retically, the unsaturated monocarbonyls resulting as secondary degradation products from linoleate hydroperoxides; i.e., deca-2,4-dienal and oct-2-enal, could upon subsequent oxidation account for α-keto heptanal and α-keto octanal. However, according to the literature the presence of the 11-hydroperoxide of methyl linoleate is questionable; hence, the origin of α-keto heptanal and/or α-keto octanal from the further oxidation of oct-2-enal is doubtful. α-Keto heptanal, which was one of the more abundant dicarbonyls present, might also be produced by direct attack at the 12, 13 double bond of the linoleate molecule.

At the present time none of the observed dicarbonyls have been directly associated with flavor defects in oxidizing lipid systems. It is evident, however, that these compounds could assume a significant role in food systems. Dicarbonyls can serve as key reactants in the Strecker degradation of amino acids as well as in nonenzymatic browning reactions, both of which are important deterioration mechanisms in food materials.

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REFERENCES

1. Patton, S., and G. W. Kurtz, J. Dairy Sci. *34*, 669–674, (1951).
2. Sinnhuber, R. O., T. C. Yu and Te Chang Yu, Food Res. *23*, 626–633, (1958).
3. Forss, D. A., E. A. Dunstone and W. Stark, J. Dairy Res. *27*, 373–380, (1960).
4. Schepartz, A. I., and B. F. Daubert, JAOCS *27*, 367–373, (1950).
5. Kawahara, F. K., H. J. Dutton and J. C. Cowan, JAOCS *29*, 633–635, (1952).
6. Lillard, D. A., and E. A. Day, JAOCS, *41*, 549–552 (1964).
7. Corbin, E. A., Anal. Chem. *34*, 1244–1247, (1962).
8. Schwartz, D. P., J. Chromatog. *9*, 187–194 (1962).
9. Wolfrom, M. L., and Arsenault, G. P., Anal. Chem. *32*, 693–695, (1960).
10. Cobb, W. Y., J. Chromatog. *14*, 512–513, (1964).
11. Cobb, W. Y., L. M. Libbey and E. A. Day, in press.
12. Dahle, L. K., and Holman, R. T., Anal. Chem. *33*, 1960–1961, (1961).
13. Yu, T. C., and R. O. Sinnhuber, Ford Technol. *11*, 104–108, (1957).
14. Keith, R. W., and E. A. Day, JAOCS *40*, 121–124, (1963).
15. Day, E. A., and D. A. Lillard, J. Dairy Sci. *43*, 585–597, (1960).
16. Shriner, R. L., R. C. Fuson and D. Y. Curtin, "The Systematic Identification of Organic Compounds. A. Laboratory Manual," 4th ed., John Wiley & Sons, New York, 1957, p. 219.
17. Schwartz, D. P., A. R. Johnson and O. W. Parks, Microchem J. *6*, 37–44, (1962).
18. Schwartz, D. P., O. W. Parks and M. Keeney, Anal. Chem. *34*, 669–671, (1963).
19. Jones, L. A., J. C. Holmes and R. B. Seligman, Anal. Chem. *28*, 191–198, (1956).
20. Hoffman, G., JAOCS *38*, 1–3, (1961).

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Methods for the Determination of Cyclopropenoid Fatty Acids V. A Spectrophotometric Method for Cottonseed Oils Based Upon the Halphen-Test Reaction

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Abstract

A spectrophotometric method of analysis for the quantitative estimation of cyclopropenoid fatty acids in cottonseed oil based upon the Halphen-test reaction has been described. Various parameters involved in the reaction have been investigated and two pigment fractions responsible for the characteristic Halphen-test cherry-red color have been isolated. The method is applicable to relatively small amts of sample material. The average deviation from the actual cyclopropenoid acid contents as determined by the stepwise HBr titration method was less than ± 0.02% in both the refined and crude oil series.

Introduction

In 1897 Halphen (1,2) found that cottonseed oil contains a minor constituent which produces a cherry-red color when the oil is heated with a mixture of amyl alcohol, carbon disulfide, and sulfur. This color test has recently been attributed to the presence of cyclopropenoid constituents, presumably malvalic and sterculic acid moieties (3–5).

Previous attempts (5–7) to develop an accurate quantitative method based upon this colorimetric test have not been entirely successful. In general, these methods have the advantage of requiring only a small amt of sample material. However, they lack precision since the color developed is a composite of several colored components, the relative proportions of which are dependent upon a number of hard-to-control parameters. This results in a variability in color response from test to test on the same sample. In addition, no authentically pure standard substance has been available which is sufficiently stable for calibration purposes, i.e., to establish a reliable, accurate relationship between the cyclopropenoid acid concn and the color intensity. A recently developed method of analysis (8) based upon a stepwise HBr titration now makes it possible to determine the cyclopropenoid acid content of cottonseed oils to within 0.01%. This titration method affords a means of setting up reliable calibration standards.

There is still a need for a method of analysis applicable to very small samples. Such a method would be advantageous, for example, in the development of a procedure for the cyclopropenoid analysis of cottonseed meals since very large amts of meal would be necessary for the isolation of relatively small amts of the fatty acid constituents.

The purpose of the present investigation was to study the various parameters involved in the Halphen-test reaction in order (*a*) to establish a set of reaction conditions under which the intensity of the color obtained for cottonseed oils by the Halphen reaction could be reproduced to within reasonable limits and (*b*) to relate the intensity of the color, as determined spectrophotometrically, to the known cyclopropenoid acid content of a series of cottonseed oils.

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Reagents and Apparatus

Reagent grade *n*-butyl alcohol (freshly distilled), carbon disulfide, morpholine, and technical grade roll sulfur were used in all analyses. The refined cottonseed oils were commercial name-brand oils purchased on the retail market. The crude oils were hexane-extracted oils and, in order to cover a wider range of concns, included six fractions extracted successively from the same batch of cottonseed meats (9). The reaction flasks were specially fabricated low-actinic glass flasks constructed by extending the neck of 50-ml Erlenmeyer flasks with 25-mm tubing terminating with a 24/40 standard-tapered joint at the top to give an overall length of approximately 8 in. Vented caps were constructed for the flasks by sealing a 1-in. length of 1-mm capillary tubing to the hollow glass stopper. The bath used was an opaque glass jar containing 10 liters of oil maintained at 110 ± 0.5C in a fume hood.

Experimental Procedures

Crude Cottonseed Oils

Ca. 0.2 g of crude cottonseed oil is introduced into each of six reaction flasks as a 5-ml aliquot of an accurately prepared solution (about 4%) of the oil in *n*-butyl alcohol. Proportionally larger samples may be used when the cyclopropenoid acid content of the oil tested is very low. The amount of 20 ml of *n*-butyl alcohol and 5 ml of a 1% solution of sulfur in carbon disulfide is pipetted into the first flask and then each of the flasks successively at about 20-min intervals. The flasks are immediately capped and clamped in the 110C oil bath to a depth of 5 in. for 2.5 hr. The caps are removed for the final 30 min of heating. Each flask is then cooled immediaetly to room temp under a stream of tap water. The colored solution is transferred quantitatively to a low-actinic 50-ml volumetric flask and made up to volume with *n*-butyl alcohol. It was found advantageous to conduct the entire operation in subdued light since the pigments were found to be light-sensitive. The absorptivity of each solution is determined without delay with a Cary Model 14M spectrophotometer with a 1-cm cell using water as a reference. The average of the absorptivities for the six specimens, *a*, read at the maximum in the curve at about 495 mμ, is multiplied by a factor to give the percentage of cyclopropenoid fatty acids expressed as malvalic acid. Since there was often a considerable variation in the individual absorptivity measurements on a given oil sample, it was found necessary to make the determinations in sextuplicate to obtain average values reproducible to ± 0.01%.

Refined Cottonseed Oils

For commercial salad oils the same procedure is followed except that 0.5 ml of a 4% solution of morpholine in *n*-butyl alcohol is added to the reaction mixture prior to the introduction of the sulfur-carbon disulfide solution.

Results

The validity of this method of analysis was established and the appropriate values for the factors were determined by applying the above procedures to a series of refined and crude cottonseed oils, the cyclopropenoid acid contents of which were first determined to within 0.01% by the stepwise HBr titration method. The absorptivities for both the refined and crude oils were found to be directly proportional to the known cyclopropenoid acid contents. The regression lines

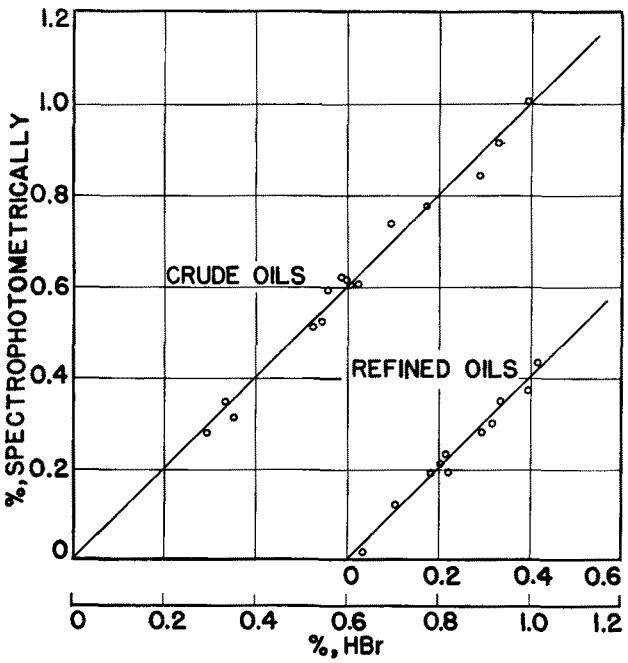

FIG. 1. Percent malvalic acid in cottonseed oils—HBr titration method vs. spectrophotometric procedure.

drawn on large scale plots are represented by the equation

$$x = fa \qquad [1]$$

where x is the cyclopropenoid fatty acid content expressed as percent malvalic acid and f is the proportionality factor. For crude oils $f = 5.12$ and for refined oils $f = 3.07$. It is possible that the value of f will be different and would therefore have to be redetermined for oils other than cottonseed oil. Because of the numerous parameters involved in the test, the duplication of results is premised on a strict adherence to the prescribed experimental conditions.

The cyclopropenoid acid concns calculated from equation [1] are in close agreement with those obtained by the stepwise HBr titration, as illustrated in Figure 1. The average deviation is ± 0.021% for the refined oil series and ± 0.015% for the crude oil series.

Discussion

The reactions involved in the Halphen test are apparently very complex. When the Halphen reaction is carried out on a refined cottonseed oil in the absence of morpholine major absorption bands are observed at about 495 and 540 mμ and a relatively weak band at about 410 mμ. The "495 mμ" peak is generally the most intense. The position of the maximum generally occurs between 495 and 500 mμ. The 540 mμ absorption may be quite intense as in Curve A, Figure 2, but usually appears as a shoulder. In this respect there is sometimes considerable difference between the curves for a given oil sample. The presence of morpholine or phosphatides in the reaction mixture causes the suppression of the absorption at 540 mμ and results in a more intense (10) and more reproducible absorption at ca. 500 mμ (Curve B, Fig. 2). The use of morpholine with crude oils is unnecessary since these oils already contain phosphatides. In fact, the presence of morpholine in crude oils often results in the formation of undesirable background absorption. Halphen reaction products having absorption characteristics of Curves A and B in Figure 2 would appear cherry-red and orange, respectively.

The intensity of the 540 mμ absorption in the test

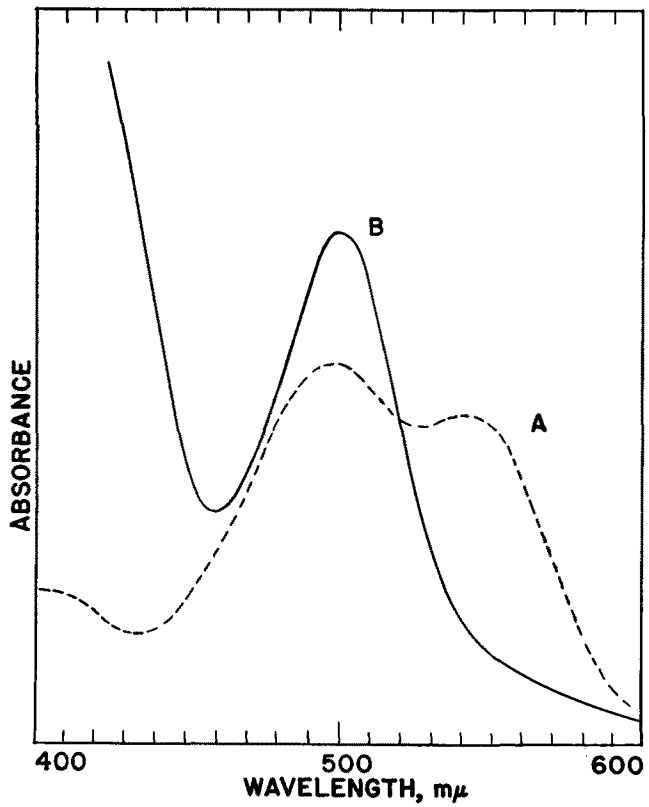

FIG. 2. Absorbance curves for refined cottonseed oil after Halphen reaction: (*A*) without morpholine, 0.5 g in 50 ml, (*B*) with morpholine, 0.2 g in 50 ml.

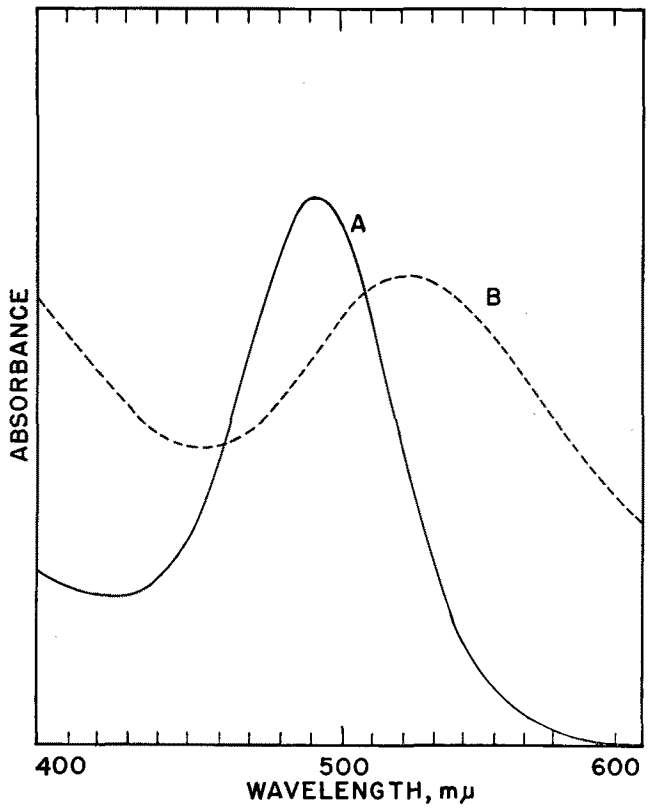

FIG. 3. Absorbance curves for isolated pigments: (*A*) orange pigment fraction in petroleum ether, (*B*) purple pigment fraction in 95% ethanol.

solution depends to a great extent upon reaction conditions such as temp, reaction time, light, solvent systems, and proportion of reagents. It is also affected by certain amine additives such as morpholine and by minor constituents present in the oil, such as phosphatides (10). However, no overall correlation could be established. Normal laboratory light tended to increase the relative intensity of the 540 mμ absorption. The test solutions when allowed to stand several weeks in the laboratory became a deep cherry-red.

Two pigment fractions, one orange and the other purple, were separated from the reaction product (without morpholine) of a methyl ester mixture—obtained by methanolysis of *Sterculia foetida* oil—containing approximately 50% methyl sterculate. The colored solutions obtained from several Halphen tests were combined (ca. 0.2 g esters per 600 ml solution) and concd by repeated water washing to remove most of the butyl alcohol. Hexane was added and water washing was continued to remove the rest of the butanol. During the final washings the purple pigment was deposited on the precipitated sulfur and on the walls of the separatory funnel. The hexane solution was drawn off and the purple pigment was washed from the separatory funnel with 95% ethanol. The solvents were removed from the combined pigment solutions under reduced pressure, leaving only the pigments and sulfur. The orange pigment was extracted with hexane, in which the purple pigment was practically insoluble. The purple pigment was then extracted with 95% ethanol. Each solution contained traces of dissolved sulfur. The characteristic absorption curves for the two pigment solutions are shown in Figure 3. The orange pigment fraction in petroleum ether exhibited an absorption max at 490 mμ and the purple pigment fraction in ethanol showed a max at 520 mμ.

By application of silicic acid chromatography to a similar Halphen reaction product, a pigment fraction was separated having an absorption max at 490 mμ in petroleum ether solution. From this, in turn, three fractions were isolated, all with an absorption max at 490 mμ but having mol wts of 320, 520, and 780 by osmometer, and constaining 7.1%, 9.2%, and 7.4% of sulfur, respectively.

In view of the complexity of the Halphen reaction and the multiplicity of color bodies formed, it is not surprising that considerable difficulty has been encountered in adapting this reaction as a basis for quantitative analyses. The effectiveness of the present procedure can be attributed in large measure to a standardization of reaction conditions and to the use of an adequate reference standard for calibration.

Though the method is not as convenient as the HBr titration method it has the important advantage of requiring a very much smaller sample. It will be particularly useful, therefore, when only small amts of sample material are available and in those instances where high levels of interfering substances preclude the use of the HBr method.

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REFERENCES

1. Halphen, G., J. Pharm. *6*, 390 (1897).
2. Halphen, G., Analyst *22*, 326 (1897).
3. Nunn, J. R., J. Chem. Soc. *1952*, 313.
4. Faure, P. K., Nature *178*, 372 (1956).
5. Shenstone, F. S., and J. R. Vickery, *Ibid. 190*, 168 (1961).
6. Mehlenbacher, V. C., Chem. Eng. News *22*, 606 (1944).
7. Deutschman, A. J., Jr., and I. S. Klaus, Anal. Chem. *32*, 1809 (1960).
8. Harris, J. A., F. C. Magne and E. L. Skau, JAOCS *41*, 309 (1964).
9. Bailey, A. V., W. A. Pons, Jr. and E. L. Skau, JAOCS *42*, 173–174 (1965).
10. Bailey, A. V., F. C. Magne, R. A. Pittman and E. L. Skau, JAOCS *38*, 505 (1961).

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