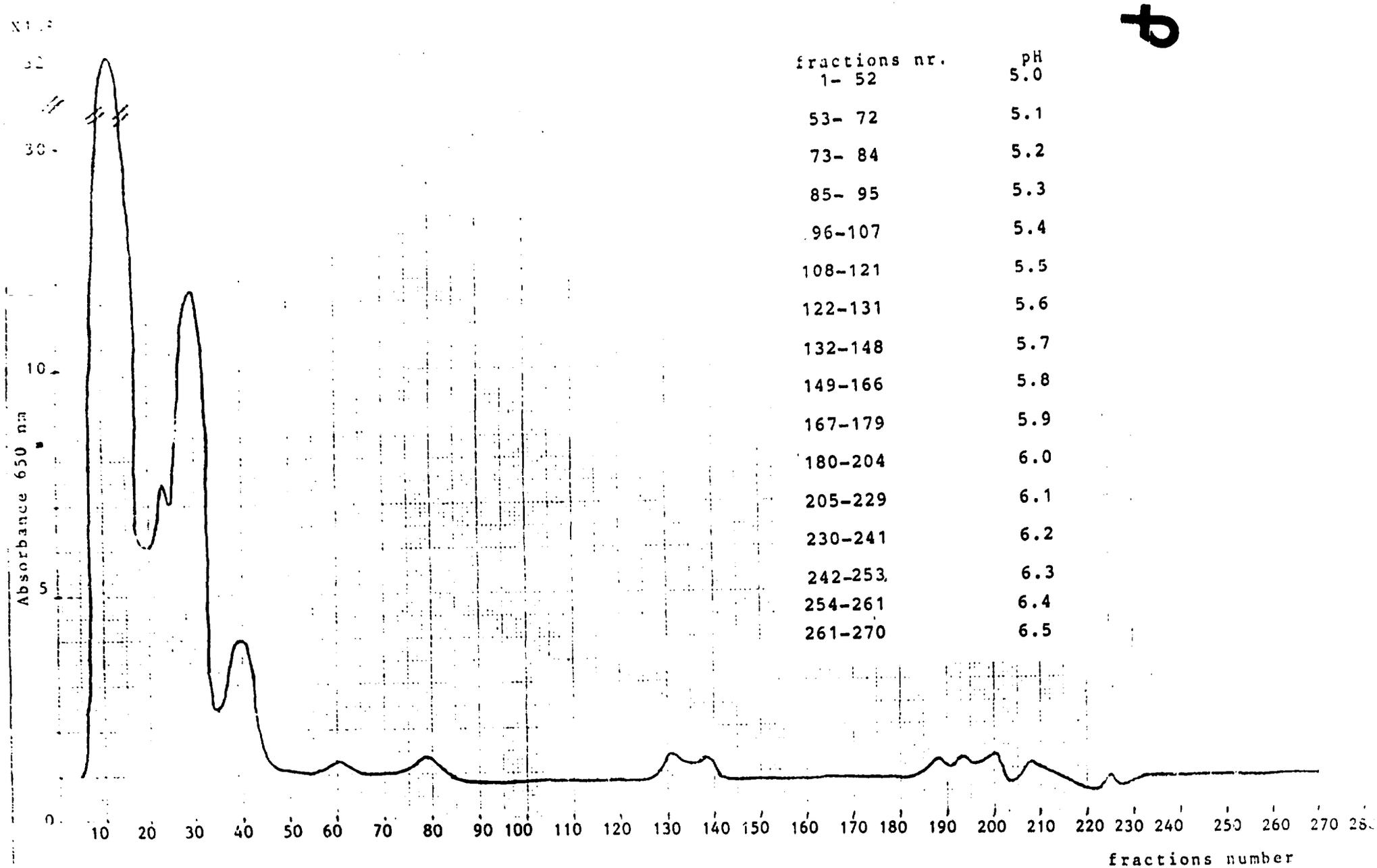
c. Detection

The protein content in the collected fractions was measured according to the modified method of Lowry (48) with absorbance reading at 650 nm.

Reagents used

Solution A: 2g of sodium and potassium tartarate and 100g of Na_2CO_3 dissolved in 500 ml of NaOH 1N and the volume completed to 1 ℓ with distilled water.

Solution B: 2g of potassium and sodium tartarate and 1g of $CuSO_4 . 5H_2O$ dissolved in 10 ml of NaOH 1N, the volume completed 100 ml with distilled water.



fractions nr.	pH
1- 52	5.0
53- 72	5.1
73- 84	5.2
85- 95	5.3
96-107	5.4
108-121	5.5
122-131	5.6
132-148	5.7
149-166	5.8
167-179	5.9
180-204	6.0
205-229	6.1
230-241	6.2
242-253	6.3
254-261	6.4
261-270	6.5

Figure 2: Chromatogram of CB-1A in Sephadex column.

Solution C: in a 1.5 ℓ round bottom flask, 100g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 25g of sodium molybdate ($Na_2 MoO_4 \cdot 2H_2O$) were dissolved in 700 ml of distilled water. A reflux condenser was then adapted and the solution was left to boil for 10 hours. It was then cooled to room temperature and 150g of lithium sulfate and 2 drops of liquid bromine were added. The mixture was then heated until boiling - point for 15 min in order to remove the excess of bromine and it was then cooled to room temperature and the volume completed to 1 ℓ with distilled water.

d. Procedure

An aliquot of 1 ml of eluted fractions was treated with 0.9 ml of solution A and kept at 50°C for 10 minutes. After cooling at room temperature, 0.1 ml of solution B was added and it was kept at room temperature for 10 minutes. 3 ml of solution C was then rapidly added and it was heated to 50°C for 10 minutes, obtaining finally a blue solution. The absorbance at 650nm was read in a glass cell (optical length of 1,0 cm) in a Micronal B-280 spectrophotometer.

e. Results and discussion

The elution profile (Figure 2) showed the presence of 4 major fractions (7 - 19, 20 - 25, 26 - 33, and

34 - 44) that by means of dialysis and liophylization gave respectively 210, 11.5, 7.6, 7.1 mg of a white solid (corresponding to 56% of the chromatographed material). All of them showed precipitation lines in the double immunodiffusion test in agar up to the minimum concentration of 1.6 ppm (the same limit observed for the sample of non-treated CB-1A). No significant difference was observed with the comparison of the spectrums in the infrared region of the non treated CB-1A sample and the principal isolated fraction (7-19): 35000-31000; 3060, 2940, 1670, 1560 cm (KBr).

The other fractions which were collected were not submitted to dialysis and liophylization due to the significantly lower absorbance values when compared to the those 4 main fractions.

3.34 Electrophoresis on Polyacrylamide Gel

a. Procedure

The electrophoresis was carried out on polyacrylamide gel according to Davis (49) and Ornstein (50).The protein content was measured according to the method described by Lowry (48).

The samples were diluted in Tris-glycine buffer,pH 8.3, and an approximate Py 200 µg per tube was applied to the gel. This quantity was considered adequate for FL-ETHER D isolate.

The electrical current was set at 2 mA per tube during the first hour and 5 mA per tube for next two hours.

The dye used was 1% solution of amido black in 7% acetic acid. The bleaching was carried out through successive washing with acetic acid 7%.

b. Results and Discussion

The electrophoretic pattern is shown in figure 3.

The CB-1A samples isolated from castor meals, FL-IND, FL-ETHER W, FL-EtOH W, FL-ETHER D, FL-EtOH D, and fraction F19, showed three very intense and well defined protein bands (a,b and c). The presence of skin in the extract did not contribute to any additional protein band and the protein pattern of the samples from meal of decorticated seeds was identical to that of samples with skin. The sample FL-ETHER W, FL-ETHER D, FL-EtOH W, FL-EtOH D, and FL-IND showed two additional protein bands (d and e) and sample F19 showed three additional protein bands (d, e and f). However, they were less intense indicating that they were in smaller concentrations.

Samples F25 and F44 showed three bands that correponded to those found in samples FL-ETHER W, FL-ETHER D, FL-EtOH W, FL-EtOH D and FL-IND (bands a, b and e), but bands b and c were less intense. Bands d, e and f were not detected. Sample F33 showed only two bands; a and b, being the latter less intense.

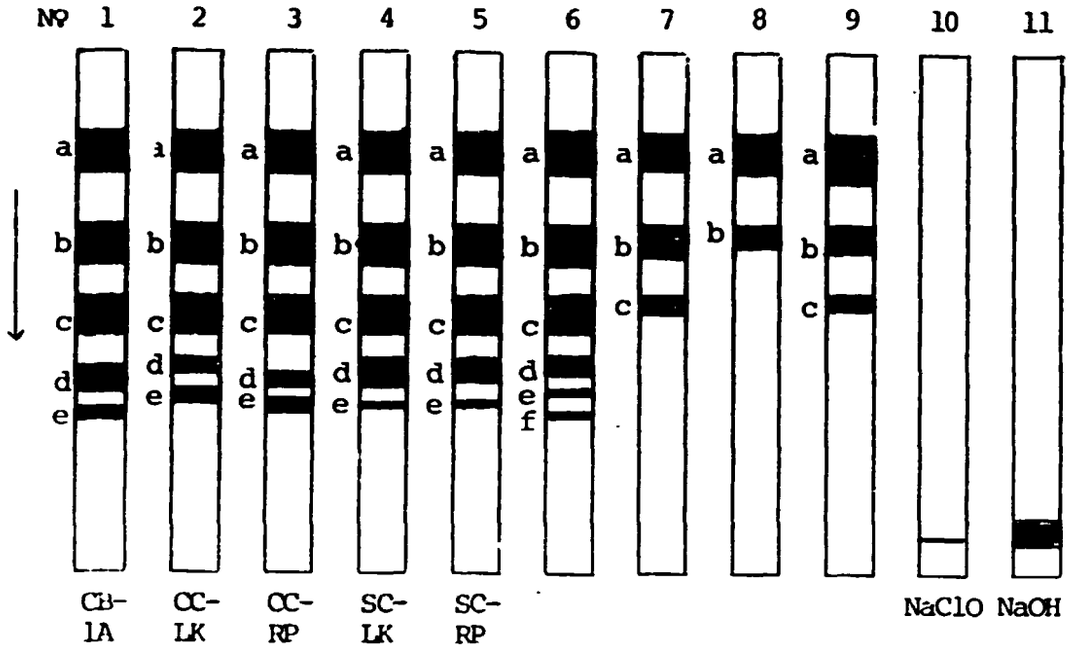


Figure 3 - Electrophoretic pattern of CB-1A samples (nº 1-5) ; fractions isolated on chromatographic separation in sephadex SP-LH50 (nº 7-9); and the products of deallergenation treatments (nº 10-11).

4. DEALLERGENATION PROCESSES

Gardner et al. (34) in 1960, reported results of probing experiments in which a number of types of treatments for the detoxification and deallergenation of castorseed cake and/or meal were investigated.

Deallergenation treatments were mainly of the practical type, which could be readily integrated or combined with conventional processes of solvent extraction. They included cooking of the castorseed cakes and meals under various conditions of moisture and temperature; dry-heat treatment at elevated temperatures; treatments with chemicals such as sodium hydroxide, hydrochloric acid, anhydrous ammonia, sodium hypochlorite, sodium chloride, ammonium sulfate, potassium permanganate, formaldehyde, urea, and their combinations. The biological treatments included aerobic fermentation and enzymatic digestion of the extracts. In all cases, the residual allergen content (CB-1A) was determined by assay of the final meal by the precipitin test, ring test, and Schultz-Dale test.

The results indicated, in general, that the different treatments were effective in reducing the content of allergen (CB-1A) to varying degrees, and, some meals of practically zero allergen content were produced as discussed below.

1) Alkaline-heat treatments (with NaOH; KOH or $Ca(OH)_2$)

A reduction on the quantity of allergen (CB-1A) of 97%-98,4% was obtained by moist-cooking the raw cake with 1% NaOH. Subsequently when the raw cake was cooked at a pressure of 20 psig with 2% NaOH, the allergen content (CB-1A) was reduced to 0%, obtaining a deallergenized meal. However when this treatment was applied directly on the meal prepared from moist-cooked raw cake the result was not the same.

Calcium hydroxide and potassium hydroxide were also used but under test conditions they were not as effective as scdium hydroxide in reducing allergen content.

2) Treatment with formaldehyde

In three of four of the formaldehyde (HCHO) treatments which were carried out, the allergen content was 0% by the precipitin test and practically 0% by the Schultz-Dale test.

The treatment conditions were:

I - with 3% HCHO and 1% NaOH;

II - " 3% HCHO and 0,9% HCl;

III - " 10% HCHO and 2% NaOH.

The results demonstrated that formaldehyde reacted with the allergenic component in some way, possibly by alkylation

and/or polymerization at the free amino group , under alkaline conditions, rendering it undetectable by both test methods.

3) Dry Heat Treatment

Meal produced from a nonalkali-cooked cake was completely deallergenized by simple dry heating to about 207°C in an elapsed period of about 125 min. When the cake was alkali-cooked, the duration of the treatment fell to 113 min, when other variables were kept constant. The treated meals, however, had a brown color and charred appearance, indicative of extensive heat damage.

4) Ammonia Treatment

The purpose of this treatment was to explore the use of ammonia as a means of further lowering the allergen content of meals which had already been partially deallergenized to about 0.8% allergen (CB-1A) content by alkaline-cooking of raw cakes followed by hexane-extraction.

The experiments were carried out under various conditions. The results of the ring and precipitin test showed that there was no significant reduction of the CB-1A content in the eight meal samples, ammoniated under the various conditions of moisture, temperature, ammonia pressure, and treatment time. However, when the ammoniated meal was stored hermetically sealed during a period of 45 days, there was a reduction on the CB-1A content,

from about 0.8% to 0.2%. This effect was due to the prolonged contact with ammonia residue. However, no additional reduction was noticed after storing for 150 days.

5) Treatment with hypochlorite

There was a 50% decrease in the allergen (CB-1A) content in two of all the experiments which were carried out. In one case, 0.9% NaOCl was applied on the cake and in the other 1% NaOCl was applied on meal pre-treated with 1% NaOH but no additional reduction in the allergen (CB-1A) content was noticed.

6) Treatment with hydrochloric acid

Treatments with 2% of hydrochloric acid in aqueous acetone at 71°C were tried in the raw cake but there was practically no reduction in the allergen (CB-1A) content.

7) Treatment with Trypsin enzyme

Trypsin digestion of the extracts was also tried in an attempt to further diminish the allergen (CB-1A) content of meals previously treated with a 0.7% NaOH solution. In this case, the allergen level was lowered to 0.2%.

8) Treatment with calcium hydroxide and other agents

Spies et al. (35), in 1962, described the conditions of temperature, time and pH for the inactivation of the castor-seed meal allergen by heating with aqueous calcium hydroxide. After inactivation, excess calcium hydroxide was neutralized with phosphoric acid.

The types of samples used were castorseed meal and isolated (CB-1A)E allergen, obtained according to the method described by the authors in previous papers (1, 12).

The tests applied on the samples to verify the efficiency of the treatments were: 1) precipitin test, in vitro method to detect the destruction of immuno-precipitating property; 2) reagin neutralization (passive-transfer method) and direct cutaneous test (skin reactivity) to detect the destruction of the allergenic properties.

Samples of castorseed meal were heated with $Ca(OH)_2$ at concentrations of 0 to 16% (based on the weight of the meal) during one hour at temperatures of 60°, 80°, and 100°C. Following this treatment, their immuno-precipitating property was tested. The results of the tests showed that: (i) at 60°C there was no complete destruction of this property at any of the concentrations of $Ca(OH)_2$; (ii) at 80°C and 100°C the destruction of this property was complete with a concentration of 8% of $Ca(OH)_2$.

The influence of the heating time was also tested using a temperature of 100°C and a concentration of $Ca(OH)_2$ of 8%. The

results of these tests showed a complete destruction of this property after 32 min. Using the same temperature and heating period, a complete destruction of the skin reactivity was found with 16% $Ca(OH)_2$.

When the isolated (CB-1A)E allergen was treated with $Ca(OH)_2$ using the same concentrations (0-16%) the same time (1 hour), and at 80°C, 100°C and 120°C, the authors concluded that:

i. at 80°C the immuno-precipitating property was destroyed with 4% $Ca(OH)_2$ but the reagin-neutralizing property was not destroyed, even at 16% $Ca(OH)_2$.

ii. at 100°C and 120°C, the immuno-precipitating property was destroyed at both temperatures with 2% $Ca(OH)_2$ and the reagin-neutralizing property was destroyed at 8 and 1% $Ca(OH)_2$, respectively.

As it was done with the meal, the influence of heating time on the immuno-precipitating, reagin-neutralizing and skin-reactivity properties was tested with the (CB-1A)E allergen at temperatures of 100°C and 120°C with 8% $Ca(OH)_2$. These results can be summarized as follows:

i. at 100°C the immuno-precipitating property was completely destroyed after 4 min of treatment and the reagin-neutralizing and skin-reactivity properties only after 32 min.

ii. at 120°C, the immuno-precipitating, reagin-neutralizing and skin reactivity properties were destroyed after 4,8 and 16 min respectively.

The allergen (CB-1A)E, in the isolated form, was subjected to another treatment that consisted of heating for one hour without $Ca(OH)_2$, at constant pH and at various temperatures. It was found that the allergenic property was destroyed with heating at 140°C, but heating at 150°C was required for complete destruction of the immuno-precipitating property.

In that work, it was observed that the relationship between destruction of the immuno-precipitating and reagin-neutralizing properties of the allergen (CB-1A)E by heating appears to depend on the pH. Thus, at pH 5.9 to 8.7 heating destroyed the reagin-neutralizing property, while at pH 10.8 to 11.9 the immuno-precipitating was impaired.

The use of other agents such as sodium chloride, potassium permanganate, ammonium sulfate, urea and aerobic fermentation had also been studied (35) on meals, but allergen destruction had not been greater than 75%, which may be considered insufficient.

In a previous report, Spies et al. (36) determined the effects of heating the castorseed allergen in solutions of pH 4 to 10 during different lengths of time on its immuno-precipitating and reagin-neutralizing properties.

Mottola et al. (37), in 1968, reported results of 54

different experiments which were carried out in a pilot plant. Castorseed meal was treated with $Ca(OH)_2$ at various concentrations (4, 6 and 8% - based on the weight of the dry meal) temperatures (100°, 120°, and 140°C) and times (15, 30, and 60 min).

The effectiveness of the antigen deactivation treatments was evaluated exclusively by a biossay technique referred to as the passive cutaneous anaphylaxis (PCA) test (Layton et al. 43). The results indicated that a deallergenization was best obtained with meal treated with 8% $Ca(OH)_2$, 140°C for 60 min. However, the amino acids cystine, serine, threonine, arginine and lysine were adversely affected by this treatment.

The authors did not guarantee that this treated meal was safe for human handling under all conditions and suggested that further work should be carried out in order to relate antigenicity as measured by the guinea pig PCA tests with allergenicity in human subjects.

Mottola et al. (38), in 1972, re-evaluated their findings of 1968 using a more sensitive and less variable biological assay procedure. In the previous work (37) the antigen reaction was systemic via the cephalic vein, whereas in the latter, the assay was based on an intradermal antigen reaction at a site previously sensitized by anticastor serum. The authors stated that excellent deallergenation was obtained by heating the isolated allergen to 120°C for 15 min. in the presence of 4% $Ca(OH)_2$.

In 1971, Mottola et al. (39) used the pilot-plant steam process for castormeal antigen deactivation. One of the

objectives of this series of experiments, was to determine whether the intra-dermal technique (40) would indicate castor antigen deactivation using low pressure steam. If deactivation was indicated, as suggested by Jenkins (44), a study on the relative effect of different levels of pressure and time would be of interest. This because previous studies with steam processing based on the intravenous biological assay technique seemed to suggest that high pressures would be required (40, 41).

Treatments consisted of 12 combinations of 10, 20, 40, and 80 psig steam pressure for 15, 30 and 60 min each. Biological assays were performed with guinea pigs using the intra-dermal technique, passive cutaneous anaphylaxis and a potency evaluation of the treated meal (Table 5).

An analysis of variance of the data (40) showed that, as expected, the process variables, time and pressure, had pronounced effect on the biological response. It also gave evidence for significant time and pressure x log dose interaction.

The overall response levels (Fig. 4) decreased with greater dilutions and longer times. A slight increase in biological response was also noted as one increases the process steam pressure from 10 to approximately 30 psig. According to the author, this response was seemingly paradoxical probably due to the two oposing reaction rates. The process might cause greater antigen extraction than destruction at these lower pressures. An evaluation of the steam process is shown in Table 5, where the

allergen potency is given as a function of the steam pressure and length of treatment.

Amino acid composition determined on selected treatments revealed significant losses in lysine content with heating. Arginine was also destroyed but remaining levels were still as good as those found in soybean meal.

Considerable antigen deactivation had been achieved using mild steam treatments, e.g., 10 psig for 60 min. The process had a mild effect upon the essential amino acid lysine.

Mottola et al. (42), in 1972, presented the results of studies on process variables of ammonia treatment carried out in a pilot plant, and the development of a practical process for the destruction of castor antigens using this reagent.

In previous laboratory studies, it was shown that it was possible to deactivate antigens and allergens using ammonium hydroxide. Four process variables considered likely to affect the deallergenation of castor meal were ammonia concentration, process temperature, quantity of liquid in the slurry and process time. The variables studied are shown in Table 6.

The antigenicity responses were determined by an abdominal intradermal injection using passively sensitized guinea pigs (40). Guinea pig response to a standard purified castor allergen preparation (CBWU) was used as the control to identify nonreactive pigs and effectiveness of the anti-castor serum (39). The preparation was also used as the standard for expressing potency of castor meals.

Table 5

Potency ratio of steam treated castor meals as a function of steam pressure and time (40).

Treatment No.	P (psig)	Time (min)	Dose (µg) equivalent 12.5 mm response	95% LCL[a]	Potency ratio[b]	95% UCL[a]
Lot A	-	-	0.347	21.6	49.9	102.0
Lot B[c]	Control	Control	3.94	1.91	4.13	8.92
0	-	-				
1	10	15	11.6	.503	1.41	3.95
2	10	30	307	.0167	.0531	.168
3	10	60	848	.00353	.0192	.105
4	20	15	13.3	.543	1.22	2.75
5	20	30	24.6	.410	.662	1.07
6	20	60	198	.0463	.0820	.145
7	40	15	23.2	.389	.702	1.27
8	40	30	17.5	.464	.928	1.85
9	40	60	971	.0140	.0168	.0201
10	80	15	794	.00763	.0205	.0551
11	80	30	1590	.00348	.0103	.0303
12	80	60	1040	.00814	.0157	.0303

[a] Abbreviations: LCL, lower control limit; UCL, upper control limit.

[b] Potency ratio expressed as µg CBWU (alcohol extract of castor seed) per milligram of castor meal. Each level equivalent to a 12.5 mm biological response. CBWU dose equivalent to 12.5 mm 0.0163 µg.

[c] Original, untreated castor meal.

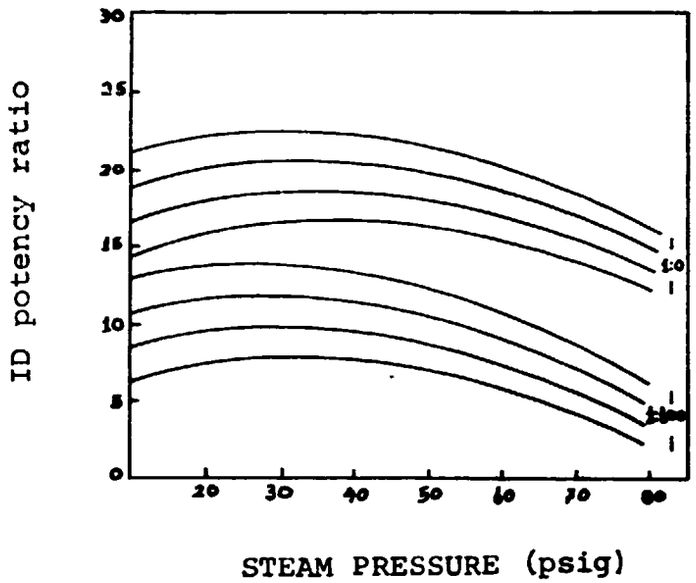


Figure 4: Intra-dermal (ID) at different steam pressure (40).

Table 6

Variables considered in an allergen inactivation process with ammonium hydroxide (42).

Exp. No.	Process Variables			
	Process temperature, °C	Process time, hr	Liquid-solids ratio	NH_3H_2O Molarity
1	100	1.5	0.1:0.2	1.6
2	80, 100, 120	1.5, 4.5	0.15:0.25	6
3	60, 70, 80	1.5	0.075:0.15	2, 4, 6
4	60, 80	1.5, 4.5	1.0:2.0	6
5	20, 50, 80	.75, 1.5	0.25:1.0	6, 10

Analysis of variance was calculated for experiments 1 to 4 (Table 5) and potencies were calculated for the significant effects. However most of the potencies did not show the significance exhibited in the analysis of variance.

Analysis of variance calculated for experiment 5 (Table 7) showed significance for the following process variables and interactions: temperature, liquid-solid x temperature, and time x temperature. However, examination of the potencies showed the effect of these interactions to be negligible compared to the large temperature effect.

The results of biological assays with guinea pigs (Table 5) using two types of meals obtained from experiment 5 (Table 7) showed that: a) at 80°C, there was antigenic response at a 1:100 dilution; b) but, at 20° and 50°C, there were antigenic responses with dilutions of up to 1:1000.

Feeding trials with poultry and cattle were also carried out using deactivated meal obtained from treatment with a 6M NH_4OH solution at 80°C during 45 min, liquid-solid ratio 1:4. However, the author did not comment on the results of this experiment. Comparison was also made with the steam treatment (40), showing good agreement in so far as the extent of antigenic destruction.

Perrone et al. (19), in 1966, in the paper entitled "Contribution to the Study of Castorseed" after a bibliographical review of the methods for deallergenation of the cake, selected 5 for further studies:

Table 7

Biological response (spot diameter in mm) for Experiment 5

Temperature, °C	Liquid-solids Ratio	Dose[a]	6M NH_4OH		10M NH_4OH	
			Time[b], 1.5 h	Time, 0.75 h	Time 1.5 h	Time, 0.75 h
20	1.4	1:0	18.2	14.8	13.1	18.6
			14.5	15.8	16.3	16.7
		1:100	15.6	12.0	10.5	15.7
			14.0	15.7	13.2	15.8
		1:1000	12.6	7.4	8.7	4.5
			10.2	4.7	5.4	5.5
	1.1	1:0	21.0	16.3	22.2	21.4
			20.5	15.9	18.5	22.2
		1:100	17.6	8.5	18.0	14.5
			16.6	10.0	9.2	12.6
		1:1000	10.0	9.4	9.3	7.5
			5.9	8.3	9.0	5.9
50	1.4	1.0	20.8	18.5	14.8	19.5
			17.9	20.6	15.8	24.7
		1:100	15.8	15.6	13.4	12.0
			15.5	16.0	13.3	11.5
		1:1000	5.2	11.3	6.5	6.3
			10.4	8.5	3.7	6.1
	1:1	1.0	14.7	16.3	19.2	19.8
			17.5	18.7	16.0	19.3
		1:100	4.5	15.7	8.0	9.1
			10.3	13.0	11.8	7.5
		1:1000	1.5	10.2	8.6	7.4
			0.0	7.7	7.0	8.8
80	1:4	1:0	14.0	7.2	10.5	14.9
			12.5	8.0	14.9	13.7
		1:100	0.0	1.0	2.0	0.0
			0.0	0.6	2.4	0.0
		1:1000	0.0	0.0	0.0	0.0
			0.0	0.0	0.0	0.0
	1:1	1:0	14.4	11.5	18.5	9.9
			14.0	11.0	16.2	13.9
		1:100	3.3	0.0	2.0	0.0
			0.0	0.0	0.6	0.0
		1.1000	0.0	0.0	0.0	0.0
			0.0	0.0	0.0	0.0

[a]Dose. Extract dilution in terms of castor meal 1.0 = 2500 µg, 1.100 = 25 µg, 1.1000 = 2.5 µg.

[b]Heating time

1) Cooking with 1% NaOH, at reflux temperature for 1 hour;

2) Cooking with 2% NaOH, at reflux temperature for 1 hour;

3) Cooking with 0,9% HCl and 3% HCHO at 80°C in a closed container for 2 min;

4) Autoclaving with steam at 125°C for 15 min;

5) Autoclaving with steam at 125°C for 30 min.

The following types of samples were used for the study:

a) Laboratory cake (ground seeds defatted with sulfuric ether Soxhlet equipment)

b) Untreated ground seeds

c) Commercial cake

Determination of allergens in the treated samples was carried out through the immunochemi. method using a gel semiquantitative procedure (46). For the preparation of the necessary antiserum for this method, CB-1A was used, obtained according to the method of Spies and Coulson (1).

According to the data on Table 8, only the treatment with HCl and HCHO completely eliminated the allergens. The autoclave treatments for 15 and 30 minutes were not so efficient, although the allergen content of the latter was reduced 1000 times. Treatment with NaOH reduced the active allergen content only about 150 times.

Determination of available lysine by Carpenter and Ellinger method, indicated that all treatments investigated produced relatively small losses in this amino acid and that lysine destruction did not appear to be a critical consideration.

By determining soluble nitrogen in the treated samples it was also shown that treatments with NaOH resulted in the smallest reduction of the nitrogen fraction which could be extracted by the various solvents used, i.e., distilled water, 70% ethyl alcohol and 0,1M borate, 9.3 pH.

Table 8. Deallergenation of castor bean products with different treatments (19).

Treatment	Sample	Allergen (%)
Control	Laboratory cake	8 - 16
Control	Commercial cake (a)	8 - 16
Control	Protein Lex (b)	0.0078 - 0.0156
1. NaOH 1%, reflux temperature, 1 hour	Defatted ground seed	0.03125 - 0.0625
2. NaOH 2%, reflux temperature, 2 hours	Defatted ground seed	0.03125 - 0.0625
3. 0.9% HCl and 3% HCHO, 80°C 2 min	Laboratory cake	absent
4. Autoclave 125°C, 15 min	Laboratory cake	0.0078 - 0.0156
5. Autoclave 125°C, 30 min	Laboratory cake	< 0.0078
	Commercial cake (c)	< 0.0078

(a), (c) - supplied by Companhia Brasileira de Óleos (Rio de Janeiro - Brazil)

(b) - commercial product produced by SANBRA (Brazil)

5. EXPERIMENTS ON DEALLERGENATION PROCEDURES

In our laboratory the following studies were carried out with CB-1A fraction of castor bean.

5.1. Treatment with sodium hydroxide

a) Room temperature

50 mg samples of CB-1A isolated using ethanolic extraction of decorticated seeds, were dissolved in 5 ml solutions of sodium hydroxide (0.1, 0.2, 0.5 and 1.0 M) These solutions were kept at room temperature (25°C) for different periods of time (0.5, 1.0, 2.0, 6.0 hours), and then they were neutralized with diluted hydrochloric acid and diluted with distilled water (if necessary) until reaching a concentration of 5,0 mg of sample/ml of solution.

The allergenic activities of the treated samples were evaluated by the double immunodiffusion test in agar and they were compared to the activity shown by a nontreated CB-1A sample of the same origin. The estimation of the CB-1A content in each sample is shown on Table 9.

Table 9 - CB-1A content (%) estimated by the double immunodiffusion test in agar, in samples treated with sodium hydroxide at 25°C.

Time (h)	NaOH (M) 0.1	0.2	0.5	1.0
0.5	100	100	100	20-100
1.0	100	100	100	20-100
2.0	100	100	4-20	4-20
6.0	100	4-20	0.8-4.0	0.8-4.0

b) Temperature of 50°C

50 mg samples of CB-1A dissolved in 5 ml sodium hydroxide (0.01, 0.05, 0.2 and 1.0 M) were put in glass ampoules, sealed and heated in a thermostatic water bath at 50°C for different periods of time (0.5, 1.0, 2.0, 6.0 and 12.0 hours). The samples were neutralized with diluted hydrochloric acid and then diluted with distilled water until a concentration of 5.0 mg of sample/ml of solution.

The CB-1A remaining in the samples (Table 10) was estimated comparing the results of the double immunodiffusion test in agar of the treated samples with that of the untreated sample.

Table 10- CB-1A (%) estimated by the double immunodiffusion test in agar, present in the sample treated with sodium hydroxide at 50°C.

Time (h)	NaOH (M) 0.01	0.05	0.2	1.0
0.5	100	20-100	4-20	4-20
1.0	100	20-100	4-20	4-20
2.0	100	4-20	4-20	0.8-4.0
6.0	100	4-20	4-20	≅ 0
12.0	100	4-20	0.8-4.0	≅ 0

C. Temperature of 75°C

The same procedure as for the treatment at 50°C was used, obtaining a content of CB-1A still present in the samples (Table 11) by the double immunodiffusion test in agar.

Table 11- CB-1A content (%), estimated by the double immunodiffusion test in agar, present in samples treated with sodium hydroxide at 75°C.

Time (h)	NaOH (M) 0.01	0.05	0.2	1.0
0.5	100	100	0.8-4.0	0.8-4.0
1.0	100	100	0.8-4.0	0.8-4.0
2.0	100	20-100	0.8-4.0	0.8-4.0
6.0	100	4-20	0.8-4.0	0.8-4.0
12.0	100	4-20	0.8-4.0	0.8-4.0

5.2. Treatment with calcium hydroxide

a) Temperature of 75°C

50 mg samples of CB-1A isolated from the ethanolic extraction from decorticated seeds, dissolved in 5 ml of $Ca(OH)_2$ solution (0.01 M and saturated solution at 25°C), were put in glass ampoules, sealed and heated in a thermostatic water bath at 75°C for different periods of time (0.5, 1.0, 2.0, 6.0, 12.0 hours). At the end of the treatment the ampoules were cooled to room temperature and their contents neutralized with 0.1M oxalic

acid and diluted with distilled water to a concentration of 5.0mg of sample/ml of solution (the aliquots for the double immunodiffusion test in agar were previously filtered or decanted).

While no reduction in the allergenic activity was observed for the treatment at room temperature, the samples of CB-1A treated at 75°C showed a reduction in the allergenic content when it was exposed to a solution of $Ca(OH)_2$ during a long period of time (Table 12).

Table 12 - CB-1A content (%), estimated by the double immunodiffusion test in agar present in samples treated with calcium hydroxide at 75°C.

Time (h)	$Ca(OH)_2$ (M) 0.01	- 0.017 (sat. sol.)
0.5	20-100	20-100
1.0	20-100	20-100
2.0	20-100	20-100
6.0	20-100	0.8-4.0
12.0	20-100	0.8-4.0

5.3. Treatment with sodium duodecyl sulfate (SDS)

50 mg samples of CB-1A, isolated from the ethanol extraction of the decorticated seeds were dissolved in 5 ml of a 0.1% and 0.01% solution of sodium duodecyl sulfate (SDS) and kept at room temperature and at 50°C for 0.5; 1; 2; 6; and, 12 hours. These solutions were then neutralized with diluted hydrochloric acid and diluted with distilled water (if necessary) until a concentration of 5.0 mg of sample/ml of solution was reached.

The allergenic activity of the treated samples was estimated by the double immunodiffusion test in agar and compared with the activity of nontreated samples of CB-1A of the same origin. They showed no redution in allergenic activity even at the most severe treatment.

5.4. Treatment with bacterial protease

20 mg samples of CB-1A, isolated from the ethanol extraction of decorticated seeds were dissolved in 2 ml potassium phosphate buffer solution pH 6.7. The bacterial protease was dissolved to reach a 0.1% solution. These solutions were kept at 50°C for varying periods of time (0.5; 1; 2; 6; 12; 24 hours). Then, the reaction was interrupted by keeping the samples in an oil bath at 100°C for 5 minutes.

The allergenic activity of the treated samples was estimated by the double immunodiffusion test in agar and compared with the activity of nontreated CB-1A samples (Table 13).

Table 13 - CB-1A content (%), estimated by the double immunodiffusion test in agar, in the samples treated with a 0.1% solution of bacterial "protease" in phosphate buffer (pH 6.7) at 50°C.

Time (h)	0.5	1.0	2.0	6.0	12.0	24.0
Content (%) CB-1A	100	100	100	100	20-100	4-20

5.5. Treatment with commercial sodium hypochlorite ("household bleach")

a) Room Temperature

Three 20 mg samples of CB-1A isolated from the ethanol extraction of decorticated seeds were dissolved with 2 ml of sodium hypochlorite solution, containing a percentage in weight of active chlorine of 2.61; 1.3; 0.52, respectively. The active chlorine was determined according to the methods of Kolthoff, et al. (51).

After variable intervals of time (0.5; 1; 2; 6; 12; 24 24 hours) at room temperature potassium iodide was added (corresponding to twice the equivalent-gram of ClO^-) and extraction with CCl_4 was carried out. Distilled water was added to obtain a solution with a concentration of approximately 5 mg/ml.

The CB-1A content of the samples was estimated by the double immunodiffusion test in agar and compared with a nontreated CB-1A sample (Table 14).

Table 14 - CB-1A content (%) estimated by the double immunodiffusion test in agar, in a sample of CB-1A treated with a commercial solution of sodium hypochlorite at 25°C.

Time (h)	NaClO (% by weight)		
	0.54	1.35	2.71
0.5	4-20	0.8-4.0	≅ 0
1.0	4-20	0.8-4.0	≅ 0
2.0	100	0.8-4.0	≅ 0
6.0	20-100	0.8-4.0	≅ 0
12.0	4-20	0.8-4.0	≅ 0
24.0	100	0.8-4.0	≅ 0

b) Temperature of 50°C

The procedure was identical to the treatment at room temperature, except that the samples were dissolved in solutions of 2.71% and 1.35%, and were sealed in glass ampoules. They were heated in a water bath at 50°C for the same interval of time.

The CB-1A content was estimated by the double immunodiffusion test in agar (Table 15).

Table 15 CB-1A content (%) estimated by the double immunodiffusion test in agar, in a sample treated with a commercial solution of sodium hypochlorite at 50°C.

Time (h)	NaClO (% by weight) 1.35	2.71
0.5	0.8-4.0	≅ 0
1.0	0.8-4.0	≅ 0
2.0	0.8-4.0	≅ 0
6.0	0.8-4.0	≅ 0
12.0	0.8-4.0	≅ 0
24.0	0.8-4.0	≅ 0

5.6. Treatment with sodium hypochlorite P.A.

a) Room Temperature

Two 20 mg samples of CB-1A, isolated from the ethanol extraction of whole seeds were dissolved in a 10,7% and 1,78% solution by weight of active chlorine of sodium hypochlorite at room temperature. After variable intervals of time (0,5; 1; 2; 6; 12; 24 hours), potassium iodide was added (corresponding to twice the equivalent -gram of the ClO^-) and extraction with CCl_4 was carried out. Distilled water was added in order to obtain a solution of approximately 5 mg/ml.

The CB-1A content of samples was estimated by double immunodiffusion test in agar and compared with a nontreated CB-1A sample (Table 16).

Table 16 - CB-1A content (%) estimated by the double immunodiffusion test in agar, in the sample treated with a sodium hypochlorite solution at 25°C.

Time (h)	NaClO (% by weight) 1.78	2.71	10.7
0.5	0.8-4.0	≅ 0	≅ 0
1.0	0.8-4.0	≅ 0	≅ 0
2.0	0.8-4.0	≅ 0	≅ 0
6.0	0.8-4.0	≅ 0	≅ 0
12.0	0.8-4.0	≅ 0	≅ 0
24.0	0.8-4.0	≅ 0	≅ 0

b) Temperature of 50°C

The procedure was the same as the treatment at room temperature. In this experiment the samples were placed in sealed glass ampoules and heated in a water bath at 50°C for the same period of time.

The CB-1A content was estimated by the double immunodiffusion test in agar (Table 17).

Table 17 - CB-1A content (%), estimated by the double immunodiffusion, test in agar, in samples treated with a sodium hypochlorite solution at 50°C.

Time (h)	NaClO (% by weight)		
	1.78	12.71	10.7
0.5	≃ 0	≃ 0	≃ 0
1.0	0.032-0.16	≃ 0	≃ 0
2.0	≃ 0	≃ 0	≃ 0
6.0	0.032-0.16	≃ 0	≃ 0
12.0	0.032-0.16	≃ 0	≃ 0
24.0	0.032-0.16	≃ 0	≃ 0

5.7. Discussion of the Results

The treatment of CB-1A samples with hydroxide solutions resulted in a reduction of the concentration of allergens (CB-1A), even when it was held at room temperature (Table 9).

Treatment during 6.0 hours with a 0.5 M or 1.0 M solution of sodium hydroxide was responsible for a 96.0 - 99.2% reduction of the concentration of CB-1A. This range of concentration has no application for the treatment of the meal, but it could be used in the treatment of effluents from the dessolventization or sterilization (autoclave) process of the meal.

The preliminary observations made during the steam flow treatment of the industrial meal, suggested that the presence of particulated material in the steam from the auto - clave and/or solvent elimination process could be responsible for the allergenic manifestation at the industrial site. In this case, the passage of these effluents through washing flasks containing sodium hydroxide in an adequate concentration ($\geq$ 2%) could be an efficient way of eliminating the allergenic material, as it permits a continuous treatment of its effluents.

The use of higher temperatures increased the eficiency of the treatment (Table 10 and 11) when compared to the treatment at 25°C (Table 9), besides the need of less concentrated solutions. A minimum reduction of 96% of the CB-1A concentration can be reached when a 0.2 M solution of sodium hydroxide at 75°C was used, with times of exposure of 30 minutes (Table 11) reaching 99.2% when a 1.0 M solution of sodium hydroxide was used.

The treatment with calcium hydroxide was effective. It was possible to evidence a minimum reduction of 96% of the concentration of CB-1A (Table 12) when a saturated solution of calcium hydroxide was used (0.017 M at 25°C) at 75°C, for a period of time greater then 6 hours. This result was significant when one compared it with a reduction of 96% when using sodium hydroxide 0.05 M at 75°C (Table 11), for the same period of time. However, the limited solubility of calcium hydroxide in water prevented the use of more concentrated solutions, which did not however discard the possibility of its use as an interesting option in the inactivating process, depending on the acceptable reduction degree of the CB-1A concentration and on the possibility of a more severe heat treatment.

During the inactivation treatment with NaOH and $Ca(OH)_2$ which caused a significant reduction of the CB-1A content and after neutralization of the samples, the discharge of a strong smell of volatile sulfur compounds was noted. As no reference to this occurence was found in the literature, a detailed study about the origin and its consequences on the inactivation processes with alcaline agents seems relevant to us.

While treatment with sodium duodecyl sulfate (SDS) did not inactivate the allergens, the use of bacterial protease in phosphate buffer (pH 6.7) at 50°C showed a partial reduction of the allergenic content of CB-1A after a prolonged treatment (Table 13).

Among the investigated treatments, the use of a hypoclorite solution as an oxydizing agent gave satisfactory results. When household bleach was used, at 2.7% solution (equivalent to 2.6% of active chlorine) a complete inactivation and/or destruction of CB-1A fraction was observed with a short treatment at room temperature (Table 14).

The use of a less concentrated solution of hypochlorite, less than 1,35%, gave only parcial inactivation, even when the treatment was carried out at 50°C (Table 15). The same result was found with corresponding treatments at 25°C (see Table 14).

In spite of the difficulties in reproducing the results of the double immunodiffusion test in agar that accompanied the preliminary treatments with a laboratory prepared NaClO solution (Tables 16 and 17), the results were promissing when compared to those of Gardner et al (34). Gardner et al (34) used a 5.25% solution of NaClO (which corresponds to 50% of active chlorine) at 77°C and obtained 50% reduction of allergenic content in the meal.

6. ROUTINE ANALYTICAL METHOD FOR DETECTION OF CB-1A FRACTION IN CASTOR MEAL

6.1. Materials

a. Equipment and glassware

1. Balance; semi-analytical with minimal reading of 0.1 g is satisfactory.
2. Buchner filtration apparatus with vacuum pump ; diameter of filter = 90 mm.
3. Filter paper, qualitative analysis grade is satisfactory.
4. Mechanical agitation apparatus rod type with stirrer.
5. Water bath; set for $80^{o}C \pm 1^{o}C$.
6. Beakers; 125 ml and 1.000 ml.
7. Graduate cylinder; 5ml.
8. Spatula; inox steel blade is preferred.
9. Dialysis bag; approx. 30 mm diameter.

b. Reagents

1. Polyethylene glycol; mw 20.000
2. Double immunodiffusion slides prepared by

specialized laboratory. Depending on demand it can be made available commercially.

3. Antiserum specific for CB-1A; prepared by specialized laboratory. Depending on demand it can be made available commercially in freeze dried form.

6.2. Procedure

1. Weigh 5g of product (castor meal, or flour , untreated or deallergenated) and place in 50 ml distilled water for 12 hours at room temperature. Shorter period could be used if accuracy is not very critical.

2. Filtrate through paper under vacuum and, resuspend the cake in 50 ml distilled water and agitate for 4 hours at 80°C. Shorter period could be used if accuracy is not very critical.

3. Repeat step 2.

4. Place the liquid portion in a dialysis bag. Make sure that no leakage is observed during dialysis.

5. Prepare 500 ml of 30% (w/v) polyethylene glycol m.w. 20.000, in a 1.000 ml Beaker and place the dialysis bag (52).

6. Wait until the volume of liquid in the bag is completely reduced (approx. 15 hours). Shorter period could be used if final volume is larger than 1 ml.

7. Cut one end of bag and take out the content in graduate cylinder. Wash the bag internally to remove all allergen and complete the volume of collected liquid to 1 ml. Larger volume could be used if high concentration of allergen is expected.

8. The amount of allergen is estimated by double immunodiffusion test on concentrated extract.

9. The antiserum was placed in the central hole and the extract (antigen) in the peripheral holes. One can use several dilutions to evaluate the approximate amount of allergen in the sample.

10. Details on double immunodiffusion tests are shown in Annex 1.

11. A negative result in 1 ml extract indicates that the allergen content in the product has 0.3 parts per million (ppm) or less.

7. CONCLUSIONS

The work carried out by this Foundation (Project nº US/GLO/77/033) during the last 18 months has shown some interesting results which enable us to conclude the following points:

1. Castorseed toxins (ricine and ricinine) can be inactivated by a technically and economically feasible treatment. Among various procedures the steam treatment under pressure has shown to be more effective and applicable. A treatment at 121°C for 10 to 15 minutes was sufficient to inactivate those toxins.

2. For industrial control of the intensity of this heat treatment, a rapid and semiquantitative method was developed which can be carried out in a modest industrial laboratory by a technician with no special skill.

3. The allergenic fraction CB-1A found in castor seed is a natural product existing in the seed itself and does not have a volatile nature. Therefore, the allergenic problems that occur near castoroil processing industries are due to the presence of castormeal or seed residues in the air.

4. The electrophoretic fractionation of CB-1A showed three distinct major fractions and three other fractions which appeared

only in small quantities. Further studies on these fractions are being carried out.

5. The experiments on deallergenation procedures comparing different parameters such as concentration, temperature, and period of treatment indicated that the following three treatments were more promissing (see also table 18):

a. Sodium hydroxide (caustic soda) - The concentration required for complete inactivation of allergens makes it difficult for industrial application on the treatment of the meal and flour. However, it can be successfully used in the treatment of effluents from the desolventization and autoclaving process of the meal. The device should have a recirculation system to make it economical.

b. Calcium hydroxide - Its limited solubility in water prevents the use of concentrated solutions. However simultaneous heat treatment makes it adequate for effluent deallergenation.

c. Sodium hypochlorite - This treatment gave satisfactory results at room temperature and relatively low concentration. Complete inactivation was achieved with a 2.7% solution in 30 minutes at room temperature.

6. A simple and quick laboratory procedure for the evaluation of residual allergens in flour and meal was developed by our

team, based on the double immuno-diffusion technique. A concentration as little as 0.3 ppm of allergens can be detected by this procedure. The method is also applicable to a purified allergen solution.

7. At this stage of development of this project, it is possible to design a detoxification plant for castorseed meal or flour. The cost of the equipment and operation seems to be less than the benefit which one can obtain by increasing the commercial value of the product. The detoxicated meal can be used as animal feed, especially for polygastric animals, such as cattle.

8. The deallergenation procedures which are economically and technically feasible are limited to the control of the environment and effluent. The device should be developed with a view of controlling the pollution of the factory environment and its vicinities.

9. Dealiergenation procedures should be investigated on castorseed meal and flour in order do reduce, up to 90 to 99%, the allergens of these products. This makes it safer for the product handlers and farmers to use them as animal feed or as fertilizer.

Table 18: Comparison of the different deallergenation treatments, choosing the greatest reduction of CB-1A obtained in the shortest time of exposure, smallest concentration of deallergenating agent and last, lowest process temperature*.

Treatment	Concentration	Temperature (°C)	Time (hr)	Reduction CB-1A content (%)
NaOH	0.2 M	75	0.5	96-99.2
$Ca(OH)_2$	0.017 M (saturation)		6	96-99.2
	0.01 M	75	0.5	0-80
SDS	Severe Treatment			No Reduction
Bacterial Protease	0.1%	50	24	80-96
(Commercial Bleach)	2.71%			
Sodium	(by weight)	25	0.5	100
Hypochlorite	1.35%		0.5	96-99.2
Sodium hypochlorite	2.71%	25	0.5	100
P.A.	1.78	50	0.5	100

* Limitation in temperature was considered less important because, in order to detoxify the meal, it has to undergo a heat treatment.

8. RECOMMENDATIONS

There are two major problems concerning the development of the castor oil producting industry:

(1) the low commercial value of castormeal due to its toxicity; and,
(2) environmental problems caused by allergenic particles (dust) in the production area and its vicinities.

The low commercial value of castorbean meal is the main reason for the existing limitations regarding the development of castorbean oil producing industries in Brazil. Castorbean meal represents more then 50% in weight of the raw-material and has a high protein content (35-40%) of good quality. Yet, it is only commercialyzed as fertilizer due to its toxicity and allergenic factor. Its value as fertilizer is low, when compared with chemical products. Therefore in order to compete with these fertilizers its market price is low, being much lower than that of other oleaginous meals.

The protein from castormeal is deficient in essential aminoacids methionine, lysine, and tryptophan. These deficiencies preclude the use of castor meal as the main source of protein for pigs and poultry, but the meal would be suitable as a concentrate for cattle.

Therefore we recommend that:

(1) the feed trial test of detoxified meal should be carried out on cattle;

(2) the feed trial test of detoxified meal should be carried out as a partial protein suplement on pigs; and

(3) the design of detoxification equipment should be developed in order to evaluate its economical feasibility. The feed trial test of detoxified castorseed meal would be compared with other oilseed meal, namely cotton seed and soybean. This test is suitable at this stage of the project in order to evaluate the comparative value of the products.

The allergens are extremely serious to the factory environment and its vicinities, especially if it is located in an urban area. Then the control of effluents is one of main concern. We therefore recommend:

(1) to design a pollution removal device through inactivation and/or removal of the allergen particle in the air, coming out of the pollution source; and,

(2) the development of methods for partial inactivation of allergens on castorbean meal in order to safeguard the health of the product handler and farmers. Any method that makes use of heat applied in the toxin inactivation is the most promissing, since we can obtain a double effect with a single step.

9. ANNEX 1

Double Immunodiffusion Test in Agar (OUCHTERLONY)

1. Type of test: it is a sorological reaction of precipitation, "in vitro", where the antigen (s) reacts with its specific antibody (a) (antiserum in the middle of a gel (agar-agarose) forming one (or more) precipitation line (p), visible to the naked eye.

2. General aim of the test: detect the presence of an antigen (s) in a distinct sample with an antiserum specific to this antigen.

3. Aim of the test in the present work: detect the presence of the allergenic antigen (s) CB-1A in the industrial castorseed cake or meal, extractable with distilled water at pH 6.5-7.0, using a specific antiserum for CB-1A from an immune rabbit (CB-Pa).

4. Technique

4.1. Preparation of the Antigen: 10.0 g of industrial castor-meal or cake are mixed with 100.0 ml of destilled water and continuously agitate during 1 hour; filter in a common paper filter and add sufficient NaCl P.A. to form an aqueous extract with 0,85% of NaCl. The saline aqueous extract is then frozen until the moment of the test.

4.2. Preparation of the gel in the glass slide:

a) Preparation of the gel (stock) mix agar or agarose with distilled water (1,5%); dissolve in a boiling water bath, allow to solidify, cut in cubes of approximately 0.5 x 1.0 cm, add enough distilled water to cover them completely and leave to rest; change the distilled water after 5-7 days (repeat this procedure during approximately 30 days).

b) Preparation of the slide: melt a sufficient quantity of cubes in a boiling water bath and add 2.5 ml of melted agar in a slide of 26 x 75 mm; leave it to solidify in Petri plates with covers containing humidified cotton wool; perforate the slide with a special device, as in figure 5:

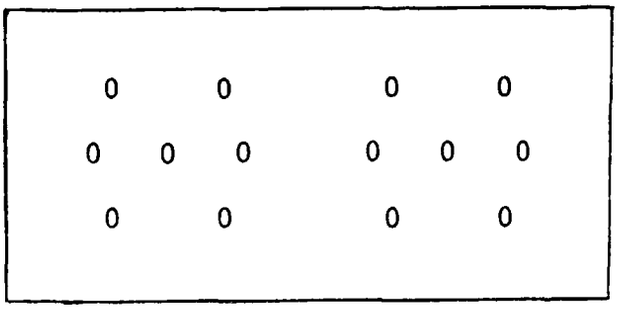


Figure 5: Sample of double immunodiffusion slides.

4.3. Procedure in the slide: fill in the central hole with antiserum and the peripherical holes with a aqueous salin extract of the castor meal or cake, in the convenient dilutions (Example - Figure 6).

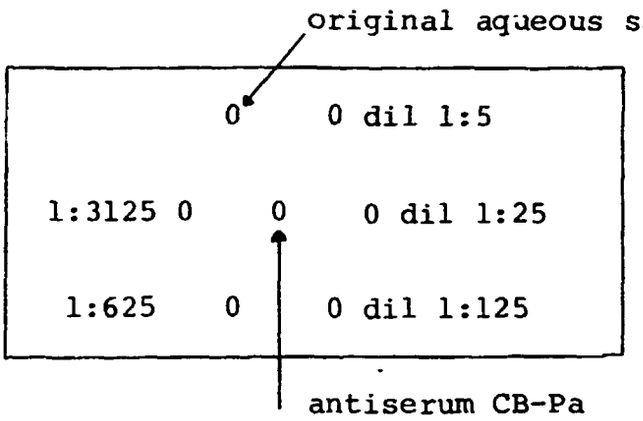


Figure 6: Example of dilution

5. Test interpretion: the presence of the allergenic antigen (s) CB-1A in the aqueous extract obtained from the industrial castorcake or meal will be revealed by the formation of one or more precipitation lines normally between the central hole and the peripherical, indicating that a reaction between the antigen (in the sample) and its antibody (in the antiserum) took place. The absence of precipitation lines can generally be interpreted as absence of the allergenic antigen (s) CB-1A. But, sometimes it is still present but not in sufficient quantity to be revealed in the test.

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