The weight and composition of livers from rats fed diets containing thermally oxidized oil were also studied, and a significantly larger liver weight–body weight ratio was found in the animals fed the diets containing the thermally oxidized oil. One hundred and ten animals which had been fed the basal diet that contained 20% thermally oxidized corn oil for periods of two to four weeks gave an average liver weight–body weight ratio of .0466. Ninety-six animals fed diets which contained 20% fresh corn oil for similar lengths of time had an average liver–body weight ratio of .0352. No difference in lipide content or total solids was noted in the livers. The livers of animals which had been fed the thermally oxidized oil diet contained 3.95% lipide and had a total solids content of 32.23% while those fed the fresh oil diet contained 4.10% lipide and had a total solids content of 32.26%. The histopathological examination of the livers from animals fed the thermally oxidized oil diet indicated very little change or none.

This increase in liver–body weight ratio has been noted in animals fed oil oxidized at 100°C. and in animals fed heat-polymerized oil. No explanation has been given, but it would appear that some change must be taking place in normal metabolism which leads to the larger liver–body weight ratio. The increased ratio was found even in animals which were transferred to a grain basal diet after three weeks on a diet containing thermally oxidized oil. Twelve male rats were fed a diet containing 20% thermally oxidized oil for three weeks and then transferred to a grain diet and kept on it until they attained a body weight of 275–340 g. A second group of 12 animals were fed the basal diet containing 20% fresh corn oil for three weeks and then transferred to the grain diet and kept on it for the same period of time as the first group. The liver–body weight ratio of the first group on a thermally oxidized oil diet was .043 while that of the second group was .0301. Further studies are necessary in order to determine the cause of the increased liver–body weight ratio.

Summary

The present results indicated that the thermal oxidation products from the polyunsaturated fatty acids, primarily linoleic acid, are responsible for much of the loss of nutritional value in thermally oxidized edible oils. Oils which have a high linoleic acid content are more likely to undergo thermal oxidative damage than those with lower linoleic contents. Also the ratio of linoleic acid to total unsaturation has some effect on the nutritive stability of the oil when it has been thermally oxidized. An oil with a high iodine value but with a low linoleic acid value appears to be more stable to thermal oxidation than an oil with an iodine value one half as great but with most of the unsaturation in the oil caused by linoleic acid.

The products formed during thermal oxidation which cause the loss of nutritional value are those which do not form urea-inclusion compounds. They are probably polymeric in nature, but thermally oxidized oils also contain carboxylic acids and carbonyl groups which might cause some of the nutritional loss observed when thermally oxidized oils are fed.

The rate of *in vitro* hydrolysis of the thermally oxidized corn oil by pancreatic lipase, also the rate of absorption from the intestine of the male rats, were found to be decreased. However the percentage of absorption in 24 hrs. was the same with both fresh and thermally oxidized oil.

The liver–body weight ratio of rats fed a diet containing the thermally oxidized oil were found to be significantly larger than the liver–body weight ratio in animals fed diets containing fresh oil. However the livers of animals fed the thermally oxidized oil diets did not differ in lipide percentage or total solid content, and histopathological investigations did not show any abnormal conditions.

REFERENCES

1. Johnson, O. C., Sakuragi, T., and Kummerow, F. A., J. Am. Oil Chemists' Soc., *33*, 433 (1956).
2. Crampton, E. W., Farmer, Florence A., and Berryman, F. Marion, J. Nutrition, *43*, 431 (1951).
3. Crampton, E. W., Common, R. H., Farmer, Florence A., Berryman, F. Marion, and Wiseblatt, J. Nutrition, *44*, 177 (1951).
4. Kaunitz, Hans, Slanetz, C. A., and Johnson, R. E., J. Nutrition, *55*, 577 (1955).
5. Kaunitz, Hans, Slanetz, C. A., Johnson, R. E., Knight, H. B., Saunders, D. H., and Swern, Daniel, J. Am. Oil Chemists' Soc., *33*, 630 (1956).
6. Sunderland, E., J. Oil and Colour Chem. Assoc., *28*, 137 (1945).
7. Bradley, T. F., J. Oil and Colour Chem. Assoc., *30*, 225 (1947).
8. Paschke, R. F., and Wheeler, D. H., J. Am. Oil Chemists' Soc., *26*, 278 (1949).
9. Paschke, R. F., and Wheeler, D. H., J. Am. Oil Chemists' Soc., *32*, 473 (1955).
10. Floyd, D. E., Paschke, R. F., Wheeler, D. H., and Baldwin, W. S., J. Am. Oil Chemists' Soc., *33*, 609 (1956).
11. Raju, N. V., and Rajagopalan, R., Nature, *176*, 513 (1955).
12. Lassen, S., Bacon, E. K., and Dunn, H. J., Arch. Biochem., *23*, 1 (1949).
13. Brice, B. A., Swain, Margaret L., Herb, S. F., Nichols, P. L., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., *29*, 279 (1954).
14. Swern, Daniel, Ind. Eng. Chem., *47*, 216 (1955).
15. Saunders, D. H., Knight, H. B., Swern, Daniel, Kaunitz, H., Slanetz, C. A., and Johnson, R. E., presented at Am. Oil Chemists' meeting, September, 1956.
16. Weinstein, S. S., and Wynne, A. M., J. Biol. Chem., *112*, 641 (1935).
17. Johnson, O. C., and Kummerow, F. A., presented at Am. Oil Chemists' meeting, September, 1956.

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The Pigments of Crude Cottonseed Oils. II. Nitrogen-Containing Pigments Derived from Gossypol

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THE GOSSYPOL in crude cottonseed oils of commercial origin exists mainly in a combined form (1), but oils containing uncombined or native gossypol can be obtained from cottonseed by mild extraction-procedures (3). Native gossypol in fresh oils undergoes rapid reaction with oil constituents to yield alkali-insoluble derivatives, but if the oils are treated immediately with p-aminobenzoic acid, an oil-insoluble Schiff base is formed instead. Crude oils treated in this manner may be stored at elevated temperatures for extended periods of time and still yield refined and bleached oils of low photometric color and normal stability (2). Apparently p-aminobenzoic acid can compete successfully for gossypol in some of the alkali-insoluble derivatives.

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These observations made it of importance to search for reactive materials in the crude oils which can combine with gossypol and therefore might be partly responsible for producing the dark color observed in oils which have been subjected to extremes of temperature during the processing operations or during storage. Accordingly concentrates of the alkali-insoluble pigments of dark, crude cottonseed oils were prepared by low-temperature, solvent crystallization of the glycerides, a technique which has been found useful for preparing tocopherol concentrates from cottonseed oils (10).

Ideal conditions exist for promoting a browning reaction during the processing of cottonseed for oil and meal; that is, there are simultaneously present, at elevated temperatures, protein, carbohydrate, and gossypol. Therefore a solid fraction obtained from the concentrate by treatment with petroleum ether was acid-hydrolyzed and examined by paper chromatography in order to separate and identify, if possible, the nitrogenous materials present.

Experimental

Cottonseed Oil Pigment Concentrate. A sample of dark, crude, solvent-extracted cottonseed oil weighing 4.8 kg. was divided into 480-g. portions, and each was dissolved in 6.1 liters of C. P. acetone. The flask was immersed in a dry-ice, acetone bath, and the solution was stirred vigorously while it was being chilled to —60°C. The glycerides and phosphatides which separated were removed after one hour by filtering in a stainless steel Buchner funnel, fitted with a metal frit bottom and an outer jacket cooled by dry-ice, acetone mixture. The combined filtrates were concentrated to about three liters by evaporating at 40°C. under reduced pressure in a nitrogen atmosphere. A small amount of solid material was obtained from the concentrate when the solution was chilled to —60°C. and filtered. This filtrate was further concentrated as described above, and the remaining acetone was removed from the residue by keeping it under high vacuum at room temperature for several hours. There were obtained 61.2 g. of a viscous, red-brown oil, having an odor strongly reminiscent of bread crust and containing 24.3% gossypol, 0.15% nitrogen, and no phosphorus.

Solid Pigment Fraction. Ten grams of the pigment concentrate were dissolved in 70 ml. of dry diethyl ether, and the solution was added over a period of 20–30 min. to three liters of vigorously stirred, low-boiling petroleum ether, b. p. 35–60°C. After one hour the precipitate, which separated, and the solvent were reduced to a thin slurry with the aid of a filter stick. The slurry was transferred to a 250-ml. centrifuge bottle, and the precipitate was collected by centrifuging at 2,000 r.p.m. for 15 min. The solid material was washed with 200-ml. portions of petroleum ether, b. p. 35–60°C., by resuspending and centrifuging until the supernatant liquid was colorless. The solid was air-dried and finally freed of traces of solvent by keeping it in vacuum over phosphorus pentoxide and mineral oil. There were obtained 1.17 g. of dark olive-green powder, which contained 27.4% gossypol, 0.43% nitrogen, and no phosphorus.

The analyses of the crude, solvent-extracted oil and the fractions obtained from it are summarized in Table I.

Absorption spectra of the original oil (in isooctane) and of the pigment concentrate (in cyclohexane) are shown in Figures 1 A and 1 B. The absorption spectra of the solid fraction in chloroform and after treatment of the solution with aniline are shown in Figures 2 A and 2 B, respectively.

TABLE I

	Gossypol	Total N[c]	Total P[d]	Free Fatty Acids
	%	%	%	%
Cottonseed oil (solvent-extracted)	1.06[a]	0.02	0.082	1.54
Pigment concentrate	24.3[a]	0.15	0.0	.....
Solid fraction	27.4[b]	0.43	0.0	.....

[a] Determined according to the methods of Pons *et al.* (7).
[b] Determined according to the method of King and Thurber (5).
[c] Determined by the Kjeldahl digestion method and using the method of Winkler (11) for absorption of the ammonia.
[d] Determined according to the method of Pons *et al.* (8).

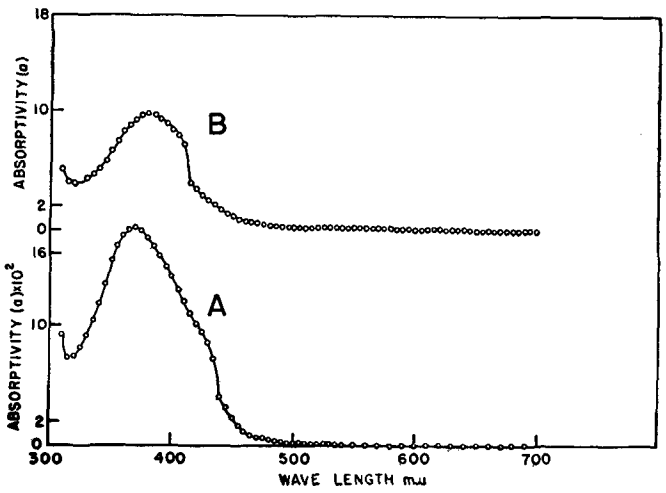

FIG 1.

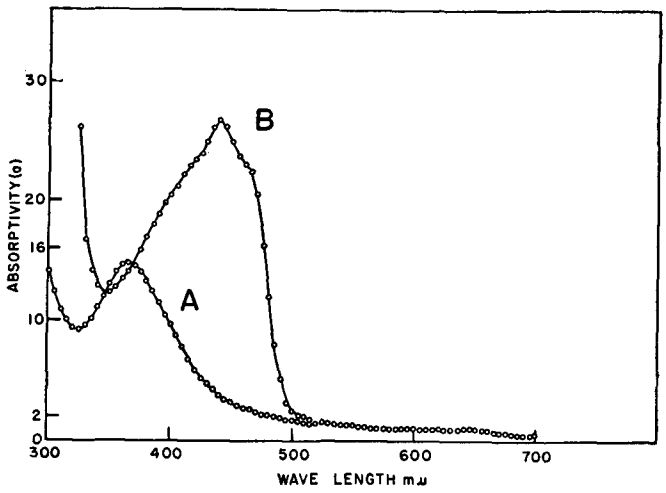

FIG. 2.

Hydrolysis of Solid-Pigment Fraction. To 225 ml. of nitrogen-free formic acid, prepared by treating one liter of the 90% acid with 2 ml. of concentrated sulfuric acid and distilling in vacuum, was added 1.51 g. of the solid-pigment fraction, and the mixture was heated under reflux for one hour. The red-brown solution was cooled somewhat, 225 ml. of 6 *N* hydrochloric acid (distilled twice in borosilicate glass equipment) were added, and refluxing was continued for

19 hrs. The cooled suspension of black solid material was filtered through a Buchner funnel fitted with a fritted glass disc, and the filtrate was evaporated to dryness at 40°C. in vacuum. A total aggregate of 6.87 g. of solid-pigment fraction was hydrolyzed in this manner. The residues from the evaporated filtrates were combined, dissolved in 10 ml. of water (distilled in borosilicate glass equipment), and aliquots of this solution were analyzed for nitrogen by the Kjeldahl method. A 71% recovery of nitrogen from the solid-pigment fraction was obtained.

Paper Chromatography of the Hydrolysate. Ten μl. of the hydrolysate were applied in 2 μl. aliquots to a circle 2 mm. in diameter on 36 x 12 cm. strips of unwashed Whatman[2] No. 1 filter paper. The spots were dried by a warm air blast between successive applications. The hydrolysate spot was flanked by a spot of a suitable mixture of known amino acids (external standards) prepared from 6 μl. of each stock solution which contained 20 μM. of each amino acid per ml. After a preliminary equilibration in the vapors of the one-phase solvent mixture[3] H or K of Hardy *et al.* (4) for 45 min., the papers were irrigated in the ascending manner for 16 hrs. The chromatograms were then dried and treated with ninhydrin (4). The treated chromatograms showed seven purple spots and one red-brown spot when solvent mixture K was used to irrigate the papers. A marked depression of the R_f values of almost all the ninhydrin-colored spots was suspected and confirmed by the method of internal standards. Nevertheless phenylalanine could be identified by its R_f value in solvent mixtures H and K, and aspartic acid was detected by the characteristic royal blue color it produces with the cyclohexylamine of solvent mixture H. Proline and hydroxyproline were apparently absent. The presence of phenylalanine and of aspartic acid was confirmed by the method of internal standards and by use of the solvent mixture H for irrigating the papers. A definite increase in the density of the colored spot was noted for both amino acids although only the phenylalanine spot gave the correct R_f value when compared with that of both the internal and external standard. This method did not permit any further positive identification by paper chromatography. Therefore the hydrolysate was desalted by ion exchange, and the desalted material was analyzed by the same technique of paper chromatography.

Desalting the Hydrolysate. Approximately 5 ml. of the hydrolysate were diluted to 25 ml. with distilled water and adjusted to pH 3.5 with 6 *N* hydrochloric acid. This solution was applied to a 2 x 5-cm. column of Dowex-50[2] resin (12% cross-linked) in the hydrogen phase and allowed to run through under slight pressure (1 ml. per minute). The resin was eluted in the manner described by Mueller *et al.* (6), and the four fractions obtained were evaporated to 0.5 ml. or less under reduced pressure in a nitrogen atmosphere at 40°C.

Glycine in Fraction 2 was identified by its R_f value in solvent H and by the characteristic red-brown color it produces with cyclohexylamine. No separate identification of any of the basic amino acids in Fraction 4 could be made although at least two were present. Visual inspection of Fraction 4 indicated that considerable quantities of sodium chloride had separated, hence the desalting was not complete.

[2] It is not the policy of the Department to recommend the products of one company over those of any others engaged in the same business. This name is furnished merely for your convenience and information.

[3] Solvent H: 1-butanol, butanone, water, cyclohexylamine (10:10:5:2, v/v); solvent K: 1-propanol, butanone, water, diethylamine (10:10:5:2, v/v).

Results and Discussion

The solid pigment fraction obtained from the crude oil contained only 0.43% nitrogen, which represents a small fraction, 3%, of the total nitrogen of the oil. The greater part of the nitrogen remained with the glycerides and phosphatides since the nitrogen of the concentrate from which the solid fraction was prepared represents about 10% of the total nitrogen of the oil. The situation with regard to gossypol is different since the gossypol of the pigment concentrate represents about 32% of the total amount in the oil and that of the solid fraction about 4%. The greatest loss of gossypol occurred in the preparation of the solid fraction from the pigment concentrate. Inspection of Table I indicates that a concentration of both gossypol and nitrogen was effected throughout the fractionation.

The absorption spectra of the crude oil, the pigment concentrate, and the solid-pigment fraction are rather similar in appearance and have a single maximum at 370–380 mμ (Figures 1 A, 1 B, and 2 A). When a chloroform solution of the solid fraction is treated with aniline, its absorption spectrum is transformed into that of dianilinogossypol with a maximum at 440 mμ (Figure 2 B). This may be interpreted to mean a displacement by aniline of the gossypol from an addition compound with some constituent(s) of the oil since the absorption maximum of pure gossypol in chloroform lies at 364–366 mμ. The liberated gossypol then reacts immediately with the aniline to form the Schiff base, dianilinogossypol.

The presence of phenylalanine, aspartic acid, glycine, and other amino acids combined with gossypol in the solid-pigment fraction supports the above contention and is not unexpected, considering the ease of formation of Schiff bases from gossypol and aliphatic amines (9).

It may be concluded that at least a part of the color of dark, crude cottonseed oils arises from the interaction of gossypol with the amino acids from the seed protein. Other factors such as the presence of oxygen, trace amounts of copper and iron, and the opportunity for the browning reaction to take place during processing, that is, simultaneous presence of carbohydrate and protein, may contribute also to color formation.

Summary

Dark, crude cottonseed oil has been shown to contain a mixture of ninhydrin-sensitive substances combined with gossypol. Phenylalanine, aspartic acid, and glycine have been obtained by acid hydrolysis of the addition compound, followed by paper chromatography of the hydrolysate. The presence of other amino acids was indicated, but their identification could not be established with certainty; proline and hydroxyproline were apparently absent.

The possibility that part of the color of dark, crude oils arises from gossypol-α-amino acid interaction is discussed.

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REFERENCES

1. Boatner, Charlotte H., Hall, C. M., O'Connor, R. T., Castillion, Leah E., and Curet, M. C., J. Am. Oil Chemists' Soc., *24*, 97–106, 276–283 (1947).
2. Dechary, J. M., Kupperman, R. P., Thurber, F. H., and O'Connor, R. T., J. Am. Oil Chemists' Soc., *31*, 420–424 (1954).
3. Frampton, V. L., and Webber, H. H., Oil and Soap, *23*, 318–323 (1946).
4. Hardy, T. L., Holland, D. O., and Nayler, J. H. C., Anal. Chem., *27*, 971–974 (1955).
5. King, W. H., and Thurber, F. H., J. Am. Oil Chemists' Soc., *33*, 169–171 (1956).
6. Mueller, G. C., Bowman, G., and Herranen, A., Anal. Chem., *27*, 1357–1358 (1955).
7. Pons, W. A. Jr., Hoffpauir, C. L., and O'Connor, R. T., J. Am. Oil Chemists' Soc., *28*, 8–12 (1951).
8. Pons, W. A. Jr., Stansbury, M. F., and Hoffpauir, C. L., Assoc. Off. Agr. Chemists' J., *36*, 492–504 (1953).
9. Shirley, D. A., and Sheehan, W. C., J. Org. Chem., *21*, 1069–1070 (1951).
10. Singleton, W. S., and Bailey, A. E., Oil and Soap, *21*, 224–226 (1944).
11. Winkler, L. W., Z. Angew. Chem., *26*, 231–232 (1913).

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Report of the Spectroscopy Committee, 1956-57

DURING THE YEAR ending with the 48th Annual Meeting of the American Oil Chemists' Society in New Orleans the Spectroscopy Committee met in two committee sessions. The first of these was held during the annual fall meeting on September 25 in the Hotel Sherman, Chicago, Ill. Six of the 10-member committee were present: N. D. Fulton, W. E. Link, Robert D. Mair, B. N. Rockwood, Donald H. Wheeler, and Robert T. O'Connor. The second meeting was held at the Roosevelt hotel in New Orleans, La., April 29, 1957, during the 48th annual meeting. Seven members, or their duly authorized alternates, were present: Mr. Fulton, S. F. Herb, K. E. Holt (representing Mr. Link), C. E. Ireland (representing Hans Wolff), Mr. Mair, Dr. Wheeler, and Mr. O'Connor. Joseph McLaughlin Jr., arrived at the 48th Annual Meeting too late to attend the Spectroscopy Committee meeting but met with the chairman at a later date.

During the past few years the committee has been considering three principal problems:

a) modification of the present A.O.C.S. Tentative Method Cd 7-48 for polyunsaturated fatty acids to simplify it and to extend its scope.

b) establishment of a method for the determination of polyunsaturated fatty acids in the presence of large quantities of conjugated constituents, *i.e.*, the determination of linoleic acid in tung oil (containing *ca.* 80% conjugated eleostearic acid).

c) investigation of the infrared absorption method for the determination of *trans* acids in the presence of nonconjugated *cis*-unsaturated and of saturated constituents.

These three problems were discussed at both meetings, and the committee has been actively engaged in collaborative work regarding them during the periods preceding each of these meetings.

Modification of A.O.C.S. Tentative Method Cd 7-48

Based on collaborative study, a completely revised edition of A.O.C.S. Tentative Method Cd 7-48 was approved by the Spectroscopy Committee and accepted by the Uniform Methods Committee. The revised procedure, which both simplifies and extends the scope of the method, is included in the Official and Tentative Methods of the A.O.C.S. as Cd 7-48 (rev. April 1956).

During the year two additional minor revisions have been suggested by members of the committee. The first is the adoption of a more accurate value for the absorptivity for computing preformed conjugated diene. The value, 119, used in the method, is based on original measurements of *trans*-10, *trans*-12-linoleic acid. Advances, particularly in infrared absorption spectroscopy, have shown that preformed conjugation in natural products is not entirely the *trans, trans*-acid, that probably little, if any, *cis, cis*-acid is present, and that very probably a mixture of *cis, trans*- and *trans, trans*-acids accounts for the measured dienoic conjugation. Recent work has shown that upon iodine treatment an equilibrium mixture consisting of 68% *trans, trans*-conjugation and 32% *cis, trans*-conjugation is obtained. Using absorptivities of 119 and 94 for these components, respectively, the absorptivity for the equilibrium mixture is 110. Following discussions of this problem, both in Chicago and in New Orleans, and with correspondence between the two meetings, it was finally agreed that the absorptivity of 110 is the most probable value and the one to be substituted for the obviously too high value of 119. This change has been recommended to the Uniform Methods Committee for adoption.

The second minor modification involves the mathematical expressions for the percentage of linolenic and arachidonic acids in the simplified procedure Cd 7-48 (rev. April 1956). Adhering strictly to the rule that "background" correction should not be made when the absorptivity is greater than 1 and should be applied when it is less than 1 results in an equation having background corrections for the alkali-isomerized portion (where the absorptivities are rather large) but not for the preformed conjugation (where they are small). Subtracting the absorption before isomerization from the value after isomerization is, in itself, a very suitable "background" correction. However, when one of these values has been "corrected," the absorptivity after isomerization is undercorrected. At the New Orleans meeting it was decided that these equations should be modified. The necessary minor changes have been recommended to the Uniform Methods Committee for adoption.

Determination of Polyunsaturated Fatty Acids in the Presence of Large Quantities of Conjugated Constituents

During the first half of the past year the committee undertook the collaborative investigation of a method for the determination of linoleic acid in the presence of large quantities of preformed conjugation, a determination outside the scope of Cd 7-48 (rev. April 1956). Five samples, three mixtures of *alpha*- and *beta*-tung oils and samples of pure *alpha*- and *beta*-eleostearic acids, were sent to each of the 10 members of the Spectroscopy Committee. Results were obtained from eight. Collaborators were asked to determine conjugated diene, *alpha*-eleostearic acid,