TABLE XIII

Summary of Results

Properties	Experiment average	Significant factors	Confidence limits (95%)
Water absorption	628	Level of addition, temp'g	±99
Icing volume	140.5	Level of addition	±3.9
White cake score	89	None	±3.5
White cake volume	1901	None	±102
Yellow cake score	94	Level of addition	±3.9
Yellow cake volume	2465	None	±88
Devil's food score	90	Mono content, tempering	±3.6
Devil's food volume	2182	None	±91

on the icing volume in a negative way. There was no interaction of factors, and the factors, *alpha* mono content of emulsifier, I.V. of emulsifier, and tempering showed no significant effects.

It is well known that maximum icing-volume is obtained with minimum or no monoglyceride added to the shortening stock. However, in order to obtain desirable icing characteristics, such as moisture retention, creaminess, and nonweeping, the use of some mono and diglycerides is indicated, but these properties are obtained with some reduction in icing volume.

In the ranges examined for the five factors, we were unable to show any significant effect on either the volume or over-all cake score of the 130% white cake, which is commonly used to evaluate shortenings and emulsifiers. In the case of the yellow cake the graphical decision method does indicate that the level of use of the monoglyceride in the shortening is significant. However, contrary to what might be expected in going from the low level, where 1.75% *alpha* mono is added to the shortening, to the higher level, where 2.5% *alpha* mono is added to the shortening, the sign of the effect is negative.

Again it is fairly well accepted by those in the prepared mix industry that as the level of monoglyceride in cake mix shortening is increased, there are two peaks. The first maximum in volume occurs somewhere around 2½% *alpha* mono, and the second slightly higher maximum at about 4 to 4½% *alpha* mono. Since we added sufficient emulsifier to give 2½% mono added, the shortening would analyze about 2.9 to 3% *alpha* mono. Apparently the levels of use were on either side of the initial maximum, which is quite peaked as compared to the second maximum.

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The Allergen Content of Castor Beans and Castor Pomace

E. J. COULSON, JOSEPH R. SPIES, and HENRY STEVENS, Allergens Laboratory,[1] Washington, District of Columbia

The allergen content of 10 varieties of decorticated, defatted castor beans, as determined by a serological method, ranged from 6.1 to 9.0%. This range of allergen content probably does not offer an encouraging prospect for the development of an allergen-free castor bean by plant breeding.

The allergen content of samples of commercial castor pomace ranged from 0.092 to 4.2%. It is apparent from these results that some current commercial milling practices are capable of reducing significantly the allergen content of castor pomace.

THE CASTOR BEAN ALLERGEN is a nontoxic, unusually stable protein (1–5) that exhibits an extraordinary capacity to sensitize individuals exposed to small concentrations of the dust from castor beans or castor pomace (6–10). Allergic diseases caused by sensitivity to castor beans or castor bean by-products appear likely to become an increasingly serious problem as the growing, transportation, and processing of the beans become more widespread in this country (11,12).

Maximal utilization of castor pomace as a source of industrial proteins depends largely on the development of a feasible method for inactivation or elimination of the castor bean allergen. Previous endeavors (13,14) to inactivate the allergen by chemical and physical methods indicate that reactions drastic enough to destroy the allergen also destroy the useful proteins. Selective plant-breeding has been proposed (15) as a possible means of diminishing and ultimately eliminating the undesirable allergenic component from castor beans. The feasibility of such a program would depend upon the demonstration of a natural variation in concentration of the allergenic component in different varieties of castor beans. The purpose of the present study was to investigate the range of allergen concentration in different varieties of castor beans in order to supply this information. Also the allergen content of a few samples of commercial castor pomace was investigated to determine the effect on the allergenic components of different, current castor-bean milling practices.

The quantitative precipitin method developed by Heidelberger and Kendall (16) was used for determining the allergen content of castor beans. This method utilizes the normal immunological protective reaction of a live rabbit to an invasion of its tissues by a foreign protein. Injection of the foreign protein, called antigen, stimulates the production of some newly formed blood proteins, called antibodies. Antibodies exhibit the unique property of reacting spe-

[1] This is a laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

cifically, *in vivo* or *in vitro,* with the antigenic protein used to generate them. Blood serum, separated from blood cells, which contain these antibodies is the antiserum to the injected antigen. When the antigen is mixed in suitable proportions with its antiserum, the antigen and antibody combine with the formation of a precipitate. This precipitin reaction has been adapted to the quantitative measurement of either antigen or antibody (*cf.* 17). Owing to the sensitivity and specificity inherent in serological reactions, the precipitin method compares favorably with chemical methods for measurement of proteins. In contrast to conventional analytical methods which differentiate proteins by their chemical and physical properties, serological methods depend upon the uniqueness of structure of individual proteins.

Experimental

Castor Bean Allergen Preparations. The principal allergen of the castor bean has been segregated into a polysaccharidic-protein fraction that has been designated by the symbol CB-1A (1,3). CB-1A was separated from defatted raw castor-bean meal on the basis of five observed properties of the castor bean allergen: it was a) water-soluble, b) stable to boiling water, c) not precipitable by basic lead acetate, d) soluble in 25% ethanol, and e) insoluble in 75% ethanol. CB-1A contained 17.0% nitrogen on an air-dry basis (3).

Fraction CB-13E represents a further step in the purification of the allergenic protein in CB-1A. Some unimportant polysaccharide and some denatured protein were eliminated from CB-1A by precipitation of the active component with picric acid and removal of the picric acid from the precipitate. The protein was then dialyzed to remove smaller molecules which passed through the membrane. Details of the preparation of CB-13E are the same as those described for preparation of the corresponding allergenic fraction CS-13E from cottonseed (18). CB-13E contained 16.5% nitrogen and 2.28% polysaccharidic carbohydrate on an air-dry basis. CB-1A and CB-13E have been characterized as mixtures of relatively low-molecular-weight proteins and polysaccharidic proteins, which were classified as natural proteoses (1,19).

Fractions CB-1A and CB-13E possess potent allergenic activity. Positive skin reactions in patients allergic to castor beans were obtained with dilutions up to $1:10^6$ of either fraction. In a normal subject, whose skin was passively sensitized with the serum from a castor-bean-sensitive patient, positive skin reactions were produced by the injection of 1×10^{-10} g. of CB-1A (1).

These fractions are also potent antigens. They produce anaphylactic sensitization and shock in guinea pigs (5) and stimulate production of precipitating antisera in rabbits (4). The allergenic and antigenic specificities of CB-1A are attributed to the protein component. This makes it possible to use serlogical methods for the identification and quantitative determination of the allergen in castor beans and castor pomace or when it occurs as a contaminate in other products (20).

Castor Bean Samples. The castor bean samples were provided for our use by L. M. Pultz, Oilseed and Industrial Crops Branch, United States Department of Agriculture. The samples included both established varieties and commercially-imported lots identified only by country of origin.

Castor Pomace Samples. Three of the castor pomace samples were obtained directly from two domestic firms that process most of the castor beans in this country. Three samples were obtained from imports through the courtesy of W. Clark Cooper, Occupational Health Program, U. S. Public Health Service.

Preparation of Samples. Castor beans in a 75-g. portion of each were decorticated by hand, macerated to a paste by mortar and pestle, and then extracted with ether in a Soxhlet extractor for 24 hrs. The air-dried, defatted samples were then ground in a small Wiley mill to pass a 40-mesh sieve. The castor pomace samples were ground to pass a 40-mesh sieve without any preliminary treatment.

For analysis 1:20 extracts were prepared by extracting 1 g. of castor bean flour or pomace with 20 ml. of buffered saline (pH 7.0) containing, as preservatives, 0.01% Merthiolate and saturation with chloroform. The extracting period was three days at 5°C. with frequent shaking of the suspension. The extract was clarified by centrifugation and filtration.

Rabbit Antiserum. Fraction CB-13E was used for the production of antiserum because it was the purest allergen fraction available in quantities sufficient for that purpose. For production of castor bean allergen antiserum, rabbits were immunized by a series of inoculations with CB-13E as described in detail in a preceding publication from this laboratory (13). The animals were then bled, the blood was allowed to clot at room temperature, and the serum was separated from the clot by centrifugation. The serum was preserved with 0.01% Merthiolate and stored in 30-ml. vials at −20°C. This antiserum gave typical precipitin curves with the allergen preparations and with unfractionated castor-bean extracts. In studies of the stability of the castor bean allergen to heat and chemical reagents, a relatively close correlation was observed between the loss of precipitating activity with this antiserum and the loss of allergenic activity as determined by passive transfer tests in human subjects (13).

Precipitin Determinations. The quantitative precipitin determinations were conducted as described by Kabat and Mayer (17). The analyses were carried out by adding 1 ml. of antiserum to each of a series of tubes containing increasing amounts of antigen in 1 ml. of buffered saline solution (pH 7.0). The contents were immediately mixed and stored 1 hr. at 37°C. and then 5 days at 5°C. The precipitates were centrifuged in a refrigerated centrifuge at 2°C., and the supernatants were carefully poured off. The precipitates were washed twice with cold saline and then transferred to micro-Kjeldahl flasks for determination of nitrogen.

Aliquots of castor bean extracts representing a range of from 0.75 to 1.4 mg. of the defatted meal were used in each analysis. In control tests with normal rabbit serum, nonspecific precipitates, probably due to ricin (21), began to appear when aliquots with more than 3.0 mg. of castor bean meal were used. Hence the range of concentrations used in the precipitin determinations of the allergen was well below the concentrations that gave measurable nonspecific precipitation. Heated castor-bean extracts

and extracts from commercial castor pomace did not precipitate normal rabbit serum.

In order to simplify the calculation of the allergen contents of castor beans, the precipitin curves for the purified allergen preparations were plotted on the solid basis, assuming that the water-, ash-, and carbohydrate-free allergen contained 19.0% nitrogen (2). For determination of the allergen content of castor beans, the nitrogen precipitated with the antiserum by an aliquot of the extract was referred to the standard precipitin curve, and the amount of allergen contained in the aliquot was read from the graph. The percentage of allergen in the sample was determined from the average derived from a minimum of 4 different, appropriately-spaced aliquots of the extracts.

Results

The precipitin curves for the allergenic fractions CB-13E and CB-1A (Figure 1) are typical of those

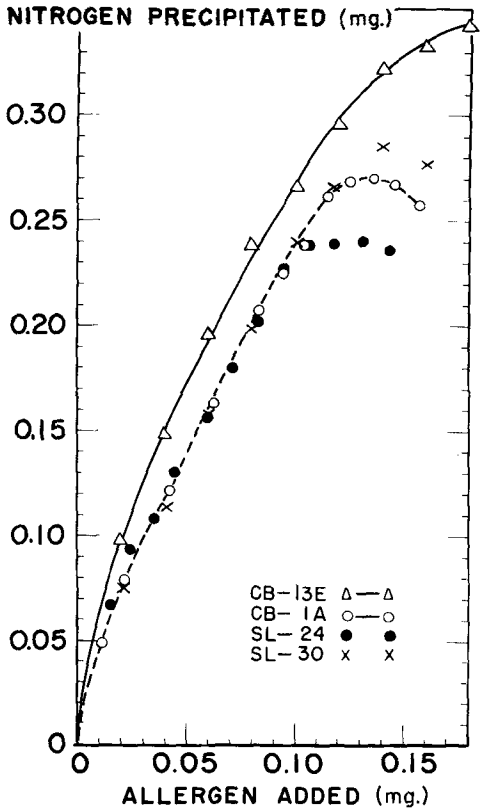

FIG. 1. The precipitin curves for castor-bean allergenic fractions (CB-13E and CB-1A) and castor bean extracts (SL-24 and SL-30) with CB-13E antiserum.

of other antigen-antibody systems. The CB-13E curve rose sharply to a maximum of 0.34 mg. of nitrogen precipitated per ml. of antiserum at about 0.18 mg. of CB-13E. Addition of more than this optional amount of CB-13E yielded progressively less precipitated nitrogen because of the formation of the soluble compounds. Fraction CB-1A precipitated a maximum of 0.27 mg. of nitrogen when about 0.13 mg. of CB-1A was added.

Maximal total nitrogen precipitated by extracts from 11 different samples of castor beans ranged from 0.24 mg. to 0.29 mg. Thus the precipitating characteristics of the castor bean extracts more nearly resembled those of CB-1A rather than CB-13E.[2] Accordingly the CB-1A precipitin curve was selected as the standard for calculation of the allergen content of castor bean extracts. The points superimposed on the CB-1A curve in Figure 1 record the results obtained when the calculated CB-1A contents of the various aliquots of two different castor bean extracts were plotted against the nitrogen precipitated with the antiserum. These results show that there was good agreement between the CB-1A standard precipitin curve and the calculated results of the 2 castor bean samples.

The maximal nitrogen precipitated by extracts from the nine remaining castor bean samples lay between the extremes of the two samples shown in Figure 1.

Effect of Heat on the Allergen Content of Castor Beans. Five ml. of a 1:20-extract of castor beans was sealed in a glass tube and heated at 100°C. for 1 hr. The copious white coagulum that resulted was centrifuged off. The nitrogen contents of the heated extract, SL-24H, and unheated extract, SL-24, were 1.24 and 1.89 mg. per ml., respectively. Figure 2

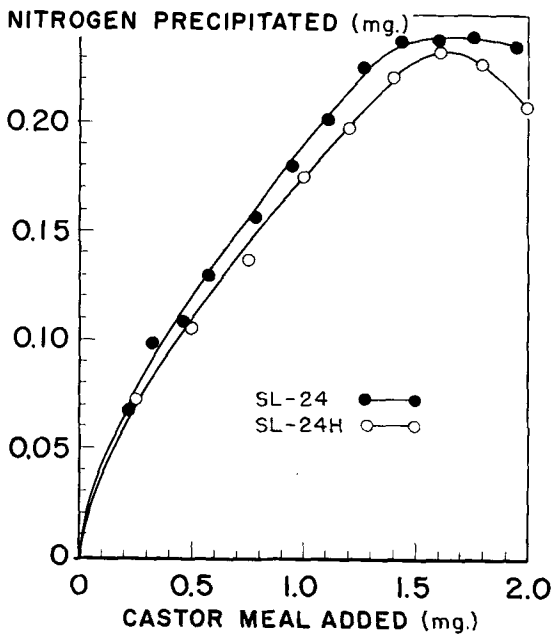

FIG. 2. Precipitin curves for unheated (SL-24) and heated (SL-24H) castor bean extract.

compares the precipitin curves with the two extracts. The precipitin curve with the heated extract indicates slight loss of activity or disappearance of part of the reactants. It is probable that some of the allergen may have been adsorbed and carried down with the coagulated proteins. These results show that

[2] The greater precipitating capacity of CB-13E is tentatively attributed to removal, during dialysis, of small but specific fractions of the allergenic protein that tend to inhibit formation of the immune precipitate. CB-1A, which represents the first major fraction in the separation of allergen from the more usual type of protein, probably contains these smaller fractions in about the same proportions as they appear in the seed.

the antiserum reacts little, if at all, with heat-coagulable proteins.

Allergen Content of Castor Beans. Table 1 records the nitrogen and the allergen contents of 11 samples, representing 10 different varieties of castor beans as determined by the precipitin method. The results are arranged in ascending order of the allergen content. The total nitrogen in the samples ranged from 10.56 to 11.67%. The allergen content ranged from 6.1 to 9.0% with an average of 7.7%. The allergen nitrogen accounted for 11 to 16% of the total nitrogen in the defatted seed and 27 to 40% of the nitrogen in the 1:20 extracts. There was no correlation between total nitrogen and allergen content or between extractable nitrogen and allergen content in these samples.

TABLE I

The Allergen Content of Decorticated, Defatted Castor Beans

Sample No.	Description	Nitrogen content	Allergen content[a]
		%	%
SL-27	Dawn, Breeders' seed, California, 1957	10.56	6.1
SL-26	Variety N-145-4 ♀, California, 1956	10.94	7.0
SL-32	From Iran, 1956	11.28	7.3
SL-34	From Australia, 1956	10.93	7.5
SL-24	Cimmaron, an inbred variety, Oklahoma, 1952	11.30	7.6
SL-33	From Argentina, 1938	11.67	7.7
SL-28	415 Hybrid, California, 1957	10.86	7.7
SL-29	Cimmaron, California, 1957	11.56	7.8
SL-30	Baker 296, California, 1957	11.35	7.9
SL-25	Variety U. S. 3/384-8-6-b, California, 1956	10.68	8.6
SL-31	From Bahia, Brazil, 1938	10.99	9.0

[a] The allergen content is reported on the solid basis, assuming that the ash-, water-, carbohydrate-free allergen contains 19.0% nitrogen (2).

To determine the reproducibility of the method, the analysis for allergen was repeated from the beginning on four samples, representing two low and two high levels of allergen. The results agreed within 1 to 4% of the originally determined values.

Allergen Content of Castor Pomace. Table II presents the nitrogen and allergen contents of six samples of commercial castor pomace. These analyses were carried out in the same way as for the raw beans. The estimated allergen content ranged from 0.092 to 4.2%. The nitrogen content of the 1:20 extracts of the pomaces ranged from 0.20 mg. to 0.83 mg. per ml. There was a direct correlation between the extractable nitrogen and the allergen content of the pomace.

TABLE II

The Allergen Content of Commercial Castor Pomace

Sample No.	Source	T.N. content of 1:20 extracts	Nitrogen content	Allergen content[a]
		mg./ml.	%	%
SL-36b	U.S.A., Sample C, 1958	0.24	6.45	0.092
SL-36a	U.S.A., Sample A, 1955	0.20	6.10	0.12
SL-37	England, 1959	0.31	6.01	1.3
SL-35	U.S.A., Sample B, 1958	0.39	5.40	2.0
SL-39	Brazil, 1959	0.72	7.30	4.1
SL-38	Brazil, 1959	0.83	7.31	4.2

[a] See footnote, Table I.

Heterogeneity of CB-1A and CB-13E. Evidence of the heterogeneity of the antigenic components of CB-1A and CB-13E was observed in the range in which both antigen and antibody could be detected in the supernatant solutions from the precipitin determinations. Hence the assays of the allergen content of the castor bean samples cannot be considered quantitatively exact. However the results were reasonable in magnitude and revealed varietal differences in castor beans that probably could not be shown by any other currently available method.

Discussion

A method was required for specific quantitative determination of the castor bean allergen to determine varietal differences in the allergen content of castor beans and to follow progressive loss of antigenic properties of the allergen following physical and chemical treatments. The antigenic properties of the castor bean allergen (4,5) as represented by CB-1A and its subfractions offered the possibility of development of an antiserum for quantitative measurement. An homogeneous preparation of allergen was not available for the development of the antiserum. However the system selected, making use of fractionated allergen preparations for preparation of rabbit antiserum and for construction of a standard precipitin curve, presented a method that gave reproducible results with raw castor meal and with castor pomace.

The allergen content of 10 different varieties of decorticated, defatted castor beans ranged from 6.1 to 9.0%. This range of allergen content probably does not offer an encouraging prospect for breeding out the allergen.

The allergen content of six samples of commercial castor pomace from foreign and domestic sources ranged from 0.092 to 4.2%. This range is equivalent to about 1 to 55% of the allergen content of solvent-extracted raw beans and reflects differences in the milling practices of different processors. It is apparent therefore that some current milling processes are capable of reducing the allergen content of castor pomace in a degree significant to the occurrence of castor bean allergy.

Results of a study of the effect of heat and hydrogen ion concentration on the precipitin reaction and reagin neutralization capacity of the castor bean allergen, CB-1A, has been reported elsewhere (13).

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Branched Carboxylic Acids from Long-Chain Unsaturated Compounds and Carbon Monoxide at Atmospheric Pressure[1]

EDWARD T. ROE and DANIEL SWERN, Eastern Regional Research Laboratory,[2] Philadelphia, Pennsylvania

Carbon monoxide at atmospheric pressure adds readily to the double bonds of certain long-chain unsaturated compounds in concentrated sulfuric acid to produce branched carboxylic acids. Unsaturated compounds studied were oleic acid, 10-hendecenoic acid, oleyl alcohol, methyl ricinoleate, and linoleic acid. A major component from the reactions of the last two compounds is the same.

Infrared spectrophotometry and gas-liquid chromatography have been the major tools employed in determining composition and structure of the products in addition to the usual chemical and physical determinations.

Both the concentration and quantity of sulfuric acid are critically important variables in determining the yields. Water is an essential reactant also and must be available throughout the reaction.

Carbon monoxide has been prepared and utilized *in situ* by a modification of Koch's (1) method in which the unsaturated compound mixed with formic acid is added to concentrated sulfuric acid or carbon monoxide from a cylinder is passed through sulfuric acid to which the unsaturated compound is added. A method for preparing methyl and butyl esters of carboxyl groups which are difficult to esterify is described.

Reaction mechanisms involving intermediate carbonium and oxocarbonium ions are proposed to account for the products.

DICARBOXYLIC ACIDS are important intermediates in the preparation of polymers, plasticizers, lubricants, and other functional fluids. At the present time the only commercial methods of preparation of dicarboxylic acids from fat sources are cleavage methods. The most important of these are ozonolysis and alkaline fusion although in the recent past chromic acid oxidation has also been employed.

One of the inherent drawbacks to a cleavage method is that it may not be sufficiently selective in its point of attack, thereby producing not just two but many products. Aside from the obvious difficulties in cleanly separating complex mixtures on a commercial scale, there is the ever-present problem that all of the cleavage products are not equally valuable and are salable only at unprofitable price levels, if at all. This is self-defeating from the fat-utilization standpoint inasmuch as dicarboxylic acids from nonfat sources are relatively low-priced substances and those prepared from fats must also be low cost to compete effectively.

An approach to which we have been giving considerable thought and attention is one in which the entire fatty molecule is employed. This demands that a) a group be introduced which is readily converted to the carboxyl group (the nitrile group is one example), or b) a direct carboxylation technique be utilized.

It has been known for some time that carbon monoxide under high pressure in the presence of the Lewis type of catalysts can be used for the direct carboxylation of alkenes and unsaturated fatty materials. The necessity for the use of high pressure and the relatively modest yields are discouraging features to such work. Our interest in carboxylation with carbon monoxide was revived by the interesting reports of Koch and Haaf (1), who showed that a wide variety of unsaturated compounds could be directly carboxylated with carbon monoxide and water at atmospheric or only moderate pressures in the presence of concentrated sulfuric acid, as solvent and reaction medium, a system with which we had worked extensively (2). Although Koch studied numerous alkenes, only two long-chain unsaturated fatty compounds were reported, namely, undecylenic and oleic acids. With the former, carboxylation was reported to give two C_{12} dicarboxylic acids, and limited characterizing data were supplied. With oleic acid, no characteristics of the products were given. The only statement concerning the reaction of oleic acid with carbon monoxide and water in the presence of concentrated sulfuric acid was that a C_{19} dicarboxylic acid was formed in good yield.

In this paper is described the direct carboxylation at atmospheric pressure of some unsaturated fatty acids, esters, and alcohols in concentrated sulfuric acid with either gaseous (cylinder) carbon monoxide or by an *in situ* method which is a modification of that described by Koch, in which formic acid is decomposed to carbon monoxide and water by the concentrated sulfuric acid while the unsaturated component is being added. As shown later in the experimental part, to obtain high carboxylation yields it is necessary to employ ratios of formic acid and sulfuric acid to double bond which are considerably different from those recommended (1). In a formal sense, as illustrated with oleic acid, the molecules of carbon monoxide and water add across a double bond to introduce a carboxyl group as a branch in the chain:

$$CH_3-(CH_2)_7-CH=CH-(CH_2)_7COOH + CO + H_2O \xrightarrow{H_2SO_4} CH_3-(CH_2)_x-\underset{\displaystyle COOH}{\underset{|}{CH}}-(CH_2)_y-COOH \quad (x+y=15)$$

[1] Presented at the annual meeting, American Oil Chemists' Society, Dallas, Tex., April 4–6, 1960.
[2] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.