milligrams per 50 ml. by the corresponding absorbance, taken from the curve.

$$\% \text{ Free Gossypol} = \frac{S \times (C\text{-}B) \times F}{10 \times V}$$

where S = volume of the final colored solution, and
V = volume of the sample aliquot,
assuming the use of a 1.0-gm. sample extracted with 50 ml. of solvent.

### Preparation of the Calibration Curve

From a burette add a series of duplicate aliquots of the stock solution of gossypol to two sets of 50 ml. volumetric flasks. Use duplicate 1, 2, 5, 10, 15, and 20 ml. aliquots. Prepare blanks and develop and measure the color as described above. Plot absorbance against milligrams of gossypol per 50 ml. Use rectilinear paper.

### Notes

1. The choice of aliquot and volumetric flask size depends upon the free gossypol expected and the sensitivity required. The following table will serve as a guide.
   The figures assume the use of 25-mm. cuvettes with a Coleman Jr. spectrophotometer.

| % Gossypol Expected | Ml. of Aliquot | Size of Volumetric (ml. of final solution) |
|---|---|---|
| 0.10 | 5 | 25 |
| 0.10-0.15 | 2 | 25 |
| 0.15-0.20 | 2 | 50 |

2. Occasionally, as a result of processing variables, some samples may exhibit strongly absorbing gossypol blanks from excessive amounts of dianilinogossypol and/or other pigments extracted by the 70% acetone. In the event that the absorbance of the gossypol blank is 0.40 or greater, smaller aliquots must be taken for solutions B and C.
3. Avoid the use of a mouth pipette for handling aniline. Redistillation should be done either in a fume hood or in a well ventilated room.

### Method—Total Gossypol

The procedure for this method is described by Pons, Hoffpauir, and O'Connor (5). The changes developed in this work are to use aniline to develop the color and to double the concentrations of the oxalic acid and barium acetate solutions. Dianilinogossypol is more difficult to hydrolyze than protein-bound gossypol.

### Summary

A modification of the Pons and Guthrie method for determining free gossypol in cottonseed materials is presented. The use of aniline, rather than p-anisidine, as the color-producing reagent is necessary if meals containing dianilinogossypol are to be analyzed correctly. Increasing the reaction temperature eliminates a serious weakness in the method and results in greater accuracy. The proposed method is applicable to all types of cottonseed meal now marketed.

A slight modification of the Pons method for total gossypol is also presented. By doubling the strength of the oxalic acid used to hydrolyze bound gossypol and by using aniline to develop the color, the method is made applicable to chemically treated meals containing dianilinogossypol.

### Acknowledgment

Grateful acknowledgment is made to the Southern Regional Research Laboratory for the pure gossypol used in this work and to C. L. Hoffpauir and W. A. Pons for their help.

REFERENCES

1. Pons, W. A., and Guthrie, J. D., J. Am. Oil Chemists' Soc., *26*, 671-676 (1949).
2. Lyman, C. M., *et al.*, Ind. Eng. Chem., Anal. Ed., *15*, 489 (1943).
3. Smith, F. H., *ibid.*, *18*, 43 (1946).
4. Carruth, F. E., J. Biol. Chem., *32*, 87 (1917).
5. Pons, W. A., *et al.*, J. Am. Oil Chemists' Soc., *27*, 390-393 (1950).

[Received April 21, 1954]

# Comparison of Methods for Determining Fatty Acid Oxidation Produced by Ultraviolet Irradiation[1]

CAROLYN B. KENASTON, KARL M. WILBUR, ATHOS OTTOLENGHI, and FREDERICK BERNHEIM,
Department of Zoology, Duke University, and Department of Physiology and Pharmacology,
Duke University Medical School, Durham, North Carolina

ULTRAVIOLET light catalyzes the oxidation of pure unsaturated fatty acids (2) and the lipids of skin (5, 15), liver slices, and mitochondria (12). The extent of such oxidation can be measured as peroxide, aldehyde, or, in the earlier stages, the degree of conjugation (2), and in the case of linoleic and linolenic acids by a colorimetric reaction with thiobarbituric acid (9). This last reaction is simple and sensitive (3, 13, 15) and has been used with tissues (1, 9, 10, 16) and for dairy products (3, 6, 13). Glavind and Hartman (7) compared the thiobarbituric acid (TBA) reaction with the Kreis test for aldehyde and the dichlorophenolindophenol reaction for peroxide on fatty acids from cod liver oil, methyl oleate hydroperoxide, epihydrin aldehyde, and benzoyl peroxide. They reported that the TBA test paralleled the aldehyde test rather closely but that there was no agreement with peroxide values, as determined by their method. On the other hand, Pool and Prater (14) found a parallel development of peroxides as determined by Wheeler's peroxide method and the substance responsible for the color in the Kreis test. These relationships have been examined further in the present study by comparing peroxide, aldehyde, conjugation, and TBA values for linolenate, linoleate, and oleate oxidized by ultraviolet light.

### Methods

Samples of methyl linolenate, methyl linoleate, and methyl oleate (Hormel Foundation, sealed under vacuum) were analyzed after exposure in thin layers to ultraviolet irradiation for various periods (G-E Precision lamp, No. 18A-T10, 6 volts, with glass envelope removed; no filter, distance 10.7 cm., 25.5-27.0°C.). Stock solutions were prepared from samples of unirradiated and irradiated esters diluted with freshly distilled methanol and were kept under oxygen-free nitrogen at 0°C. until analysis. Aliquots (17.9 mg.) of irradiated and unirradiated esters were analyzed by each of the methods given below in rapid succession to minimize differences in degree of autoxidation.

[1] Supported by a grant from the Atomic Energy Commission.

The following analytical methods were used:

*Peroxide.* Lundberg and Chipault's method (11) with the following modifications. The dental dam covering the reaction flask was replaced by a rubber stopper with two glass tubing outlets: one for gassing, which reached the bottom of the flask; and the other for addition of samples, which was flush with the bottom of the stopper. Thin-walled rubber tubing was attached to their outer ends. Both outlets were clamped off after gassing the flask with nitrogen, and additions were made by introducing the needle of a 1-ml. syringe through the rubber outlet directly into the flask.

*Aldehyde.* Method of Pool and Prater (14). Optical density was measured in a Beckman spectrophotometer at 550 mμ at slit width 0.022.

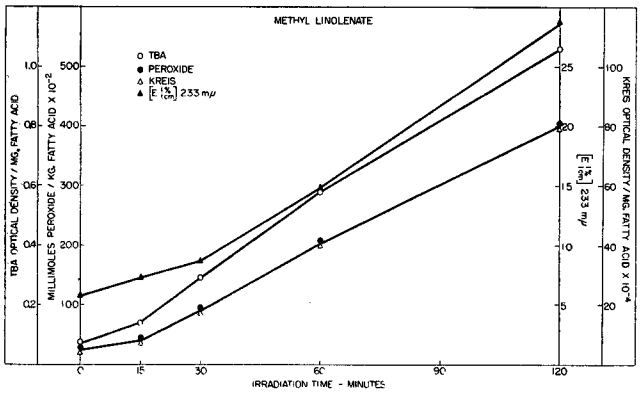


FIG. 1.

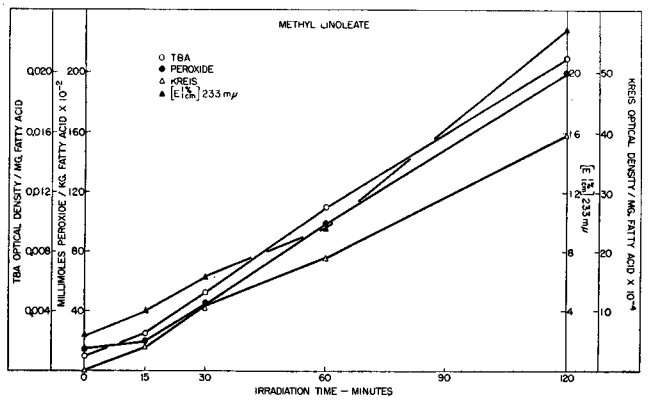


FIG. 2.

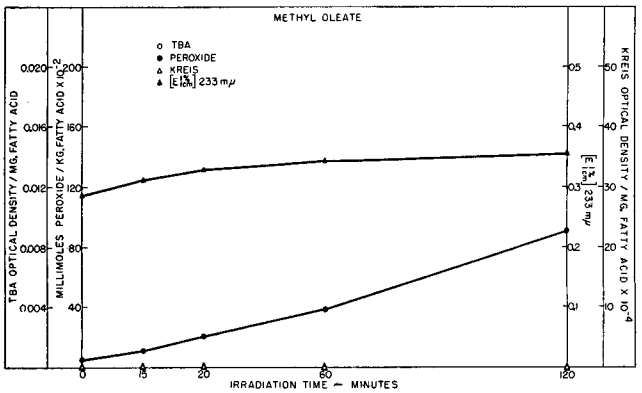


FIG. 3.

FIGS. 1, 2, 3. Oxidation of methyl linolenate, methyl linoleate, and methyl oleate with ultraviolet light. For each time period aliquots of 17.9 mg. were removed and measured by each of the four methods for determining oxidation products (see Methods for details).

*Conjugation.* Method of Brode *et al.* (4).

*TBA Reaction.* (15). Heating was carried out for 15 min. Optical density was measured at 534 mμ at slit width 0.026. Because of the sensitivity of the TBA method, methyl linoleate and methyl linolenate were diluted 10 times and 100 times, respectively. The Beer-Lambert law is followed in the concentration ranges employed.

## Results

The oxidation of methyl linoleate and methyl linolenate (Figures 1 and 2) irradiated with ultraviolet light for various periods was measured by analysis of peroxide, aldehyde, conjugation, and TBA chromagen. With increasing periods of irradiation, values obtained by all four methods increased in parallel and in direct proportion to the period of irradiation. Conjugation was almost completely of the dienoic type for both esters. A comparison of TBA values of linolenate and linoleate for corresponding periods of irradiation shows that the TBA reaction is 60-100 times more sensitive for linolenate than for linoleate. Under the conditions of irradiation employed the peroxide, aldehyde, and conjugation values of linolenate were approximately 1.2-2.2 times those for linoleate. The sensitivity of the TBA reaction for linolenate at any given peroxide value was found to be 30- to 80-fold greater than for linoleate. This selectivity of the TBA test confirms earlier findings (15).

TBA and aldehyde values for methyl oleate irradiated under the same conditions increased so little that the values as plotted on the same scale as the others in Figure 3 are essentially zero. Peroxide and dienoic conjugation values showed greater increases with the same periods of irradiation (Figure 3). The magnitude of peroxide formation, while small, is such that it cannot be attributed directly to linoleate and linolenate present as contaminants (0.02% dienoic conjugated acid present) (8).

## Discussion

These experiments have further defined the limits of the TBA test. It is the most sensitive of all the chemical methods used for the oxidation products of linolenic and linoleic acids, but, like the Kreis test, it is relatively insensitive to the oxidation products of oleic acid. The values obtained with the TBA test parallel those obtained with the other methods in showing an increased concentration of oxidation products of linolenic and linoleic acids with increase in time of ultraviolet irradiation. Thus the TBA test as used on tissues and other biological products appears to be a reliable method for estimating the oxidation products of linolenic and linoleic acids present. The mechanism of the test as previously suggested is probably the condensation of thiobarbituric acid with aldehydes formed when the oxidation products are heated. The colored compound produced is proportional to the concentration of these products in a fairly wide concentration range.

## Summary

The thiobarbituric acid (TBA) reaction for fatty acid oxidation has been compared with Lundberg and Chipault's method for peroxides, the Kreis test for aldehydes, and with the degree of conjugation, using fatty acid esters exposed to ultraviolet light for various periods. The TBA test paralleled the other methods for methyl linolenate and methyl linoleate but was essentially negative for methyl oleate oxidation.

The sensitivity of the TBA test for linolenate was 30-80 times that for linoleate at the same peroxide values. The TBA test appears to be a reliable method of estimating the oxidation products of linolenic and linoleic acids in tissues and other biological material.

REFERENCES

1. Abramson, H., J. Biol. Chem., *178*, 179-183 (1949).
2. Bergström, S., and Holman, R. T., Adv. in Enzymology, *8*, 425-457 (1948).
3. Biggs, D. A., and Bryant, L. R., Canad. J. Technol., *31*, 138-145 (1953).
4. Brode, W. R., Patterson, J. W., Brown, J. B., and Frankel, J., Ind. Eng. Chem., Anal. Ed., *16*, 77-80 (1944).
5. Dubouloz, P., Dumas, J., and Vigne, J., Comp. rend. soc. biol., *144*, 1080-1081 (1950).
6. Dunkley, W. L., and Jennings, W. G., J. Dairy Sci., *34*, 1064-1069 (1951).
7. Glavind, J., and Hartman, S., Acta Chem. Scand., *5*, 975-976 (1951).
8. Gunstone, F. D., and Hilditch, T. P., J. Chem. Soc., 1022-25 (1946).
9. Kohn, H. I., and Liversedge, M., J. Pharmacol. Exptl. Therap., *82*, 292-300 (1944).
10. Kuck, S. K. D., Arch. Biochem., *26*, 351-354 (1950).
11. Lundberg, W. O., and Chipault, J. R., J. Am. Chem. Soc., *69*, 833-836 (1947).
12. Ottolenghi, A., Bernheim, F., Wilbur, K. M., and Kenaston, C. B., unpublished data (1954).
13. Patton, S., and Kurtz, G. W., J. Dairy Sci., *34*, 669-674 (1951).
14. Pool, M. F., and Prater, A. N., Oil and Soap, *22*, 215-216 (1945).
15. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., Arch. Biochem., *24*, 305-313 (1949).
16. Zauder, H., J. Pharmacol. Exptl. Therap., *101*, 40-46 (1951).

[Received May 26, 1954]

# Summary of the Collaborative Work on Total Nitrogen

Joint Committee of Association of Official Agricultural Chemists and American Oil Chemists' Society

*Objective.* The study was intended to provide the basis for elimination of numerous variations in the procedure for total nitrogen in fertilizers and feeds. Specifically, the work compared copper with mercury as a catalyst, compared boric acid with standard acid for absorption of ammonia, and gave some comparison of the heating devices used in digestion of the sample.

*Collaborators.* Of the 27 laboratories which participated in the work, 12 had expressed a preference for copper as the catalyst (Group 1), 11 others had expressed a preference for mercury (Group 2), and the remainder had indicated no preference. Four preferred boric acid over standard acid and used it in analyzing all samples. The participating laboratories were as follows:

Harry R. Allen, Agricultural Experiment Station, University of Kentucky, Lexington, Ky.
B. W. Beadle, Geo. W. Gooch Laboratories, Los Angeles, Calif.
A. T. Blackwell, Davison Chemical Corporation, Baltimore, Md.
W. A. Bridgers, Southern Testing Company, Wilson, N. C.
P. D. Cretien, Texas Testing Laboratories, Dallas, Tex.
H. A. Davis, Department of Agriculture and Biological Chemistry, University of New Hampshire, Durham, N. H.
E. A. Epps Jr., Department of Agriculture and Immigration, Baton Rouge, La.
C. W. Gehrke, Department of Agricultural Chemistry, Agricultural Experiment Station, Columbia, Mo.
C. W. Gourley, Beacon Milling Company, Cayuga, N. Y.
E. R. Hahn, Hahn Laboratories, Columbia, S. C.
G. C. Henry, Law and Company, Atlanta, Ga.
T. H. Hopper, Southern Regional Research Laboratory, New Orleans, La.
W. J. Ingram, Department of Agriculture, Salem, Ore.
L. A. Koehler, State Laboratories Department, Bismarck, N. D.
J. C. Konen, Archer-Daniels-Midland, Minneapolis, Minn.
H. R. Kraybill, American Meat Institute, Chicago, Ill.
R. F. Larsen, Feed and Fertilizer Laboratory, Department of Agriculture, Boise, Idaho.
C. A. Lathrap, Curtis and Thompkins Ltd., San Francisco, Calif.
C. L. Manning, Fort Worth Laboratories, Fort Worth, Tex.
C. V. Marshall, Department of Agriculture, Ottawa 2, Canada.
J. R. Mays Jr., Barrow-Agee Laboratories, Memphis, Tenn.
V. E. Munsey, Dept. Health, Education and Welfare, Food and Drug Administration, Washington, D. C.
B. O. Pattison, Pattison's Southwest Laboratories, Harlingen, Tex.
Willis Richerson, Department of Agriculture, Oklahoma City, Okla.
E. H. Tenent, Woodson-Tenent Laboratories, Memphis, Tenn.
W. S. Thompson, Department of Agriculture Laboratories, Reynoldsburg, O.
E. R. Toby, Agricultural Experiment Station, Orono, Me.

*Samples.* Ten samples were selected as follows: cottonseed meal, soybean meal, dried blood, digester tankage, meat scrap, fish meal, commercial fertilizer, commercial feed, ground hoofs, and S-benzylthiuronium chloride (m.p. 172°C.; N content, calculated 13.82%). This compound, recommended by Ogg and Willits [Ind. Eng. Chem., Anal. Ed., *18*, 334 (1946)], was prepared in the Purdue Laboratory by the method of Danbury [J. Am. Chem. Soc., *58*, 1004 (1936)] and was purified by recrystallizing three times from alcohol.

*Directions to Collaborators.* Laboratories were asked to number each burner to be used for digestion of samples, determine the time required to bring 250 ml. of water to a rolling boil and record the number of the burner used for each sample digested. With each catalyst, copper and mercury, samples were to be analyzed in duplicate and the analysis repeated at least one week later, for a total of four analyses per catalyst. Standard acid and standard alkali were to be prepared independently, then checked, one against the other. Boric acid was compared with standard acid on two samples (7 and 8). Collaborators were given detailed digestion procedures which were identical except for catalyst (copper *vs.* mercury). The mercury procedure was as follows:

Place weighed sample (0.7 to 2.2 g.) in digestion flask. Add 0.7 g. HgO (or 0.65 g. metallic Hg), 10 g. powd. $K_2SO_4$ (or anhyd. $Na_2SO_4$) and 25 ml. con. $H_2SO_4$. If sample larger than 2.2 g. is used, increase $H_2SO_4$ 10 ml. for earh gram of sample. Place flask in inclined position and heat gently until frothing ceases (if necessary, add small amount of paraffin to reduce frothing); boil briskly, until solution clears and then for at least 30 min. longer.

Cool, add *ca.* 200 ml. of water and 25 ml. of sulfide or thiosulfate soln. to ppt. Hg (thiosulfate or sulfide may be mixed with the NaOH before addition to flask). Mix thoroughly and cool to 25°C. or below. Add a few Zn granules to prevent bumping, tilt flask, and add layer of NaOH (25 g. solid reagent or sufficient soln. to make contents strongly alkaline) without agitation. Immediately connect flask to distilling bulb on condenser (end of condenser is immersed in standard acid or 50-75 ml. boric acid soln. in receiver) and rotate to mix contents thoroughly, then heat until all $NH_3$ has distilled (at least 150 of distillate). Titrate excess std. acid in distillate with std. alkali soln. (Me red indicator), or if boric acid is used in receiver, titrate direct with std. acid (bromocresol green—Me red or methylene blue—Me red indicator).