Attempts to obtain a good saponification number for the combined fraction failed, due to the extreme difficulty of accomplishing complete saponification of wax esters (*5*). However, a modification of the method of Chibnall *et al.* (*3*) gave sufficient saponification for isolation and identification of the products. Five-tenths gram of the combined ester (melting point 68 to 70° C.) was placed in 100 ml. of ethyl alcohol to which had been added 3.25 grams of sodium. The mixture was refluxed for 8 hours and then was extracted with 3 portions of Skellysolve B. These extracts were combined and concentrated to 50 ml., and the resulting solution was poured into 50 ml. of a 10% ethanolic potassium hydroxide solution. After refluxing for one hour, 3 grams of calcium chloride were added and refluxing was continued for 2 hours. Water was added and the mixture was extracted with hot benzene. The benzene extract was evaporated to dryness and the residue recrystallized from acetone.

The alcohol thus isolated had a melting point of 81.5–2.5° C. Its acetate was prepared and had a melting point of 65.5–6.0° C. The alcohol was converted by chromic acid oxidation to a fatty acid melting at 85–7° C. From these data, it was concluded that the alcohol component of the wax ester probably is a mixture similar to the free alcohol mixture (Table III).

The saponification mixture, from which the alcohols had been removed by benzene extraction, contained a precipitate of the calcium salts of the fatty acids liberated from the ester. This precipitate was removed by filtration and was washed twice with boiling benzene. The precipitate was acidified with 10% hydrochloric acid, the fatty acids were extracted with ether, and the ether solution was evaporated to dryness. After recrystallization from acetone, the fatty acids melted at 79 to 84° C. Further purification was accomplished by agitating the fatty acids with ethanolic sodium hydroxide to form the insoluble sodium soaps. The precipitate was washed with ether and acidified. The fatty acids then were extracted with ether and the ether solution was evaporated to dryness. The residue had a melting point of 84–6° C. (compare with data of Table III). Repetition of this technique did not change the melting point.

The neutralization equivalent of the purified fatty acid was 430. The neutralization equivalent of octacosanoic acid is 424, and that of triacontanoic acid is 452. The melting point and neutralization equivalent indicate that the fatty acid component of the wax ester probably is a mixture of *n*-hexacosanoic, *n*-octacosanoic, and *n*-triacontanoic acids.

Discussion

Warth (*9*) has reported the following composition for carnauba wax: esters, 80%; free alcohol, 12%; paraffins, 1%; lactone, 3%; and resins, 4%. From the present study, the composition of sorghum grain wax appears to be: esters, 49%; free alcohols, 46%; and paraffins, 5%. Sorghum grain wax may contain other components, because minor constituents might have been lost during isolation and resolution.

The components of the two waxes which perhaps cause the principal differences in their physical properties would seem to be esters and free alcohols. If the percentage of free alcohols in sorghum grain wax could be reduced, the properties of the modified wax might be more similar to those of carnauba wax. This study indicates that the free alcohols can be removed with little difficulty. Removal of either of the other major components from the wax would be more difficult.

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Literature Cited

(1) Blair, E. H., Mitchell, H. L., Silker, R. E., *Ind. Eng. Chem.* **45,** 1104 (1953).
(2) Bunger, W. B., Kummerow, F. A., *J. Am. Oil Chemists' Soc.* **28,** 121 (1951).
(3) Chibnall, A. C., Piper, S. H., Pollard, A., Smith, J. A. B., *Biochem. J.* **25,** 2095 (1931).
(4) Koonce, S. D., Brown, J. B., *Oil & Soap* **21,** 231 (1944).
(5) Markley, K. S., "Fatty Acids," Interscience, New York, 1947.
(6) Piper, S. H., Chibnall, A. C., Hopkins, S. J., Pollard, A., Smith, J. A. B., *Biochem. J.* **25,** 2072 (1931).
(7) Piper, S. H., Chibnall, A. C., Williams, E. F., *Ibid.*, **28,** 2175 (1934).
(8) Pollard, A., Chibnall, A. C., Piper, S. H., *Ibid.*, **25,** 2111 (1931).
(9) Warth, A. H., "Chemistry and Technology of Waxes," Reinhold, New York, 1947.

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VEGETABLE OIL EXTRACTION

Toxicity of Amine-Extracted Soybean Meal

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EXPERIMENTS designed to identify a possible factor in soybean meal that could reverse the antimalarial activity of *m*-chloridine (*14*) led to the discovery that soybean-meal residues after extraction with organic amines were highly toxic for chicks. Hexane-extracted soybean meal is widely used as feed for domestic animals with no ill effect, but soybean meal extracted with trichloroethylene is toxic for cattle (*7, 9, 11, 13, 15*), sheep (*5*), chickens (*2, 4, 8*), and guinea pigs (*3, 10*). The toxic substance produced by trichloroethylene extraction of soybean meal may be *S*-(dichlorovinyl)-L-cysteine (*6*). Commercial preparations of trichloroethylene contain an inhibitor, sometimes an organic amine, to prevent the corrosive action of degradation products. It is therefore of interest that residues from the extraction of soybean meal with organic amines were far more toxic for the chick than residues from extraction with trichloroethylene.

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Materials and Methods

Except for experiments described in Tables IV and V, day-old New Hampshire Red chicks were used throughout. They were fed variants of a purified diet designed by Briggs *et al.* (*1*), which is adequate for the growth of chicks. This diet is composed of casein, gelatin, methionine, and glucose, and is supplemented with adequate vitamins and minerals. Test substances were added to the diet by replacing an equal weight of glucose (Cerelose). Unless otherwise stated, chicks were weighed on days 0, 6, and 10, the last being the final day of the experiment.

The soybean meal used was a com-

Residues of soybean meal, after extraction with primary, secondary, or tertiary organic amines, were toxic for the chick when incorporated, at levels of 20 to 40%, in an otherwise adequate diet. Approximately 80% of chicks fed amine-extracted residues of soybean meal died within 10 days. Chicks fed residues after extraction with acetone, ethyl alcohol, or trichloroethylene survived and appeared normal. Those fed the extracts (except the butylamine extract) were normal. Triethylamine-extracted cottonseed meal, black-eyed peas, soybean protein, zein, and Cerelose were toxic in varying degrees. Triethylamine-extracted gelatin and casein were well tolerated. Twelve amino acids failed to react with triethylamine when refluxed in the solvent for 24 hours; L-lysine monohydrochloride under the same conditions showed evidence of change in composition.

mercial preparation which had been hexane-extracted and toasted. All extractions, with the exception of the pilot experiments in Table I, were done in a large Soxhlet apparatus and were run long enough to allow at least 20 changes of solvent in the upper chamber. The soybean protein was partially purified and contained approximately 6% carbohydrates, less than 2.5% salts (determined as ash), 90% protein (N × 6.25), and less than 0.5% fat.

Results

The result of extracting 100 grams of soybean meal with 200 ml. of eight organic solvents is shown in Table I. Extracts were dried to a constant weight. Considerably more material was extracted by nitrogen-containing solvents, except triethylamine, than by non-nitrogen-containing organic solvents. There were large differences in the amount of material extracted by the various amines.

The data in Table I were used as a basis for compounding diets in which extracts and residues were tested. In large-scale experiments the residues after extraction were air-dried to constant weight, so there was some assurance that most of the residual-free solvent had been driven off. The gummy extracts, however, were not completely freed of solvent or water even after prolonged efforts at evaporation.

The results of feeding chicks test diet supplemented with residues and extracts of soybean meal are shown in Table II. Residues of soybean meal after extraction with organic amines were associated with the death of about 80% of the chicks in the 10-day period. Deaths occurred as early as the fourth day and most chicks were dead by the end of the first week. With so few survivors, weight changes are meaningless. On the other hand, chicks survived and appeared normal when the diet was supplemented with the residues of soybean meal after extraction with acetone, ethyl alcohol, and redistilled trichloroethylene, or with any of the extracts of soybean meal, except *n*-butylamine.

Table III summarizes results obtained when chicks were fed diets supplemented with natural substances extracted with triethylamine. Extracted cottonseed meal was as toxic as soybean meal, indicating that the toxicity of amine-extracted materials is not limited to leguminous plants. Triethylamine-extracted black-eyed peas were not as toxic as soybean meal or cottonseed meal.

Soybean protein extracted with triethylamine was toxic in two experiments. In one the mortality rate was similar to that observed in chicks fed extracted soybean meal; in the other, most chicks survived the experimental period but gained little or no weight. The difference in the response in these two experiments cannot now be explained.

Not all proteins behaved as did soybean proteins. Triethylamine-extracted zein was only slightly toxic, in that the chicks gained weight poorly. Triethylamine-extracted gelatin and casein were well tolerated. Cerelose extracted with the amine inhibited the growth of chicks significantly.

To determine whether there was a chemical reaction between amino acids and triethylamine, producing any toxic product, 2 grams each of 13 amino acids were refluxed individually for 24 hours in 20 ml. of triethylamine: glycine, DL-phenylalanine, L-glutamic acid, L-cystine, L-leucine, DL-serine, DL-tyrosine, L-lysine monohydrochloride, L-arginine monohydrochloride, DL-methionine, L-proline, DL-threonine, and DL-tryptophan. With the exception of L-lysine monohydrochloride, all amino acids were recovered quantitatively unchanged in appearance or melting point. With lysine 1.93 grams of a tan-colored substance were recovered; from the colorless filtrate 0.01 mg. of a yellow oil with a sharp acid odor was recovered.

Varying levels of triethylamine-extracted soybean meal were tested in chick diet (Table IV). The chicks gained weight normally at levels of 2 and 8% triethylamine-extracted soybean meal, but at the 20% level a significant reduction in rate of weight gain was observed over a 4-week period. The inhibition of weight gain was only slightly reversed in diets further supplemented with dried liver, dried yeast, or ten times the usual amount of vitamin D.

Table I. Material Extracted from Typical Sample of Hexane-Extracted Soybean Meal by 200 Ml. of Organic Solvents

(24-hour refluxing)

Solvent	Wt. of Extract, G.
Acetone	1.13
Trichloroethylene	1.14
Dioxane	1.78
Isopropyl alcohol	2.06
n-Butylamine	13.60
Diethylamine	5.18
Triethylamine	1.20
Pyridine	7.46

Table II. Results of Feeding Chicks Diets Containing Various Residues and Extracts of Soybean Meal

Diet Supplement	% Diet	No. of Expts.	Total Chicks	Deaths (10 Days)
Soybean meal	41	7	210	3
Acetone residue	39	1	30	0
Ethyl alcohol residue	39	2	60	0
Trichloroethylene residue	40	3	90	1
Pyridine residue	34	1	25	20
Triethylamine residue	39	1	25	20
Diethylamine residue	38	1	24	20
Butylamine residue	34	1	30	20
Acetone extract[a]	1.5	1	30	0
Ethyl alcohol extract[a]	1.5	2	60	0
Trichloroethylene extract[a]	1.0	4	120	1
Pyridine extract[a]	4.5	2	60	2
Triethylamine extract[a]	1.5	2	60	2
Diethylamine extract[a]	2.5	1	30	1
n-Butylamine extract[a]	7.0	1	30	7

[a] All extracts were concentrated under vacuum to a thick sirup but not dried to constant weight. They were fed at levels equivalent to 41% of soybean-oil meal.

Table III. Results Obtained with Chicks Fed Diet C-2 Containing Natural Substances Extracted with Triethylamine Substituted for Glucose

Diet	Day 6 to Day 10,[a] Av. Wt. Gain	10 Days, No. Died/ No. Started
+ 20% soybean protein	+14.6	0/60
+ 20% extracted soybean protein	+ 1.0	27/60
+ 20% gelatin	+13.0	0/30
+ 20% extracted gelatin	+14.3	1/30
+ 20% casein	+16.5	0/30
+ 20% extracted casein	+14.6	0/30
+ 20% zein	+24.2	0/30
+ 20% extracted zein	+11.3	0/30
+ Cerelose	+10.0	0/30
+ extracted Cerelose	+ 1.4	0/30
+ 40% cottonseed meal	+16.7	0/30
+ 40% extracted cottonseed meal	− 1.8	21/30
+ 40% black-eyed peas	+15.9	0/30
+ 40% extracted black-eyed peas	+ 6.4	4/30
+ 40% soybean meal	+23.1[b]	

[a] Based on only chicks that survived. [b] Average gain of chicks in 12 experiments.

Table IV. Results with Triethylamine-Extracted Soybean-Oil Meal Fed to Female New Hampshire Chicks

(6 chicks per group. Supplements added to purified diet at expense of glucose)

Expt. No.	Supplement to Diet C-2[a]	Grams at 4 Weeks (±S.E.)	No. Dead at 4 Weeks
1	None	316 ± 23	0
	2% TEA-extracted soybean meal	330 ± 11	0
	8% TEA-extracted soybean meal	300 ± 19	0
	20% TEA-extracted soybean meal	174 ± 24[b]	0
	0.5% triethylamine	343 ± 24	0
2	None	362 ± 30	0
	20% TEA-extracted soybean meal	130 ± 26	3
	20% TEA-extracted soybean meal + 5% dried liver + 5% dried yeast + 10 times vitamin D	179 ± 26	1
	20% regular soybean meal	426 ± 17	0

[a] Soybean meal and supplements added at expense of glucose.
[b] At autopsy birds on 20% TEA-extracted soybean meal had a very high (89%) incidence of enlarged proventriculus and enlarged glands within proventriculus.

Table V. Results with Osborne-Mendel Female Rats Fed Triethylamine-Extracted Soybean Meal

(5 animals per group)

Supplement to Purified Rat Diet[a]	Weight, Grams		No. Rats Dead at 4 Weeks
	0 weeks	4 weeks	
50% soybean meal	42	175	0
50% TEA-extracted soybean meal	40	50	1

[a] Purified diet contained casein, sucrose, minerals and all vitamins required for growth. Soybean-oil meal added at expense of sucrose.

It is also significant that 0.5% triethylamine in the diet did not inhibit growth.

Triethylamine-extracted soybean meal was tested in diets fed to rats (Table V). At a level of 50% of the diet, only one of five rats failed to survive 4 weeks, but the remainder gained almost no weight.

Discussions

These experiments demonstrate that the residues of natural products after extraction with triethylamine were toxic for chicks and rats. Triethylamine-extracted soybean meal is also toxic for guinea pigs (*12*).

No simple chemical explanation would define the reactions leading to toxic products, especially as tertiary amines yielded products as toxic as those produced by primary and secondary amines. Furthermore, the toxic substances were not soluble in the amines, with the possible exception of *n*-butylamine. No attempt was made to extract the toxic substances with non-nitrogen-containing organic solvents or inorganic solvents. Residual-free amines can be ruled out as an explanation of toxicity because of the results reported in Table IV, because the extracts were dried to constant weight, and because there was probably more free amine in the incompletely dried extracts than in the residues. Starvation because of distaste cannot surely be ruled out, because paired feedings were not done which chicks. However, in pair-fed guinea pigs, the inhibition of growth is not due to failure to eat (*12*).

The explanation of the toxicity is probably not as simple as the combination of amine and proteinaceous materials: Not all amine-extracted proteins were toxic; amine-extracted glucose was toxic; the soybean protein used had some carbohydrate and other impurities; and no reaction occurred between any of 13 amino acids, with the possible exception of lysine, and triethylamine after refluxing for 24 hours.

The toxicity observed with amine-extracted glucose is possibly different from that associated with amine-extracted protein and the toxicity of amine-extracted soybean meal may be a combination of both toxicities.

Perhaps the most difficult fact to explain is that the tertiary amines, normally considered unreactive, produced substances as toxic as the primary and secondary amines. This may mean that amines function only as catalysts.

Many critical experiments have yet to be done to determine the nature of the toxic substances and of the toxicity.

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Literature Cited

(1) Briggs, G. M., Spivey, M. R., Keresztesy, J. C., Silverman, M., *Proc. Soc. Exptl. Biol. Med.* **81,** 113 (1952).
(2) Eveleth, D. F., Goldsby, A. I., *J. Am. Vet. Med. Assoc.* **123,** 38 (1953).
(3) Eveleth, D. F., Holm, G. C., *Ibid.*, **122,** 377 (1953).
(4) Hill, E. G., Misra, K. P., Canfield, T. H., Johnson, E. L., Perman, V., Pritchard, W. R., Sautter, J. H., Schultze, M. O., *Poultry Sci.* **35,** 686–92 (1956).
(5) Holm, G. C., Eveleth, D. F., Dinusson, W. E., *J. Am. Vet. Med. Assoc.* **122,** 380 (1953).
(6) McKinney, L. L., Weakley, F. B., Eldridge, A. C., Campbell, R. B., Cowan, J. C., Picken, J. C., Jr., Biester, H. B., *J. Am. Chem. Soc.* **79,** 3932 (1957).
(7) Picken, J. C., Jr., Jacobsen, N. L., Allen, R. S., Biester, H. E., Bennett,

P. C., McKinney, L. L., Cowan, J. C., J. AGR. FOOD CHEM. **3,** 420 (1955).
(8) Pritchard, W. R., Davis, O. S., Taylor, D. B., Doyle, L. P., *Am. J. Vet. Research* **17,** 771–77 (1956).
(9) Pritchard, W. R., Rehfeld, C. E., Sautter, J. H., *J. Am. Vet. Med. Assoc.* **121,** 1 (1952).
(10) Pritchard, W. R., Sauer, F., Rehfeld, C. E., Perman, V., Sautter, J. H., Wada, S., Schultze, M. O., *Am. J. Vet. Research* **17,** 448–54 (1956).
(11) Sautter, J. H., Rehfeld, C. E., Pritchard, W. R., *J. Am. Vet. Med. Assoc.* **121,** 73 (1952).
(12) Sherman, J. F., Taylor, D. J., Bond, H. W., Greenberg, J., Nadel, E. M., unpublished data.
(13) Stockman, S., *J. Compt. Pathol. Therap.* **29,** 95 (1916).
(14) Taylor, D. J., Greenberg, J., *Proc. Soc. Exptl. Biol. Med.* **90,** 551 (1955).
(15) Twiehaus, M. J., Leasure, E. E., *Vet. Med.* **46,** 428 (1951).

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ALCOHOLIC FERMENTATION

Reduction of Aldehydes during Alcoholic Fermentation. Application to Processing of Heads

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Aldehydes are effectively reduced by the action of yeasts during alcoholic fermentation; this provides a simple and effective method for processing heads separated during the distillation of wine into brandy. Acetal was used as the aldehyde source to characterize the ability of 14 species or strains of fermentative yeasts to reduce aldehydes. All yeasts were able to complete the fermentation of grape musts after acetal additions equal to 0.23% aldehyde and all except two at the 0.46% added aldehyde level. The effectiveness of reduction varies, but in all cases of completed fermentations amounts to 90% or more of the quantity added with a corresponding supplement of ethyl alcohol production.

NUMEROUS MINOR CONSTITUENTS are contained in fermented alcoholic liquids. These minor compounds may be regarded as impurities or as flavor materials, depending on their nature and concentration and the intended use of the product. The compounds less volatile than ethyl alcohol accumulate at the top of concentrating columns during distillation. Customarily a low-boiling fraction, termed heads, is separated from the main product during the distillation of wine into brandy.

In the production of brandy, the principal impurities in the heads fraction are acetaldehyde, acetal, ethyl acetate, and acetaldehyde–sulfurous acid, if sulfites have been used during fermentation. In the California wine and brandy industry, sulfur dioxide or bisulfites are commonly used during fermentation, especially in the distilling material used for the production of neutral brandy for addition to dessert wines.

The heads fraction generally contains less than 1% of volatile compounds, but in plants that use an aldehyde-concentrating column it may contain as much as 10 to 15% aldehydes. In either case, ethyl alcohol constitutes the major portion of the liquid, and its recovery in usable form has been a problem in processing procedures. Various procedures, including chemical treatment and further concentration by distillation, have been and still are in use. They generally cost too much or are ineffective in removal of impurities.

Acetaldehyde and its compounds are generally the most objectionable impurities. They impart sharp odors and hot tastes to alcoholic beverages. Ethyl acetate may often constitute a larger percentage of the nonalcohol compounds than the aldehydic ones, especially from liquids fermented without sulfites. However, it is less objectionable and can be considered to have a higher tolerance in alcoholic beverages.

This report includes studies underlying a procedure for the successful removal of the aldehydic portions of heads by reduction during alcoholic fermentation. Heads collected during distilling operations are recycled into subsequent active fermentations of distilling material. Under suitable conditions removal or reduction of aldehydes is complete and a corresponding supplement of alcohol is produced from the aldehydes.

The ability of yeast to reduce numerous aldehydes including acetaldehyde was summarized by Harden (*9*). Acetaldehyde added to a fermenting medium should be reduced to ethyl alcohol in view of the well-established concept that acetaldehyde is the direct precursor of ethyl alcohol in alcoholic fermentation (*2*). Genevois, Peynaud, and Ribéreau-Gayon (*4*) and Ribéreau-Gayon, Peynaud, and Lafon (*11*) have shown that progressive additions of acetaldehyde to fermenting grape juice or must produce increased amounts of minor constituents, including acetic, lactic, and succinic acids and 2,3-butylene glycol, as well as ethyl alcohol. Gade (*3*) patented a process for recycling the forerun or heads from distillation of fermented sulfite liquor into subsequent fermentations. Sundman (*12*) recommended the addition of heads to subsequent sulfite liquor fermentations in order to bind the free sulfite in the liquor and inhibit the formation of more aldehydes, and thus increase the yield of ethyl alcohol.

Guymon and Nakagiri (*5*) showed that the aldehydes in heads from brandy distillations were removed by adding them to fermenting grape musts, most effectively when addition was delayed until the end of the yeast growth phase. These authors (*6*) also reported on the separate effects and limiting concentrations of acetaldehyde, acetal, and ethyl acetate, three principal components of heads, added to grape must both before and during fermentations. These results were applied to the processing of heads in pilot scale and wine industry experiments by Guymon and Pool (*8*).

A particular strain of *Saccharomyces cerevisiae* var. *ellipsoideus*, a wine yeast called Montrachet and widely employed in the California wine industry, was used in these studies. Its behavior constitutes a basis of comparison with that of 13 other yeast species or strains.

Acetal affects fermentations in the same manner and degree as acetaldehyde, if the concentrations used are expressed on an equivalent aldehyde basis (*6*). Hence, acetal, somewhat easier to prepare and handle quantita-