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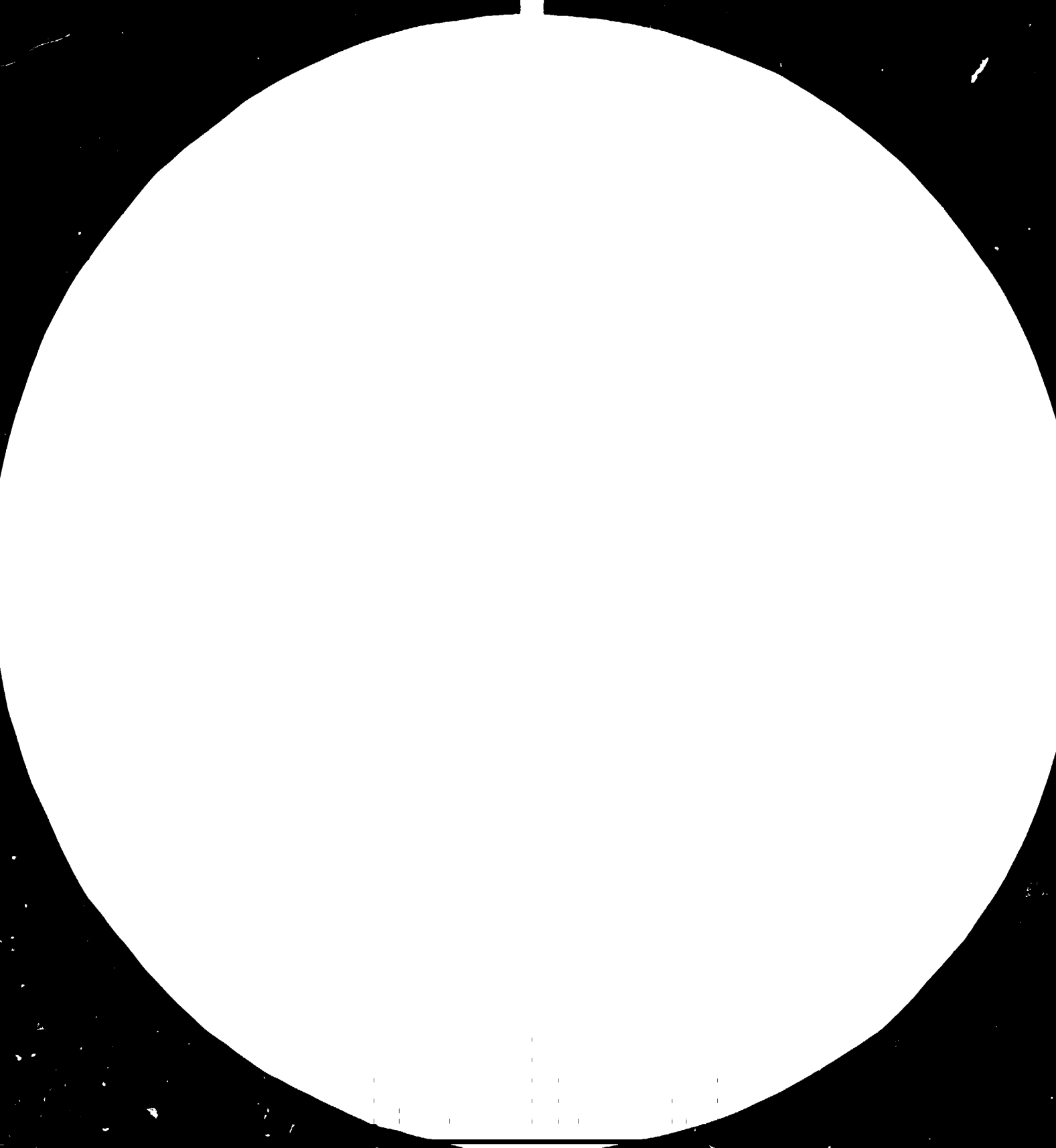

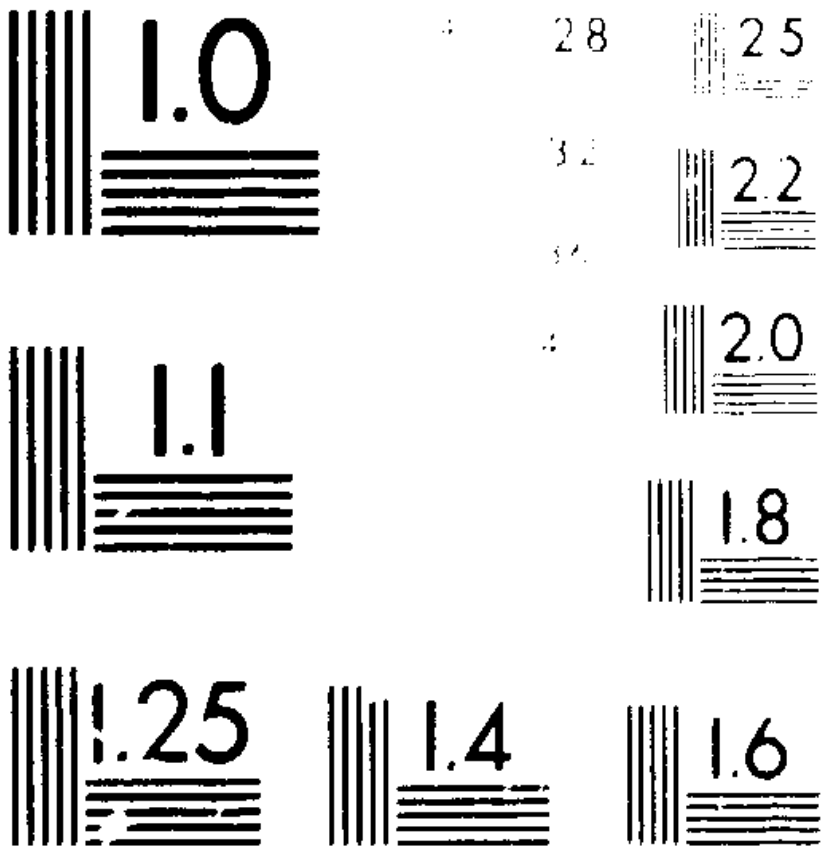

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Final Report

on

The Development of A Castor meal Detoxification and Deallergenation Technology Phase Two: The Definition and Specifications of A Suitable Technology for Application in Industry

UNIDO Contract No. 83/37
UNIDO Project No. US/GLO/77/033
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FOREWORD

This contract research was officially initiated on May 17, 1983; however, due to unforeseen difficulties in purchasing castor seeds, the actual research began early in September 1983. All attempts to import the needed quantities of the seeds (about 5 tons) by the Northrup King Seed Company, one of the largest seed companies in the United States, and by Alnor Oil Company, a castor seed and oil importer/exporter in New York, who had previously promised the delivery of 5 tons of castor seeds by the end of June 1983, failed.

Fortunately, however, the United States Department of Agriculture allowed us to purchase one hundred pounds of one-year-old seeds from its seed stocks in Yuma, Arizona, toward the end of August. This enabled us to begin laboratory-scale experiments in early September. In early February, 1984, we were able to obtain three tons of freshly harvested castor seeds from many small Texas farmers, with the assistance of the U.S. Department of Agriculture and Northrup King Seed Company. This was followed by an additional two-ton purchase in June, 1984, to complete all planned experiments.

At one time or another, the following individuals were involved in various aspects of the research for its successful completion.

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I. INTRODUCTION

The castor seed processing industry has a serious disadvantage, compared to other oilseed processing industries, because castormeal has a very low economic value. Although rich in protein, castormeal at best can only be used as a fertilizer, far below its potential value, because of its toxic properties.

Generally, two major products, oil and meal, are produced when oilseeds are processed. Economics of most oilseed processing plants, therefore, are based on sales of two valuable major products. This is not the case for castor seed processing operations, which produce castor oil as the only income-generating product. By necessity, the toxic meal has to be considered a low-value by-product or, very often, a waste. This fact affects industrial castor seed processing economics very unfavorably. It has caused stagnation and even decline of world-wide production of castor oil, as well as loss of castor meal, a potentially valuable product, for the producing country's national economy. Reclamation of castormeal for potential use as an animal feed component is an important world-wide challenge which needs to be met.

The castor seed industry as a whole (processors, traders and user alike) is deeply concerned about risks of serious

allergic reactions caused by the very potent allergen, CB-1A, which occurs naturally in castormeal. This concern is naturally shared by public health officials of all countries engaged in castor seed processing operations and castormeal handling. Means must be found to eliminate health risks and make castormeal a product safe for world-wide utilization as an animal feed component.

The only way to effectively achieve this goal is through development and application of a practical castor seed detoxification and deallergenation technology. A preliminary feasibility study was conducted under sponsorship of the United Nationals Industrial Development Organization (UNIDO) during 1981-1982. UNIDO then wished to undertake a comprehensive development project, based on results obtained from the feasibility study, to make a practical detoxification and deallergenation technology available for industrial application. A low-cost, technologically-sound detoxification and deallergenation method, when appropriately applied, will substantially increase the commercial value of the castormeal by permitting it to be traded as a protein feed ingredient instead of only a fertilizer.

II. OBJECTIVES

The ultimate goal of the project was to develop a practical, low-cost, technologically-sound detoxification and deallergenation technology which can be used for industrial production of castormeal safe for use as an animal feed component.

The following specific objectives were outlined in the contract:

1. To reproduce the results obtained in the preceding laboratory scale experiments with regard to removal and/or destruction of ricin and ricinin by using appropriate pilot-scale equipment (steaming at 120°C for three hours).
2. To make use of the toxicity test method, as described in the laboratory research report dated June 1982, for measuring detoxification results, and to comment on its practical usefulness.
3. Having in mind its practical application in the castor seed processing (pressing and solvent extraction) industry, to modify the detoxification method to define the minimum effective temperature and reaction time, and also to meet the techno-economic requirements of industrial operations (pressure heating). In this context, use of pilot scale equipment was considered essential.
4. Upon having defined the optimum detoxification (removal of ricin and ricinin) technology, to measure and determine the residual CB-1A (allergen) content

of the detoxified cake or meal by making use of the analytical test method described in the laboratory research report dated December 1982. Also, to use this control method in all other relevant activities and to comment on its usefulness.

5. Based on the results obtained in preceding research work (laboratory report dated December 1982), to carry out further laboratory scale and essential pilot plant scale experiments to detoxify castor seed press cake or meal by using practical, acceptable agents such as calcium hydroxide, ammonium hydroxide, and ammonia gas.

6. To discuss and evaluate the results obtained, and, based on appropriate conclusions derived therefrom, to define the optimum detoxification and deallergenation methods and technologies in view of obtaining castor seed cake or meal product that is free from ricin and ricinin and ready for use as an animal feed component, and which does not contain any harmful allergenic (CB-1A) substances.

7. By relevant pilot scale operations, to verify effectiveness of the detoxification/deallergenation method recommended under objective 6, and to support its technical efficiency by relevant figures and data.

8. To outline the optimum combined castor seed cake or meal detoxification and deallergenation technology which can be made effective in operational castor

seed processing factories, either by modifications of existing equipment, or in the form of an additional compact equipment unit to be added.

9. To prepare relevant technical documentation on the basis of which the technical modifications can be conducted, and/or relevant equipment can be constructed ready for installation and demonstration, in connection with a castor seed processing factory in a developing country yet to be selected.

III. SCOPE OF WORK

The scope of this project was limited to the nine specific objectives stated in the "Objectives" above. Animal feeding tests for toxicological evaluation of products specifically were not included in the project. The degree of detoxification and deallergenation was evaluated by only the immunochemical and chemical methods specified. UNIDO is therefore advised to seek assistance from competent animal nutritionists and toxicologists to accurately assess the nutritional and toxicological properties of products before making its final recommendations to prospective users.

Also, this project is not intended to obtain detailed information necessary for designing a production plant. Although much of the information generated from this project can be used for such purposes, additional information may be needed to ensure proper design and operation of an economically viable full-scale production plant.

IV. EXPERIMENTAL

IV.1. Raw Material and Chemicals

IV.1.1. Raw Material

The first 100 pounds of one year-old castor seeds were purchased from the U.S. Department of Agriculture Seed Stocks in Yuma, Arizona, toward the end of August 1983. These seeds then were used for all preliminary laboratory-scale experiments. In February 1984, three tons of fresh seed were purchased from various farmers in Texas, then an additional two tons in June from the same sources. All pilot plant-scale experiments were carried out using these composite castor seeds.

IV.1.2. Chemicals

All chemicals used in the experiments, except hexane and ethyl alcohol, were of reagent grade. Hexane and ethyl alcohol were of technical grade, since they currently are used in the industry for extraction of oils from various oilseeds.

IV.2. Experimental Procedures

To accomplish the stated goals and objectives, this research was divided into three sections: re-evaluation of the detoxification and deallergenation processes and the analytical methods developed in Phase One of the study; evaluation of various operational steps involved in the conventional pre-pressing/solvent extraction processes to determine their effectiveness in detoxification and deallergenation of castor seeds; and evaluation of the use of extruders, in conjunction with selected chemicals, to simultaneously detoxify and deallergenate castor meals.

IV.2.1. Re-evaluation of the Phase One Study Results

IV.2.1.1. CB-1A Allergenecity Testing Methods

IV.2.1.1.1. Preparation of CB-1A

IV.2.1.1.1.1. Preparation of Defatted Castormeal

Defatted castormeal was prepared for use in preparation of purified CB-1A. Care was taken to minimize the exposure of all products to extreme temperatures, in order to denature the allergens as little as possible.

Fifty pounds of whole castor seed were cracked using a cracking roll. Hulls then were separated from kernels by air aspiration. The dehulled kernels were flaked to a average thickness of approximately 0.012 inch, using a flaking roll. The flakes were extracted, using 99% ethyl alcohol, at room temperature for five hours with occasional agitation. The flake-to-alcohol ratio was 1:30 (w/v). The extraction was repeated five times, with fresh ethyl alcohol each time, to ensure complete removal of oil. After each extraction, the alcohol was separated from the meal by filtering the slurry through cheesecloth (usually four-layer thickness). The meal was air-dried at room temperature in a forced-air hood, then stored in a freezer in a tightly sealed glass bottle.

IV.2.1.1.1.2. Separation and Purification of CB-1A

The allergen CB-1A was separated from the defatted castormeal and purified according to the procedure outlined by Spies and Coulson (Spies, J. R. and Coulson, E. J., J. Amer. Oil Chem. Soc. 65: 1720, 1943). Detailed steps of the procedure are shown in Figure 1.

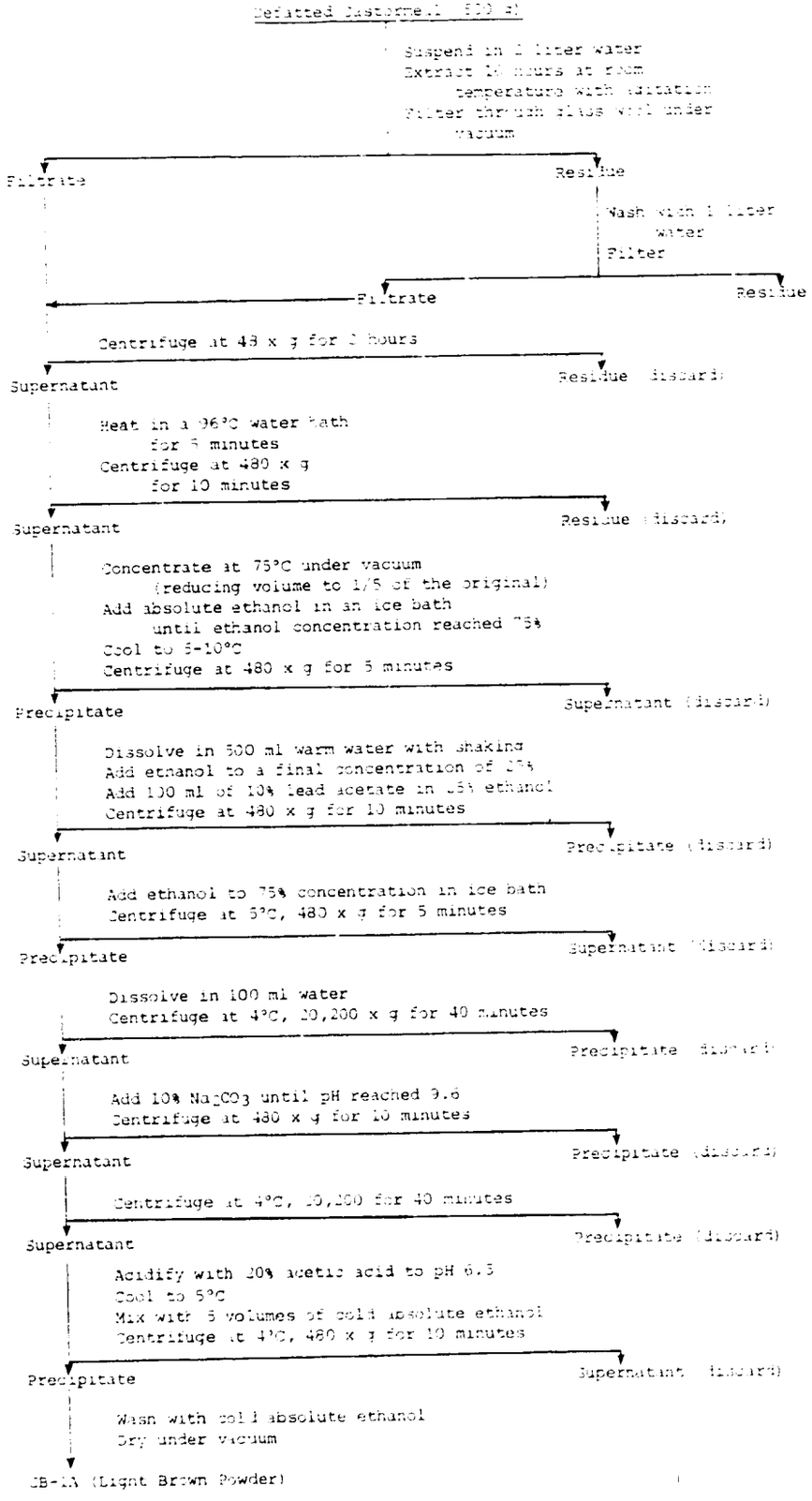


Figure 1. Steps involved in separation and purification of JB-1A.

IV.2.1.1.1.3. Identification of the Purified CB-1A

Disc polyacrylamide gel electrophoresis was performed with 8% gels using a pH 8.3 phosphate buffer as the running buffer, but without sample gels. The sample was loaded on top of the gel, and the electrophoresis carried out at a current of 4 mA/gel. The detailed steps involved in the electrophoresis were described by Davis (Davis, B. J., Ann. N. Y. Acad. Sci. 121: 404, 1964).

IV.2.1.1.1.4. Allergenic Effects of the Purified CB-1A on Rats

An animal test was performed with rats (body weight range of 128-154 g) to determine if the purified CB-1A had any allergenic effects. Three different solutions, i.e., saline solution alone as a control, crude defatted meal extract in saline solution, and saline solution of the purified CB-1A, were injected into the abdomens of different rats. Symptoms developed immediately after the injections were observed. The amount of saline solution injected was approximately 0.5-0.7 ml, containing 10-15 mg protein.

Subcutaneous skin tests also were performed to determine the allergenic effects by applying the purified CB-1A on the skin of rats.

IV.2.1.1.2. Preparation of the CB-1A Anti-serum (Antibody)

The purified CB-1A was dissolved in the physiological saline solution with the antibody-formation stimulator, "Complete Adjuvant". Approximately 10-20 mg CB-1A was injected subcutaneously into each of three rabbits (body weight 4-5 kg) once a week. After three weeks, blood serum was separated from

the blood, and tested for antibody formation using an immunodiffusion technique against the purified CB-1A. The antibody formed was then separated and stored frozen for use in subsequent experiments.

IV.2.1.1.3. Detection of Allergenecity by Immunodiffusion Technique

Allergenic effects of castor seed products were determined by the immunodiffusion technique of Ouchterlony, as summarized in the Final Report of the Phase One study (December 1982).

IV.2.1.2. Ricin Toxicity Testing Methods

IV.2.1.2.1. Hemagglutin Reaction Method

Toxicity of ricin and other toxic substances was determined by the red blood cell agglutination test. The procedure of Gardner et al. (Gardner, H. E., Jr., D'Aquin, E. L., Koltun, S. P., McCourtney, E. J., Vix, H. L. E. and Gastrock, E. A., J. Amer. Oil Chem. Soc. 37: 142, 1960) was slightly modified to prepare red blood cell corpuscle and sample extract and to carry out the hemagglutinin test.

IV.2.1.2.1.1. Preparation of Red Blood Cell Corpuscle

Citrated whole blood was separated into red blood corpuscle and plasma fractions by centrifugation at 800 x g for 5 minutes. The corpuscle then were washed with a physiological saline solution (0.85% sodium chloride in demineralized water) 3 times and the washed corpuscle were diluted to 10 times its volume with saline solution. The steps involved in the

preparation of red blood corpuscle for use in ricin agglutination test are shown in Figure 2.

IV.2.1.2.1.2. Preparation of Castormeal Extract

Castormeal (0.5 g) was suspended in 49.5 g physiological saline solution with vigorous shaking for one hour.

IV.2.1.2.1.3. Hemagglutinin Test

Ten test tubes were placed in a rack and 0.9 ml of saline solution was pipetted into the first tube and 0.5 ml each in subsequent tubes. To the first tube was added 0.1 ml meal extract, and the content mixed using a Vortex mixer. One-half (0.5 ml) of this mixture was transferred to the second tube and the content mixed. The same procedure was repeated until all ten tubes were prepared. Then, 0.5 ml of red blood corpuscle solution was added to each tube and mixed vigorously. The dilutions of meal extraction in the tubes were 1:10, 1:20, 1:40 and so on. The contents of the tubes were centrifuged at 500 x g for 2 minutes, and the appearance and characteristics of the red blood agglutination were observed after shaking the tube gently. The following ratings were then made:

+4: Complete agglutination with no dispersion by gentle shaking.

+3: Complete agglutination with some breaking up by gentle shaking.

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

+1: Agglutination, but easily dispersed to visible agglutinated particles.

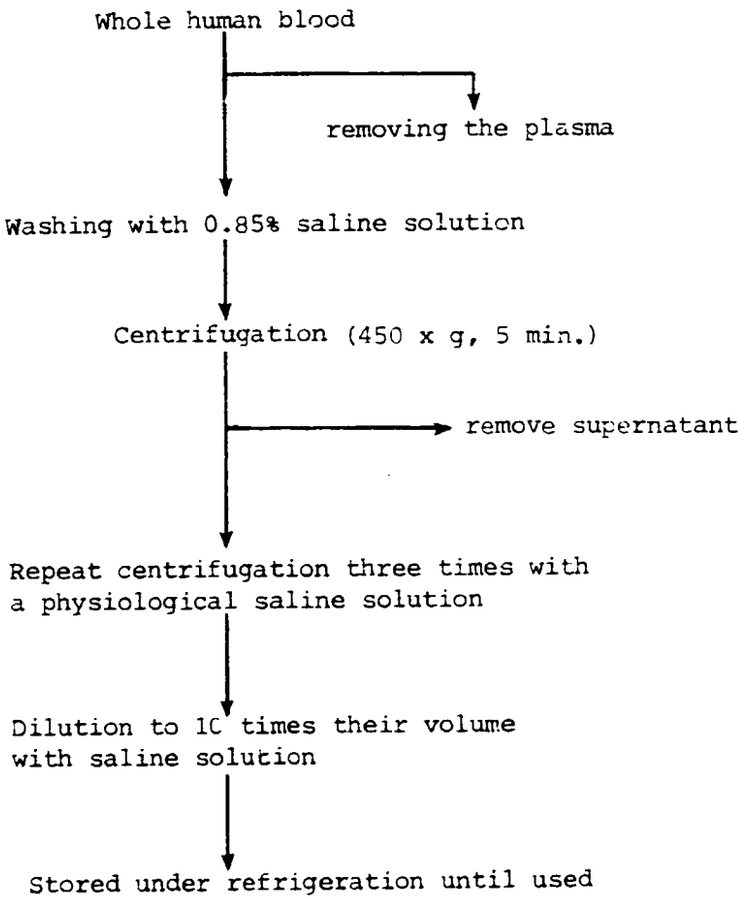


Figure 2. Preparation of red blood cell corpuscle for use in ricin agglutination test.

The highest dilution showing +1 rating was defined as the "titre". The value of titre represents the amount of ricin present in the sample extract. The procedures used for ricin agglutination test are summarized in Figure 3.

IV.2.1.2.2. Biuret Method

The Biuret tests were carried out according to the procedures outlined in the Progress Report No. 8 of the Phase One Study (December 1980). The cake or meal was first ground finely to pass through a sieve with 0.59 mm openings. Five grams of this sample was extracted with 50 ml distilled water for two hours with agitation, and then filtered through a Whatman No. 4 filter paper.

The sample (1 ml filtrate) was then reacted with the Biuret reagent (4 ml) at room temperature for 30 minutes, and the intensity of the color was measured at 540 nm and compared to those of standard solutions 1, 2 and 3.

IV.2.1.3. Heat Treatment of Castormeal

Defatted castormeal was heat treated to determine its effects on CB-1A and ricin. Two different heating methods were used.

IV.2.1.3.1. Dry Heat Treatment

Several 500 gram batches of defatted castormeal were spread on four layers of cheese cloth and placed in an electrically heated forced-air convection oven, pre-heated to 120°C. After the oven temperature returned to the preset temperature, samples were removed from the oven at time intervals of 10, 20, 30, 50 and 90 minutes. Protein

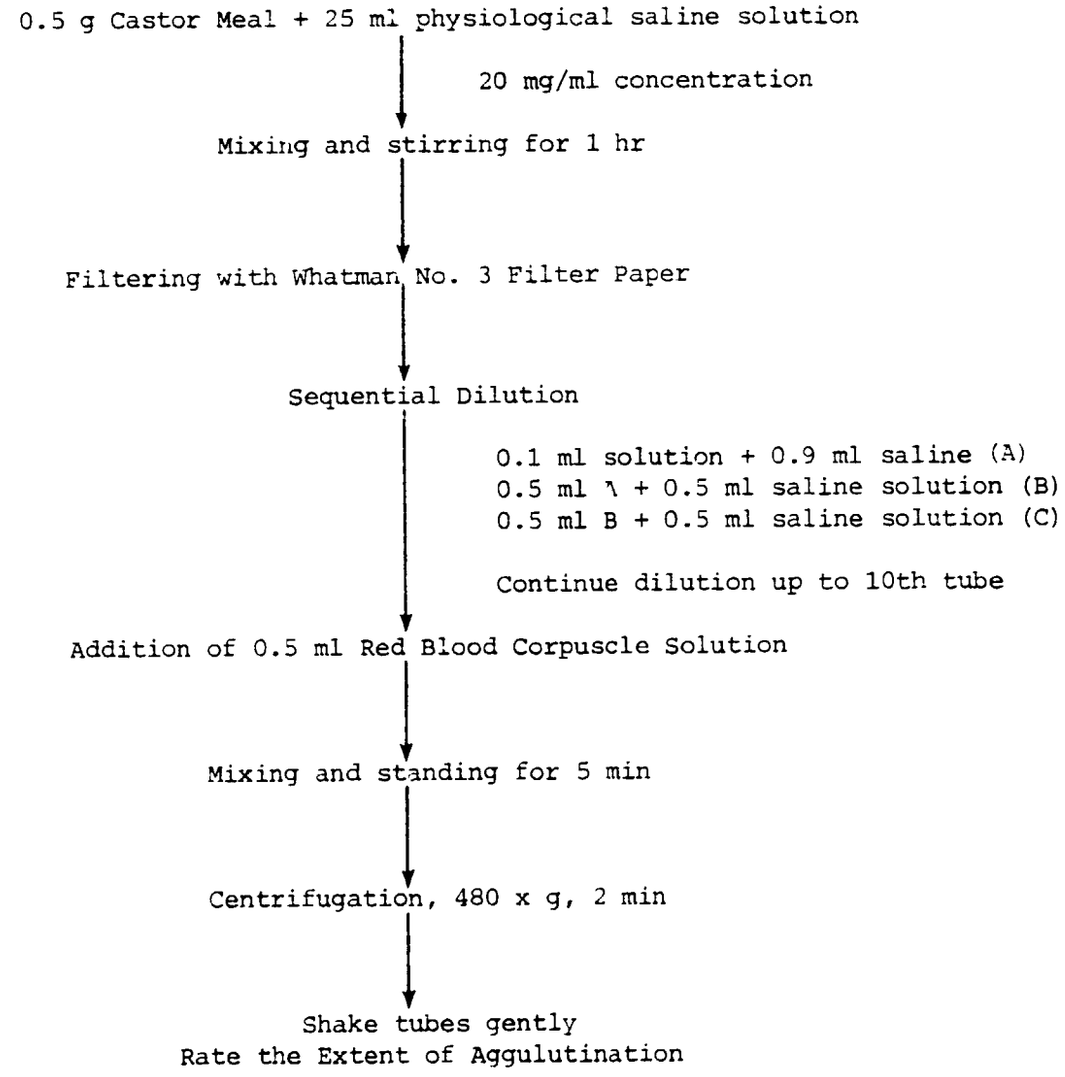


Figure 3 . Procedures used for ricin agglutination test.

solubility, hemagglutinin reaction and CB-1A precipitin reaction were determined for each sample.

IV.2.1.3.2. Wet Heat Treatment

Defatted castormeal (about 500 grams) was wrapped with four layers of cheese cloth and autoclaved at 120°C for 10, 20, 30, 50 and 90 minutes. Autoclaving time was measured from the time when the autoclave temperature reached 120°C. The autoclaved castormeal was analyzed for protein solubility, hemagglutin reaction and CB-1A precipitin reaction.

IV.2.2. Evaluation of Conventional Castor Seed Processing Steps

Conventional, castor seed processing involves many steps to produce oil and meal. Some of the important steps where heat can be applied to the seed or meal include pre-conditioning, pre-pressing, solvent extraction and desolventization. Therefore, each step has been evaluated for its effectiveness on ricin and CB-1A. The overall procedures used for pilot scale production of defatted castormeal are presented in Figure 4.

IV.2.2.1. Pre-conditioning

Pre-conditioning was performed by holding 50 pounds of castor seeds at 250°F in a Bauer 22" cooker for about 15 minutes. Enough water (about one pound of water per 50 pounds of seeds) was sprayed on the seed prior to pre-conditioning so that the final moisture content of the pre-conditioned seed was approximately 4%. The cooker was pre-heated by steam to about 300°F before adding the seeds.

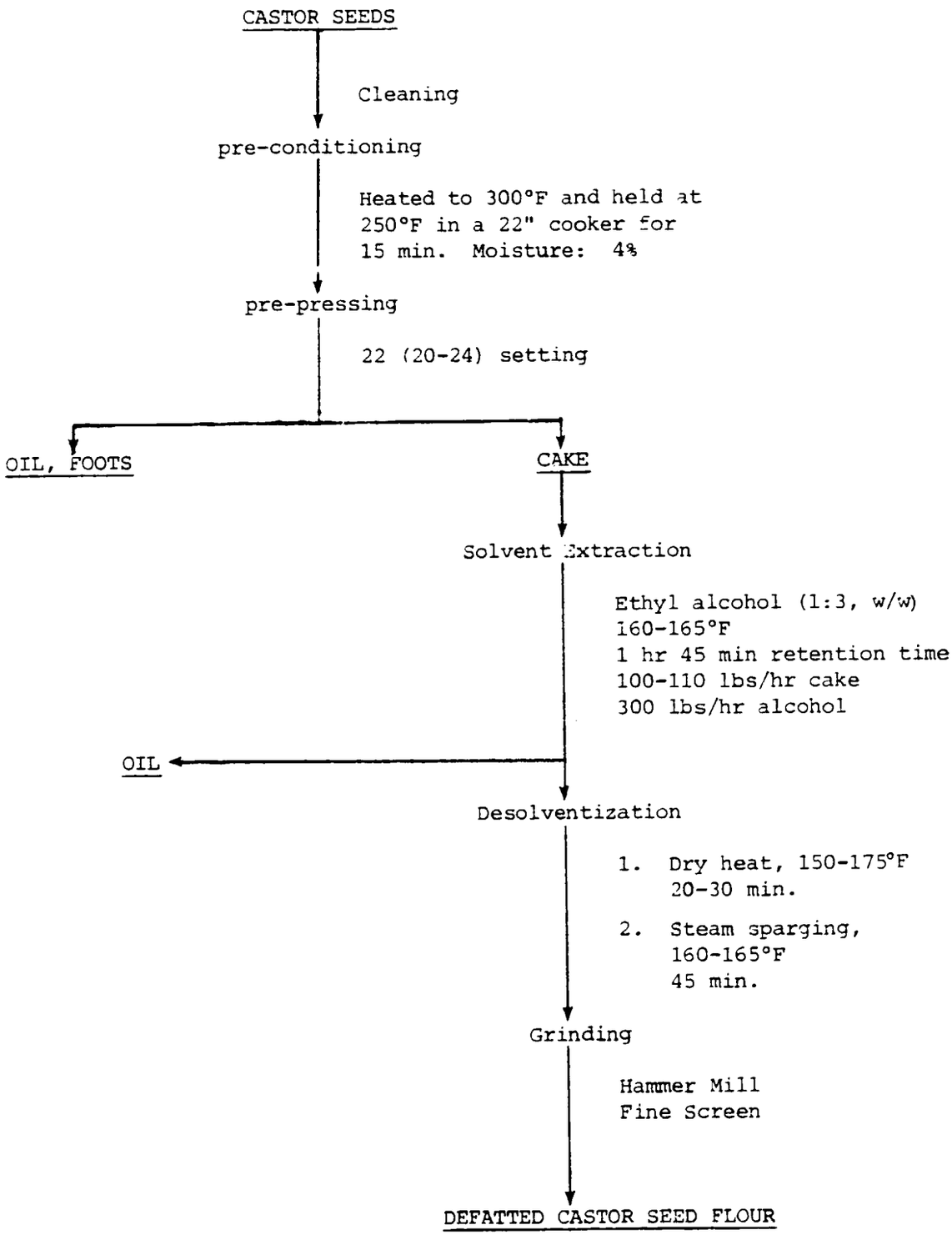


Figure 4. Procedures used for pilot scale production of defatted castormeal.

To prepare samples for pilot scale runs (1.5 tons for each run), preconditioning was carried out in two steps: castor seeds were pre-heated in a large gas-heated Proctor-Schwartz oven to about 200°F, and then transferred to the cooker to complete the pre-conditioning step. Also, for some experiments, castor seeds were dehulled prior to pre-conditioning.

IV.2.2.2. Pre-pressing

The pre-conditioned castor seeds were pre-pressed using a Simon-Rosedowns' expeller at a throughput of approximately 100 pounds per hour. The highest temperature reached during the pre-pressing was about 315-340°F.

IV.2.2.3. Solvent Extraction

For preliminary experiments, a series of 10 pound capacity batch type extractors were used. The press cakes were loaded into the batch extractor and soaked with either 99% ethyl alcohol or hexane for 24 hours at temperatures of 160-165°F for alcohol and 110-120°F for hexane. At the end of the extraction, the solvent was drained from the extractor and replaced with new solvent for a second 24-hour extraction. A total of three extractions were carried out for each sample. The defatted meal then was air-dried by exposing it to air under the sun.

For pilot scale experiments, a Crown continuous countercurrent extractor was used at a meal-to-solvent ratio of about 1:3 (w/w). Approximately 100 pounds press cake and 300 pounds alcohol or hexane were continuously delivered into the extractor every hour to continuously extract the residual oil

in the cake. The temperature was maintained as close to the boiling point of the solvent (160-165°F for alcohol and 110-120°F for hexane) as possible throughout the extraction. The average retention time of the cake in the extractor was approximately 90 minutes for hexane extraction and 105 minutes for alcohol extraction.

IV.2.2.4. Desolventization

For preliminary experiments, desolventization was carried out by spreading the wet meal on a flat surface and exposing it to the sunlight in an open air. This method was used to prepare defatted castormeal samples for all laboratory scale experiments.

For larger scale experiments, a Crown desolventizer/toaster (32" x 8', 3 deck) was used. The desolventizer was operated at temperatures of 150-175°F for dry heat desolventization and at 160-165°F for steam sparging desolventization. Average retention times of the meal in the desolventizer were approximately 20-30 minutes for dry heating and 45 minutes for steam sparging.

The desolventized castormeal was ground into a flour, 60 mesh or smaller, using a hammermill.

IV.2.3. Evaluation of Chemical Treatment and Extrusion

The possibility of using an extruder as a short-time, high-temperature chemical reactor was explored using a Wenger X-20 extruder.

IV.2.3.1. Extrusion Conditions

The finely ground defatted castormeal was first adjusted to a final moisture content of 20% by adding measured amounts of water while mixing. When chemicals were used, they were dissolved in or mixed with water before mixing with the meal. In any case, the final moisture content of the meal prior to extrusion was adjusted to 20%.

Conditions used for extrusion of castormeal, whether it was treated chemically or not, are summarized in Table 1. After extrusion, all extrudates were dried to an average moisture content of about 8% in a Wenger drier.

IV.2.3.2. Type and Concentration of Chemicals

The types and concentrations of chemicals used in conjunction with extrusion are listed in Table 2.

TABLE 1

Conditions Used in Extrusion of Defatted Castormeal

Parameter	Condition 1	Condition 2
Main Drive Speed, rpm	300	400
Head Temperature, °F		
7th	300	315
6th	285	300
5th	270	285
Feeder Speed, rpm	7	11.5
Mixer Speed, rpm	75	150
Additional Water to Screw	Yes	Yes
Direct Steam Injection	No	Yes
Production Rate, lbs/hr	500	600

TABLE 2

Types and concentrations of chemicals used in conjunction with extrusion [a/]

Chemical	Concentration (%)	
	Condition 1	Condition 2
NaOH	1.0	2.0
NaOCl	1.0	1.0
H_2O_2	1.0	2.0
NaOH + NaOCl	0.5 + 0.5	1.0 + 1.0
$NaHCO_3$	1.0	2.0
Control	none	none

[a/] Based on the final moisture content of 20% prior to extrusion.

V. RESULTS AND DISCUSSION

V.1. Proximate Compositions

Castor seeds were purchased in three separate shipments from various sources in Texas and Arizona, due to difficulties of obtaining them from a single source. However, their proximate compositions were identical, and representative data are summarized in Table 3. On the average, the castor seeds consisted of 28% hulls and 72% kernels. Of the kernels, proteins comprised about 23%, and oil, 64%.

V.2. Re-evaluation of the Phase One Study Results

In order to affirm the validity and practical usefulness of the CB-1A allergenecity and ricin toxicity testing methods, and the effects of heat treatments on CB-1A and ricin, as proposed in the Phase One Study Reports, some of the experiments were repeated in the laboratory following the proposed experimental procedures and conditions as closely as possible.

V.2.1. CB-1A Allergenecity Testing Methods

V.2.1.1. Confirmation of the Purified CB-1A

When CB-1A allergens were extracted from the defatted castormeal and purified according the procedures outlined in Figure 1, a light brown solid was obtained. This light brown solid was later confirmed as concentrated CB-1A using disc polyacrylamide gel electrophoresis. The purified CB-1A showed three intense and well-defined protein bands, as shown in Figure 5. These gel patterns were identical to those reported by previous workers.

TABLE 3

Proximate Composition of Castorseed

Fraction	% Composition
Hulls	28.0
Kernels	72.0
Moisture	4.2
Protein	22.8
Oil	64.1
Ash	2.9

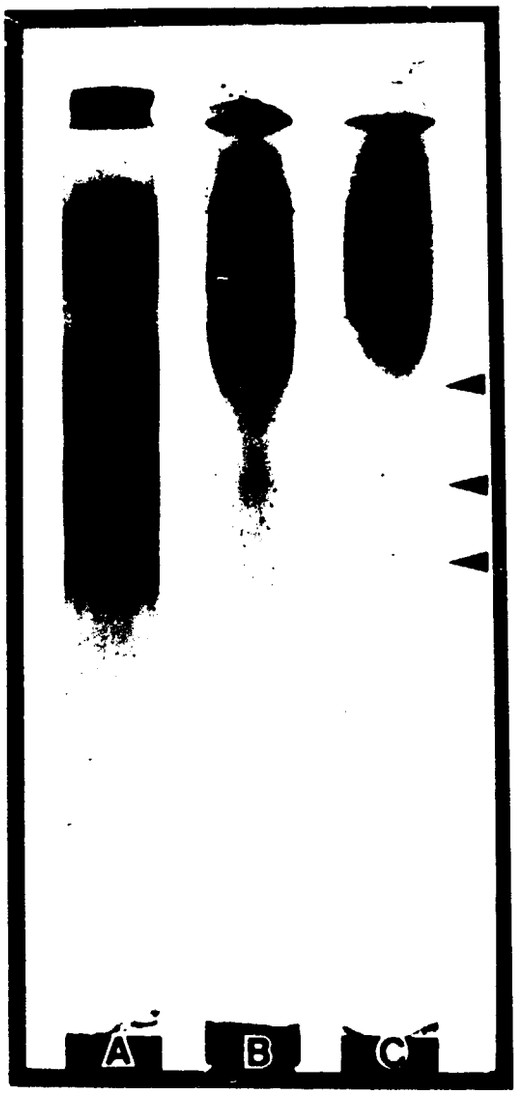


Figure 5. Disc polyacrylamide gel electrophoretic patterns for A: purified 31-1, B: heat treated 31-1, C: autoclaved defatted meal extract. Arrows indicate the protein bands responsible for the activity.

The acute allergenecity of the purified CB-1A preparation was also confirmed indirectly by rat studies. The rats, injected with crude extracts of castormeal, showed loss of vitality of movement, irregular respiration and heartbeat, and closed eyes. The rats injected with the purified CB-1A resulted in more serious symptoms than those injected with crude extracts. In all cases, however, the rats recovered from the symptoms completely after one day. Injection of rats with saline solution alone or bovine serum albumin in saline solution did not cause any of the symptoms mentioned above.

These observations indirectly demonstrated that the crude extracts of castormeal or the purified CB-1A contained substances that are responsible for allergenic and/or toxic effects to rats.

Subcutaneous skin tests were also performed on rats, but the allergenic reaction was not clear enough for documentation.

V.2.1.2. Immunodiffusion Technique

The precipitin reaction between antibody and antigen is a very sensitive in vitro immunochemical method to determine the reactivity of protein-like substances. The immunodiffusion technique proposed for allergenecity determination of CB-1A has proven to be a sensitive method one can use for the purpose. We must, however, recognize the facts that this method is time consuming, requiring preparation of pure CB-1A and its antiserum (antibody), and that interpretation of the resulting precipitin reaction pattern for quantitation of the allergenecity reduction is very difficult. In the absence of

any other acceptable alternative method, the immunodiffusion method was used throughout the study. As evidenced in the following sections, use of this method with utmost care will produce results very useful in monitoring the rate of allergenecity reduction during processing of castor seeds.

V.2.2. Ricin Toxicity Testing Methods

Two methods were proposed: first, the more sensitive but time consuming hemagglutinin reaction method, and second, a simpler but less sensitive Biuret method.

V.2.2.1. Hemagglutinin Reaction Method

This method is based on the ability of toxins, such as ricin and ricinin, to coagulate the red blood cell corpuscle. Although time consuming, this method has proven itself to be a relatively simple, accurate method for determining the ricin toxicity of castor seeds and products. For these reasons, this method was used throughout the study as the primary analytical method to monitor processing effects on ricin toxicity reduction.

V.2.2.2. The Biuret Method

This method was proposed as a simple quality control method to monitor the processing effects on ricin toxicity. The principles behind this method are as follows. Since ricin is known to be a type of protein, heat treatment will reduce the toxicity by denaturing the protein. The degree of protein denaturation is directly related to solubility of the protein, and, therefore, the toxicity reduction should be proportional to the protein solubility reduction as measured by the Biuret method.

In theory, this method should be adequate to estimate ricin toxicity if the rate of ricin denaturation were at least equal to that of other proteins in castor seeds. Although not enough experimental data are available at this time to establish such a relationship with confidence, the data presented in Table 4 tend to support this hypothesis.

One of the major concerns with this method was the uncertainty that whether the reduction of protein solubility, as measured by the Biuret method, would also reduce the ricin toxicity proportionately. To answer this question, hemagglutinin tests were carried out on a constant protein basis by using different dilution factors based on the Biuret test results. As shown in Table 4, the titre for all hemagglutinin reaction was at No. 6 tube, which corresponded to a dilution of 1/16000. This observation reasonably demonstrates that the Biuret method can be used to estimate the ricin toxicity. For this reason, the Biuret test data are presented along with the hemagglutinin data through this study. However, the Biuret method does not seem to be any simpler than the hemagglutinin method described earlier.

V.2.3. Effects of Heat Treatment on CB-1A and Ricin

The Phase One Study Report suggested that heating the castormeal at 120°C for 30 minutes would destroy both ricin and CB-1A. To confirm these findings, castormeal was heat treated by two different methods in the laboratory.

V.2.3.1. Effects of Dry Heat Treatment

TABLE 4

Hemagglutinin Reaction on a Constant Protein Quantity Basis

Heating Time (Min)	Biuret Test (O.D.)	Dilution [a]	Hemagglutinin Reaction Tube # (Dilution)
Control	0.153	0.1:0.9	6(1/16000)
10	0.027	0.567:0.433	-
20	0.030	0.51:0.49	-
30	0.041	0.37:0.63	-
50	0.045	0.34:0.66	-
90	0.057	0.268:0.732	-

[a] Extract: Water in milliliters.

As summarized in Table 5, dry heating of the solvent-defatted castormeal in an electrically heated forced-air convection oven, even as long as 50-90 minutes, did not reduce the CB-1A activity appreciably. Under the same conditions, the titre for hemagglutinin reaction was at the No. 4 tube, which corresponds to a dilution of 1/4000. However, the protein solubility was actually increased as the castormeal was heated longer, from about 87% for unheated sample to about 90-94% after heating for 50-90 minutes.

The immunodiffusion patterns showing the effects of dry heat treatment on CB-1A precipitin reaction are shown in Figure 6. A clear precipitin line is absent from the pattern for castormeal samples treated with dry heat for 50-90 minutes (patterns E and F).

V.2.3.2. Effects of Wet Heat Treatment

Effects of wet heat treatment of defatted castormeal using an autoclave on CB-1A, ricin and protein solubility are summarized in Table 6. At least 20-30 minutes of autoclaving was required to obtain a negative CB-1A precipitin reaction. The titre for hemagglutinin reaction was at the No. 6 tube, corresponding to a dilution of 1/16000. The protein solubility dropped to a low of about 16% after 10 minutes of autoclaving, then increased gradually to reach 35% after 90 minutes of heating.

The immunodiffusion patterns showing the effects of wet heat treatment on CB-1A precipitin reaction are also shown in Figure 6. A precipitin line is not visible for castormeal samples heated longer than 20-30 minutes (patterns H and I).

TABLE 5

Effects of Dry Heating on Protein Solubility, Hemagglutinin Reaction and CB-1A Precipitin Reaction

Heating Time (Min)	% Protein Solubility (pH 7.0)	Hemagglutinin Reaction Tube # (Dilution)	CB-1A Precipitin Reaction
Control	86.9	6(1/16000)	+
10	85.8	5(1/8000)	+
20	87.8	5(1/8000)	+
30	89.9	5(1/8000)	+
50	90.3	4(1/4000)	±
90	94.4	4(1/4000)	-

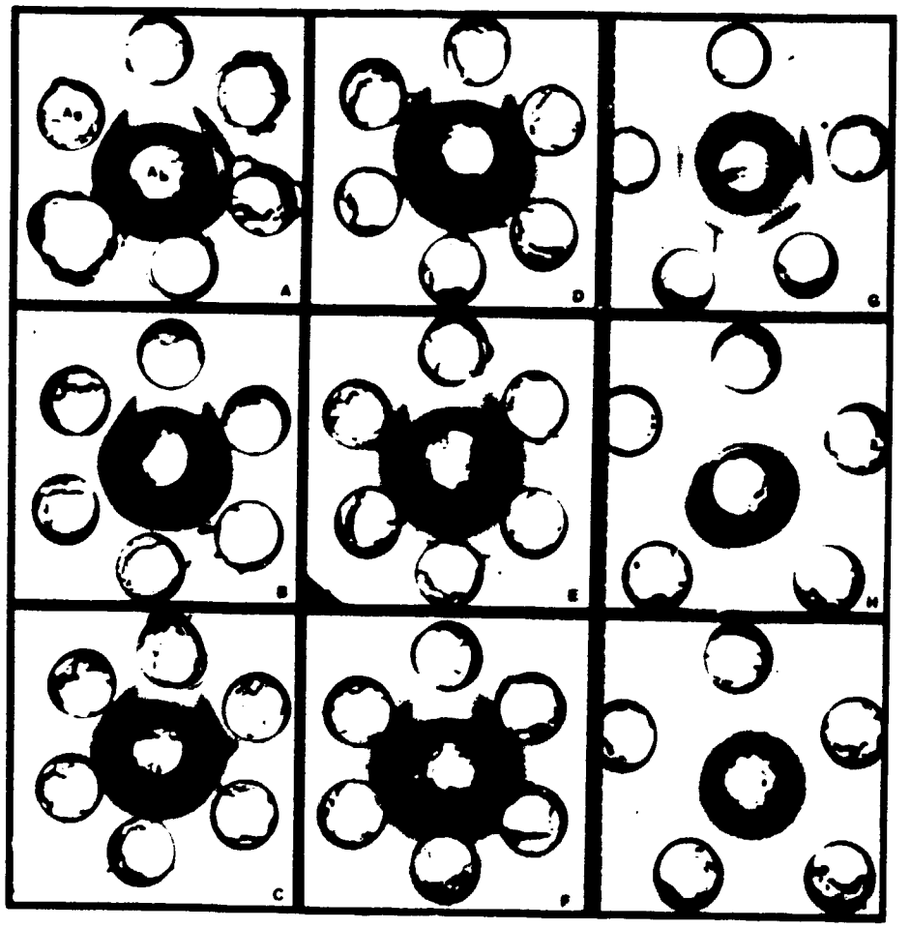


Figure 6. Immunodiffusion patterns showing the effects of dry (B-F) and wet (G-I) heat treatments of solvent extracted castormeal on CB-1A. A: control, B and G: 10 minute heating, C and H: 20 minute heating, D and I: 30 minute heating, E: 50 minute heating, F: 90 minute heating, Ab: antibody (antiserum) of the purified CB-1A) and Ag: antigen (heated castormeal extract).

TABLE 6

Effects of Wet Heating on Protein Solubility, Hemagglutinin Reaction and CB-1A Precipitin Reaction

Heating Time (min)	% Protein Solubility (pH 7.0)	Moisture (%)	Hemagglutinin Reaction Tube # (Dilution)	CB-1A Precipitin Reaction
Control	88.0	7.9	6(1/16000)	+
10	15.8	13.9	-	+
20	17.6	12.4	-	±
30	21.9	14.2	-	-
50	23.4	16.4	-	-
90	35.3	22.3	-	-

The data in Tables 5 and 6 clearly demonstrate that dry heat treatment can destroy ricin completely if it is subjected to heat treatment at high enough temperatures, but the wet heat treatment is more effective in destroying CB-1A. Even then, however, at least 20-30 minutes of autoclaving is required to completely destroy CB-1A.

V.3. Evaluation of Conventional Castor Seed Processing Steps

During the re-evaluation of the Phase One Study Results, it was confirmed that heat treatment of defatted castormeal indeed destroys both CB-1A and ricin. Therefore, in this phase of the study, each conventional castor seed processing step where heat can be applied has been investigated to determine if any of the steps could be used to effectively destroy both CB-1A and ricin simultaneously.

V.3.1. Effects of Pre-Conditioning on CB-1A and Ricin

Table 7 summarizes the effects of various processing steps on protein solubility as measured by the Biuret method, ricin toxicity as measured by hemagglutinin test, and CB-1A. Dehulling, flaking and pre-conditioning followed by solvent extraction did not produce negative hemagglutinin reaction unless the defatted meal was wet heated again for 30-50 minutes. The pre-conditioning step subjects castor seeds to a temperature in excess of 250°F for at least 15 minutes, but the data in Table 7 clearly demonstrate that the pre-conditioning step alone cannot be effectively used to destroy ricin toxicity, although the tube number for titre can be changed. Pre-conditioning also showed no significant effects on the Biuret protein solubility or CB-1A precipitin reaction.

TABLE 7

Effects of Processing Conditions on Agglutination and CB-1A

	Test	Unheated	Weat Heated	
			30 min	50 min
Dehulled, flaked, defatted meal	Biuret HA CB-1A	0.396 6 (1/16,000) +++	0.074 Negative +/-	0.088 Negative -
Whole seed, flaked defatted meal	Biuret HA CB-1A	0.278 5 (1/8,000) +++	0.059 Negative -	0.062 Negative -
Whole seed, preconditioned, flaked, defatted meal	Biuret HA CB-1A	0.313 4 (1/4,000) +++	0.048 Negative +++	0.056 Negative +/-
Whole seed, preconditioned prepressed, defatted meal	Biuret HA CB-1A	0.079 Negative +++	0.054 Negative -	0.063 Negative -
Whole seed, prepressed, defatted meal	Biuret HA CB-1A	0.115 Negative +++	0.044 Negative +++	0.050 Negative -

Pictures showing the hemagglutinin reaction are presented in Figure 7 (pictures A through D).

V.3.2. Effects of Pre-pressing on CB-1A Ricin

As shown in Tables 7 and 8, pre-pressing followed by solvent extraction has significant effects on both protein solubility and hemagglutinin reaction. All pre-pressed cakes exhibited negative hemagglutinin reaction and considerably low Biuret protein solubilities. This observation is also clearly shown in Figure 7 (pictures E through H). The data presented in Tables 7 and 8 and Figure 7 clearly demonstrate that pre-pressing using an expeller might be sufficient to destroy ricin completely.

The data in Table 7 also show that CB-1A activity was still strong, even after the pre-pressing of the seeds. However, in actuality, the CB-1A activity was reduced considerably by the steps of pre-pressing followed by solvent extraction, as shown in Figure 8. Comparison of the intensity of the precipitin lines produced by the pre-pressed solvent extracted castormeal samples, with those of the meal produced by solvent extraction alone without the pre-pressing step, clearly shows at least ten-fold reduction in the CB-1A activity in the pre-pressed meals (compare samples (S) against the control (C) in Figures 8-A and 8-B). However, the residual CB-1A remaining in the meal was sufficient to give strong positive precipitin reaction shown in Table 7. Pre-pressed solvent extracted samples produced at different dates produced similar effects (compare samples S in Figures 8-A and 8-B).

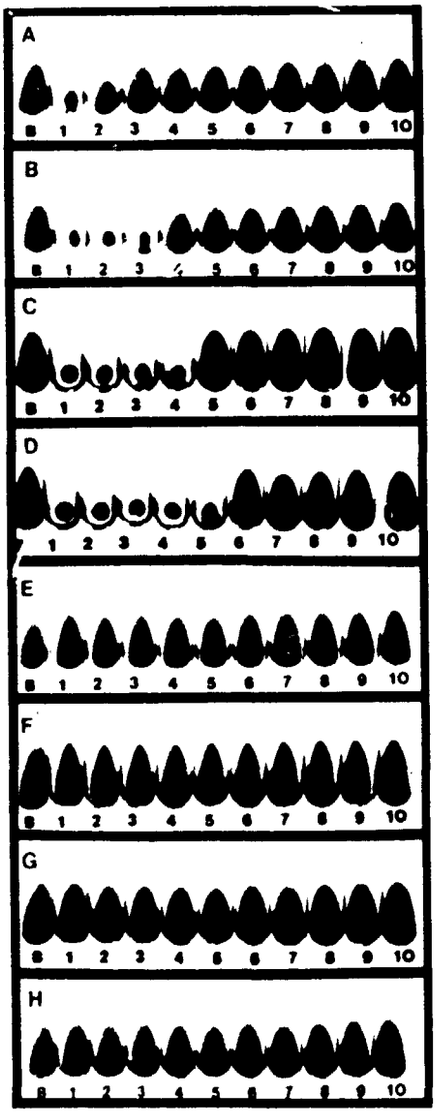


Figure 7. Effects of [illegible]
[illegible]

TABLE 8

AGGLUTINATION TESTS (RATINGS) OF CASTOR MEALS

Sample / Dilution factor	Test tube Number 1 1/500	2 1/1000	3 1/2000	4 1/4000	5 1/8000	6 1/16000	7 1/32000	8 1/64000	9 1/128000	10 1/256000
Whole seed	4+	2+	1+*	1+	1+	1+	1+	1+	1+	1+
Kernel (undefatted)	4+	4+	3+	1+*	1+	1+	1+	1+	1+	1+
Defatted flour with hulls	4+	4+	4+	3+	1+*	1+	1+	1+	1+	1+
Defatted flour without hulls	4+	4+	4+	4+	3+	1+*	1+	1+	1+	1+
Pre-pressed cake (A)	No agglutination									
Pre-pressed cake (B)	No agglutination									
Solvent extracted flour (A)	No agglutination									
Solvent extracted flour (B)	No agglutination									

* the titre; the highest final dilution showing a 1+.

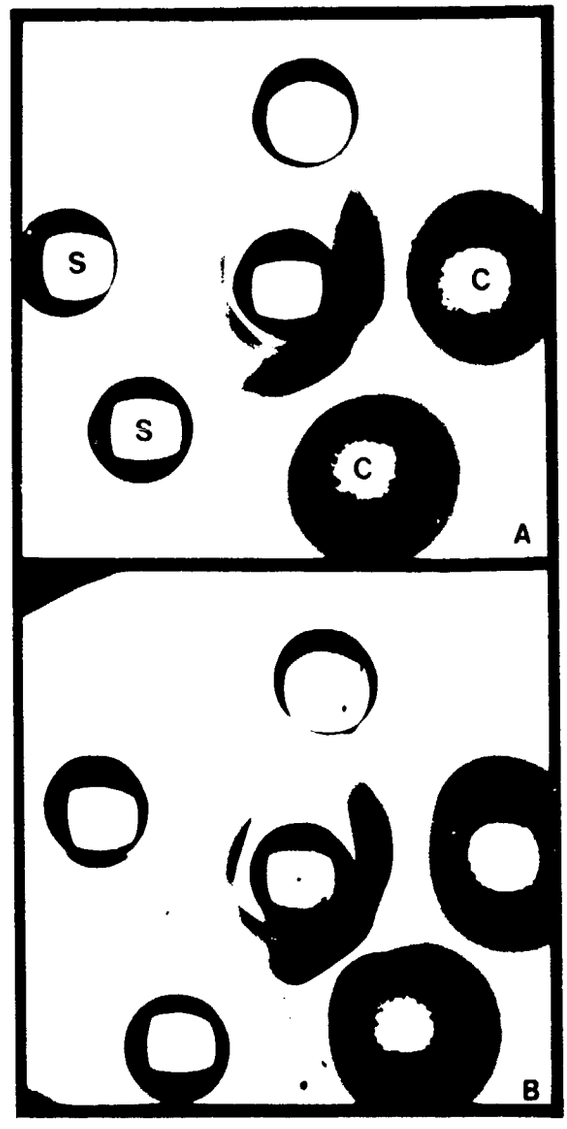


Figure 8. Effects of pre-pressing, solvent extraction and desolventization on CB-1A. A: dry heat desolventized; B: steam sparging desolventized, C: solvent extracted control, and S: sample.

V.3.3. Effects of Solvent Extraction on CB-1A and Ricin

As already shown in Tables 7 and 8, solvent extraction alone has only limited effects on CB-1A, ricin and protein solubility. However, as will be discussed later in the extrusion section, ethyl alcohol extraction is more effective than hexane extraction in destroying the residual CB-1A remaining in the pre-pressed solvent extracted meal using extrusion cooking in the presence of selected chemicals.

V.3.4. Effects of Desolventization on CB-1A and Ricin

Desolventization was carried out in a Crown 30" 3-stack desolventizer/toaster using either dry heating method or steam sparging method. Neither method, however, had any significant additional effects on CB-1A beyond those from the pre-pressing step. As expected, hemagglutinin reaction was no longer detectable after desolventization since ricin was supposedly destroyed completely during the pre-pressing step as discussed earlier in Tables 7 and 8 and Figure 7.

V.4. Effects of Chemical Treatment and Extrusion on Residual CB-1A

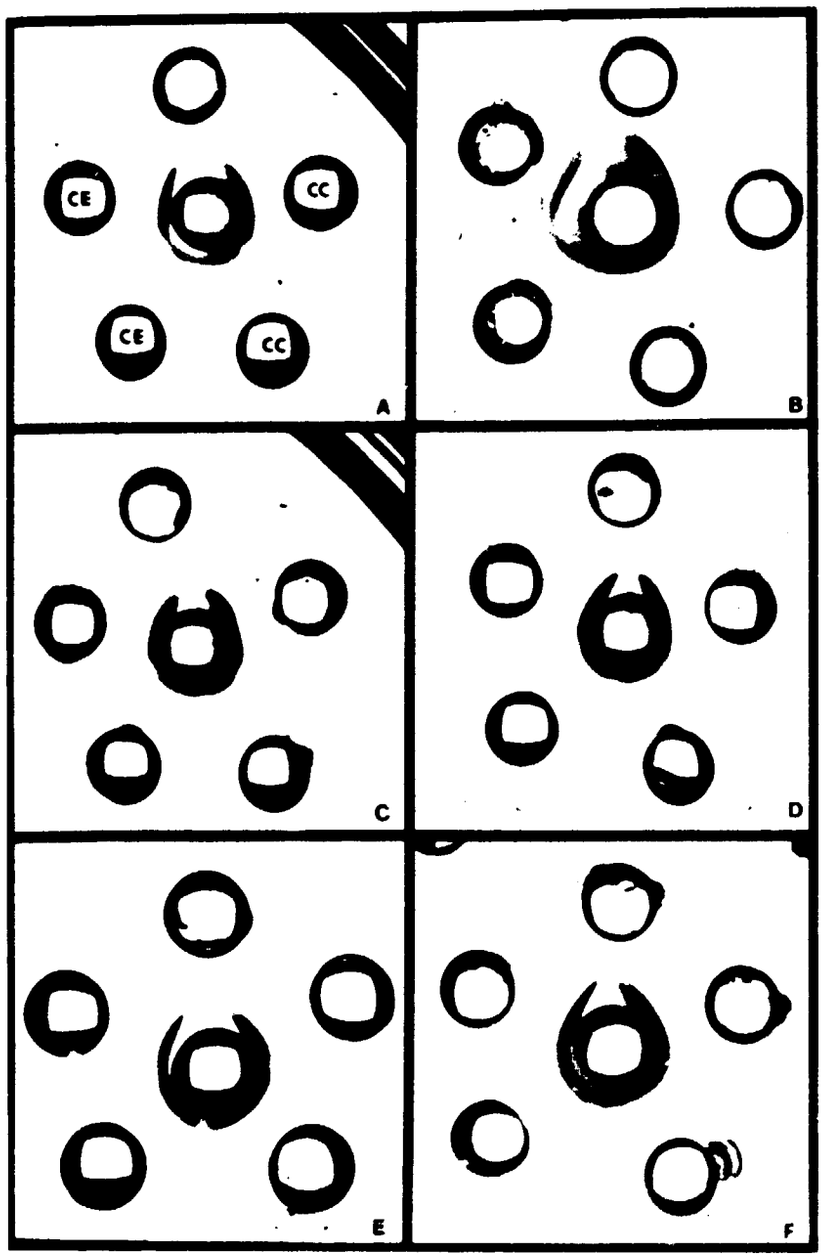
As discussed earlier, when castor seeds were processed through pre-conditioning, pre-pressing, solvent extraction and desolventization, ricin was destroyed completely and the hemagglutinin reaction was no longer detectable in the meal. Also, the CB-1A content of the meal has been reduced by as much as 90%, but the amount of the residual CB-1A remaining in the meal is still sufficiently large to cause a strong positive precipitin reaction.

In an attempt to further reduce the residual CB-1A content in the meal, feasibility of utilizing selected chemicals has been explored. Since most of the chemical reactions are known to be accelerated at high temperatures, feasibility of using an extruder as a high-temperature short-time reactor was also evaluated.

As shown in Figure 9, chemical treatments at 1% levels, without extrusion, had a minimum effect on the residual CB-1A (compare "C" of pictures A-E with that of picture F). The intensity of the precipitin lines was slightly reduced, but the reduction was very small.

Figure 9 also shows that the combined chemical treatment and extrusion, under the extrusion condition 1 in Table 1, did not reduce the residual CB-1A content of the meal significantly (compare "CE" and "CC" of pictures A-F). Identical results were obtained for all samples, regardless of the solvent extraction and desolventization methods used to produce the samples (i. e., alcohol or hexane extraction and dry heat or steam sparg , uesolventization).

Figure 10 shows the effects of extrusion alone (without added chemicals) on the residual CB-1A contents of alcohol (pictures A-E) and hexane (pictures F-J) extracted meals. The results demonstrated that extrusion alone did not reduce the residual CB-1A content at all (compare "NE" of pictures A-D with picture E for alcohol extracted meal and "NE" of pictures F-I with picture J for hexane extracted meal).


CE
CC
CE
CC
A
B
C
D
E
F

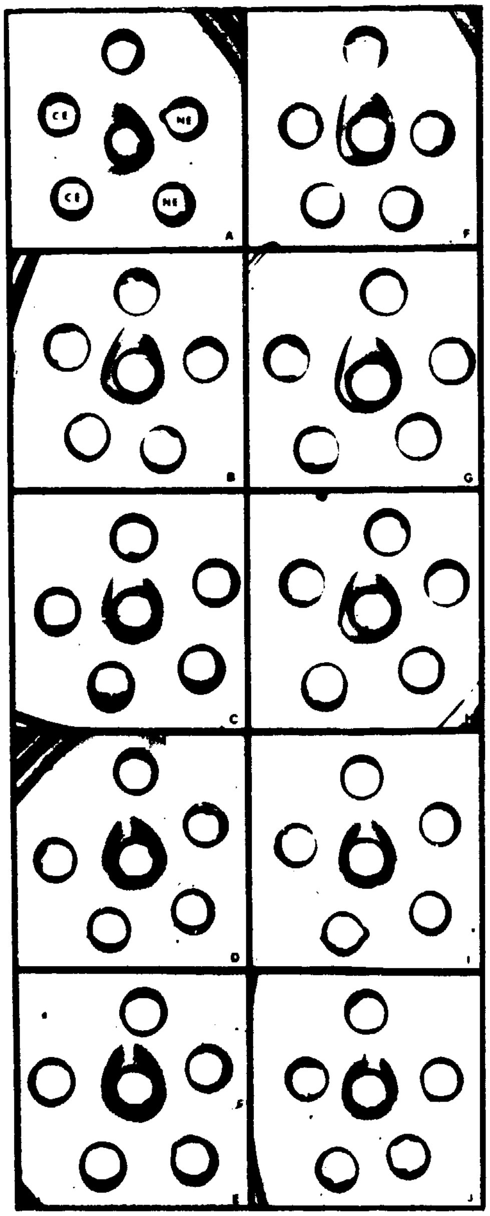


Figure 1[illegible]. Immunodiffusion patterns showing extraction of [illegible] of pre-pressed, air-dried (A-E) and hexane (F-J) extracted and desolventized defatted cottonseed meal on Ch-IA during extraction in the presence of [illegible]. A and F: [illegible] NaOH, B and G: 2[illegible] NaHSO[illegible], C and H: [illegible], D and I: [illegible] NaOCl, E and J: [illegible], NE: nonchemically treated and extracted samples, and CE: extracted [illegible].

Figure 10 also shows the effects of the combined chemical treatment and extrusion of alcohol and hexane defatted castormeals at 2% chemical concentration, on the residual CB-1A content. For this series of studies, the extrusion was carried out under extrusion condition 2, shown in Table 1. Extrusion of the alcohol defatted castormeals with 2% sodium hydroxide eliminated the CB-1A precipitin reaction completely (compare "CE" and "NE" in picture A and "CE" of picture A with "CE" of picture E). The same treatment also reduced the residual CB-1A in the hexane defatted castormeals, but the degree of reduction was somewhat smaller than the alcohol defatted meals.

Two other chemicals, 2% sodium bicarbonate and the mixture of 1% sodium hydroxide and 1% sodium hypochlorite, also reduced the residual CB-1A content from both alcohol and hexane defatted castormeals upon extrusion; however, the reduction was somewhat smaller than the 2% sodium hydroxide treatment. There is, an important note to make in connection with the 2% sodium bicarbonate treatment. When 2% sodium bicarbonate treated meals were incubated for 5 hours, as specified in the commonly used immunodiffusion procedures, no precipitin reaction was observed; however, when the incubation was extended overnight, a small amount of precipitin reaction occurred. The reasons for this delayed reaction are not known at this time; however, this observation suggests that care must be taken in carrying out the immunodiffusion tests and in interpreting the data thus obtained.

Treatment of castormeals with 2% hydrogen peroxide or 1% sodium hypochlorite did not affect the residual CB-1A content appreciably.

A dilution technique was used to determine the degree of residual CB-1A reduction through extrusion of chemically treated castormeals. In this technique, the castormeal extract, prepared from the meal fat resulted from pre-conditioning, pre-pressing, solvent extraction and desolventization, was diluted to 1/2, 1/5, 1/10, and 1/50 of its original concentration, and the intensities of the precipitin lines formed by the chemically treated and extruded samples were compared with those of the diluted extracts. The immunodiffusion patterns of this examination are shown in Figures 11 (alcohol extracted meal) and 12 (hexane defatted meal).

Table 9 presents the tabulated data from these two figures, in combination with the information from Figure 8. Extrusion of 2% sodium hydroxide treated alcohol defatted castormeal reduced the residual CB-1A content completely, while the same treatment was 90% effective on hexane defatted meal. these values correspond to 100% and 99% reduction of CB-1A originally present in the castorseed, respectively, assuming that the CB-1A reduction by pre-pressing solvent extraction and desolventization steps was about 90%.

Likewise, extrusion of castormeal treated with mixed chemicals (1% sodium hydroxide and 1% sodium hypochlorite) reduced the residual CB-1A content by 90%, corresponding to 99% reduction of CB-1A present in the castor seed. Although some

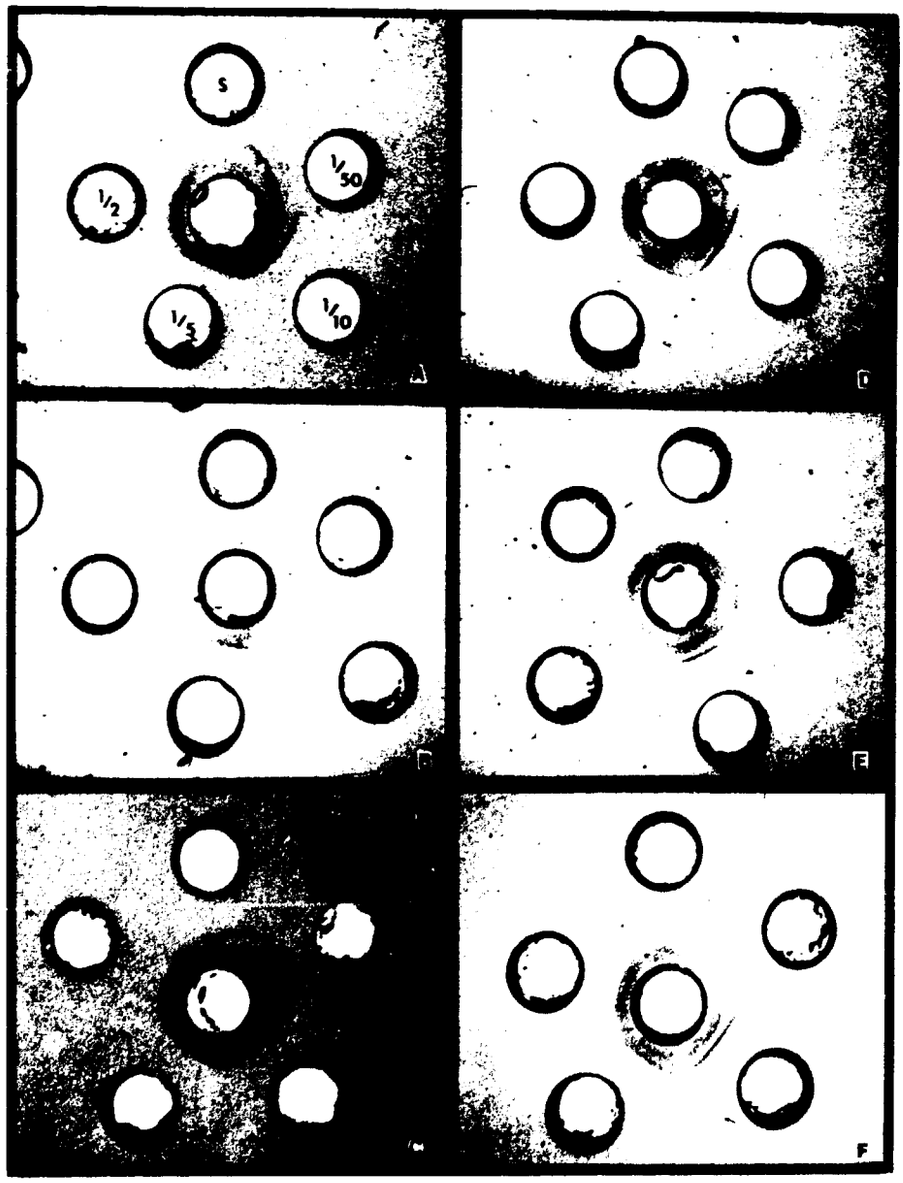


Figure 11. Estimation of the relative CB-1A concentrations remaining in the meal after extraction of chemically treated alcohol defatted cottonseed using dilution technique. A: 2 NaOH, B: 2% $NaHCO_3$, C: 1 [illegible] 1% NaCl, D: 1% NaCl, E: H_2O_2, F: control, and S: sample. Numbers indicate the dilution factors of the pre-treated, alcohol extracted and protein-free cottonseed extract.

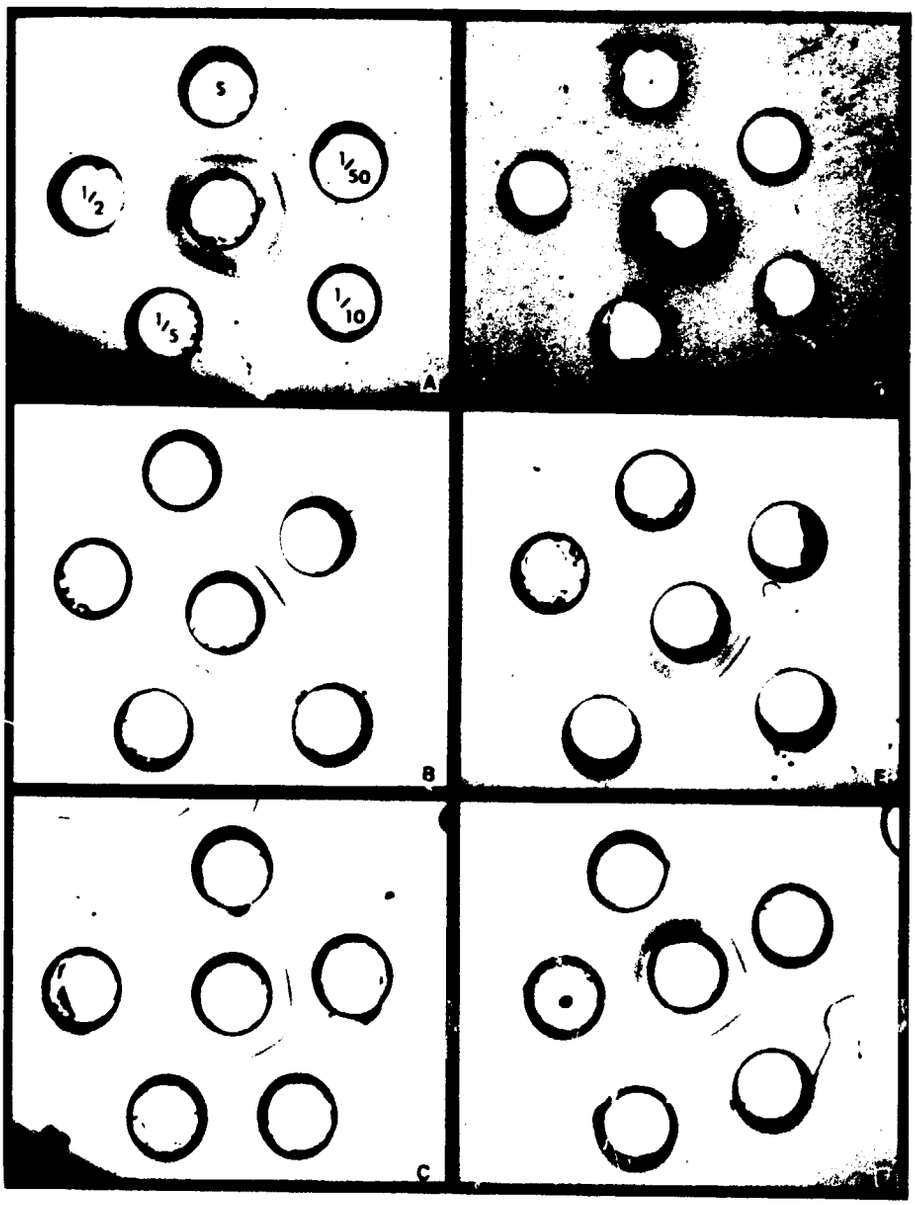


Figure 12. Estimation of the relative CP-1A concentrations [illegible] in the meal after extraction of chemically treated [illegible] using the "serum" agar dilution technique. A: 2% [illegible], B: 2% $NaHCO_3$, C: 1% [illegible] + 1% NaCl, D: 1% NaCl, E: 2% [illegible], F: control, and S: sample. Numbers indicate the dilution [illegible] pre-treated, hexane extracted and desolventized [illegible].

TABLE 9

Effects of Chemical Treatments of Defatted Castormeal on CB-1A during Extrusion [a/]

Chemical Treatment	% Reduction of CB-1A Activity			
	Hexane Defatted		Alcohol Defatted	
	From Residual	From Total	From Residual	From Total
2% NaOH	90	99	100	100
1% NaOCl	50	95	50	95
2% H_2O_2	50	95	50	95
1% NaOH + 1% NaOCl	90	99	90	99
2% $NaHCO_3$	50	95	90	99
Control	50	95	50	95

[a/] Moisture content of the defatted meal was adjusted to 20% prior to extrusion.

of the chemical treatments reduced CB-1A content by only 50%, this amount of reduction was significant when considered in terms of total CB-1A reduction. In all cases, the total reduction was at least 95%.

As expected, none of the chemically treated and extruded castormeal samples exhibited hemagglutin reaction, as shown in Figure 13.

At this point, we should address ourselves to the important question "How much reduction in total CB-1A content is sufficient and acceptable?" Obviously, this question can only be answered by comprehensive animal feeding tests, which have not been carried out in this study. However, the Phase One Study Reports suggested, based on their animal testing data, that 90-99% reduction of CB-1A would be sufficient to make the product safe for handling and feeding to animals. If these findings were correct, any of the procedures outlined in this report can be used to produce acceptable detoxified and deallergenated castormeals for animal feeding.

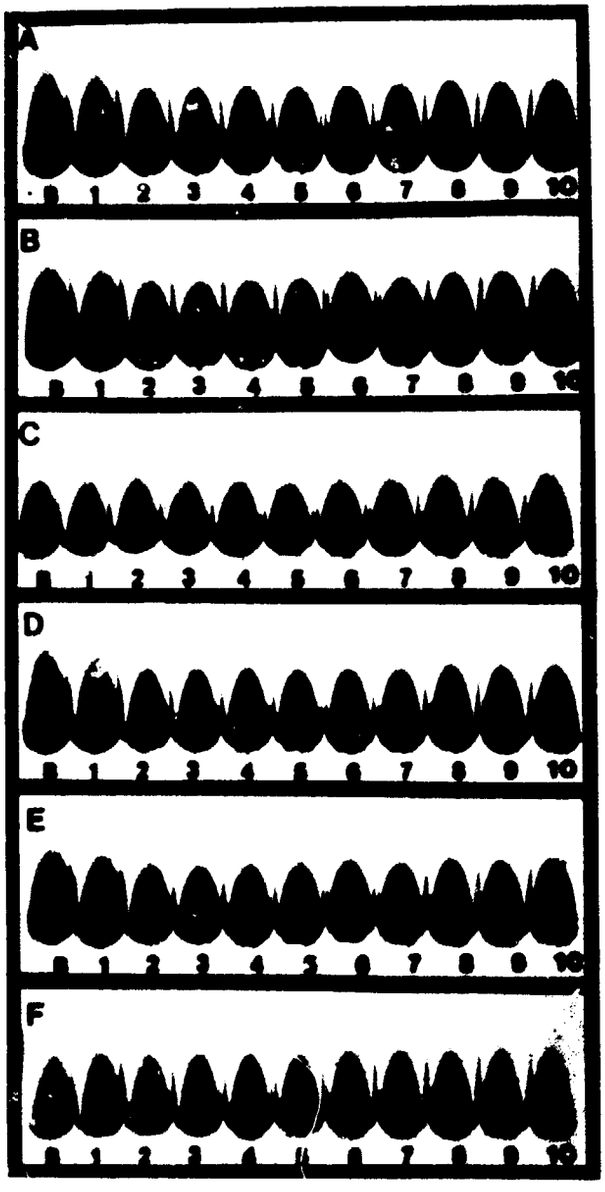


Figure 13. Effects of chemical treatments and extrusion of the pre-processed, soy-oat extracted and desolventized cast-removal on benzoylation reaction. A: 1% NaOH, B: 1% $NaBO_2$, C: 1% NaOCl, D: 0.5% NaOH + 0.5% NaOCl, E: 1% H_2O_2, and F: control.

VI. CONCLUSIONS

The research conducted by the Food Protein Research and Development Center has generated sufficient data to make the following conclusions.

1. Castor seed toxins (ricin and ricinin) can be completely detoxified during the pre-pressing step using a commercially available expeller.
2. The amount of castor seed allergens (CB-1A) can be reduced to about 10% of the original contents, using conventional castor seed processing steps, i.e., pre-conditioning, pre-pressing, solvent (either alcohol or hexane) extraction and desolventization (either dry heat or steam sparging).
3. The residual CB-1A remaining in the meal after the conventional processing of castor seeds can further be reduced by 50-100% by continuously treating the meal with selected chemicals using a commercially available extruder. This additional step results in products with 95-100% of its original CB-1A content reduced.
4. Pre-conditioning of castor seeds at temperatures of 250-300°F, even with added moisture, does not seem to affect the toxins or allergens appreciably. However, pre-conditioning does make the pre-pressing step easier and more efficient.
5. Castor oil can be extracted effectively from the press cake either by alcohol or hexane; however, destruction of the residual CB-1A remaining in the

meal after the solvent extraction seems easier for alcohol extracted meals than hexane extracted meals.

6. Desolventization technique seems to have little effect on residual CB-1A in the meal; however, the steam sparging desolventization technique is slightly more effective than the dry heating technique.
7. The immunodiffusion technique seems very sensitive in detecting the residual CB-1A content of meal; however, further work is needed to make this technique more reliable in quantitating CB-1A content.
8. The hemagglutinin reaction technique seems sensitive enough for use in toxicity measurements. Also the time required to carry out the test is not prohibitively long.
9. The Biuret method also seems to work well in measuring the toxicity; however, the relationship between the Biuret readings and toxicity is still unclear. Moreover, the time required for the Biuret test does not seem to be any shorter than the hemagglutinin reaction technique.

VII. RECOMMENDATIONS

The major problem facing the castor seed industry is the low economic value of the meal due to its toxicity and allergenecity as it is currently manufactured. Therefore, any improvements in processing steps which will produce castormeals with less toxicity and allergenecity will undoubtedly make significant contributions to the sagging castor seed industry.

Based on the experimental results obtained during the course of this study, as well as the results obtained during the Phase One Study, the following recommendations are made:

1. Set up a demonstration plant at a site where a conventional castor seed processing plant exists to test and make improvements on the procedures developed, and to produce enough products for use in animal feeding tests.
2. Carry out further experiments using a larger production scale extruder, such as the Wenger X-25, which has a production scale of 2500-3000 lbs per hour, to determine if the current information is scalable to larger production equipment, and to identify equipment which will meet the desired production capacity.
3. Carry out in-depth basic studies to more clearly understand the nature of castor seed toxins and allergens so that a set of fool-proof methods for detoxification and deallergenation can be developed.

4. Carry out in-depth animal feeding tests to verify the chemical methods of detoxification and deallergenation through physiological means.