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[Received July 21, 1964—Accepted December 22, 1964]

Analysis of Cyclopropenoid and Cyclopropanoid Acids in Fats and Oils

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Abstract

The analysis of cyclopropenoid acids may be considered, from a historical standpoint, to have started with the discovery of the Halphen test. Although this test as originally conceived was utilized as a means of detecting adulteration of premium edible oils with cottonseed oil, it has since been shown to be a characteristic test for cyclopropenoid fatty acids and has been adapted with various modifications as a quantitative colorimetric test for these substances. More recently, spectrophotometric methods particularly in the IR region have been applied to the analysis of these substances. The 9.8 μ band, characteristic of the cyclopropane, and the 9.91 μ band, characteristic of the cyclopropene group, as well as the 11.0 μ band, characteristic of some of the noncyclic degradation derivatives, have been utilized. Gas-liquid chromatography (GLC) has been applied to the methyl esters of cyclopropanoid and hydrogenated cyclopropenoid acids. The reactivity of the cyclopropene ring toward hydrohalogens has been the basis of several analytical methods developed for use with cyclopropene acid-containing oils. Both aqueous and nonaqueous solutions of hydrohalogens have been employed. The hydrohalogenation methods are the most precise methods currently available for these analyses but only GLC has the inherent potential of identifying the specific cyclopropenoid or cyclopropenoids involved.

Introduction

THE OBSERVATIONS of the last decade (11,12,13,15, 23,26,31,41) which show that the occurrence of cyclopropenoid and cyclopropanoid fatty acid moieties in natural products is not as uncommon as once believed, and the current interest in their biological significance have focused attention on the need for reliable methods of detection and estimation. In this discussion the methods have been arbitrarily grouped in two categories: Chemical Methods and Instrumental Methods. Cyclopropanoid analytical methodology will not be considered separately because of the very limited work reported in this area, but will be discussed when appropriate along with cyclopropenoid methods.

The determination of cyclopropenoid fatty acids may be considered from a historical standpoint to date back to 1897 with the disclosure of the Halphen test (19). This colorimetric test was originally believed to be specific to cottonseed oil and was employed to detect the presence of cottonseed oil as an adulterant in premiumn-type edible oils. This test has more recently been observed with numerous other oils derived from seeds or fruits of the *Malvaceae, Sterculiaceae, Tiliaceae,* and *Bombacaceae* families (5, 11, 38a). Faure (15) showed that a positive Halphen test was observed with sterculic acid, the predominant constituent acid moiety in *Sterculia foetida* oil. This acid was characterized by Nunn (36) as a C_{19} acid containing a cyclopropenyl group involving the ninth and tenth carbon atoms of the aliphatic chain. The analogous cyclopropanyl derivative, dihydrosterculic acid, does not give a Halphen test (4a,9,41a). These facts established that the Halphen reaction is characteristic of the cyclopropenyl group in this acid. Other naturally occurring acids of this type which give a positive Halphen test are malvalic and bombacic acids (9, 31, 40, 41).

Chemical Methods

Halphen Test

The conventional Halphen color test (38) calls for heating for several minutes at 75-80C a mixture consisting of two parts of the oil under examination, one part of amyl alcohol, and one part of a 1% solution of sulfur in carbon disulfide followed by a ½ hr heating period at 110-115C. The color developed at low cyclopropenoid levels, 0.1 to 1% malvalic acid, may range from orange to red (26), frequently even in replicate tests on the same oil (34). Variation of the color intensity is also a frequent occurrence. Although these variances do not pose any problem in a qualitative test, they make the method highly unreliable, as Mehlenbacher (34) showed, in quantitative applications. The color developed with high cyclopropenoid concns, on the other hand, is so intense that visual differentiation is virtually impossible unless a dilution approach is used with the color-reaction product. Such a method, reported by Shenstone et al. (41), involved an adjusted dilution

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of the Halphen color to virtual extinction and the intercomparison of the dilution requirement of the unknown with that previously established for the reaction product of a standard concn of sterculic acid. The dilution ratios establish the cyclopropenoid concn in the unknown. These investigators (40) report a detectable limit of 10 ppm for the Halphen color reaction.

Spectroscopic data (4) have shown that the variation in the color obtained by the Halphen reaction is caused by the fact that a number of pigments are formed in varying proportions. The spectrophotometric curves show broad absorption bands at about 495 and 540 mμ and usually a weak band at 410 mμ. The red color results from an increase in the 540 mμ absorption. By fractionation of the Halphen reaction products of cyclopropenoid methyl esters, it was found possible to isolate various pigments or pigment fractions (4). One gave a purple solution in 95% ethanol and had an absorption maximum at 520 mμ. Another, giving an orange solution in petroleum ether and exhibiting an absorption maximum at 490 mμ, was shown by further fractionation to be a mixture of at least three compounds differing in mol wt and sulfur content, but giving the same absorption maximum. It is not at all surprising, in view of the multiplicity of pigments and the variability of their proportions why the test has not been very successful from a quantitative standpoint.

Modified Halphen Test Methods

Investigations aimed at stabilizing the color response (4, 8) have shown that light, temp, reaction time, and solvent systems, as well as other factors, some yet unknown, are all parameters affecting this problem. Bailey et al. (3) have reported, for example, that a Halphen-positive cottonseed oil refined by the AOCS official neutral oil method (37) gives a lower and less uniform color response than the parent crude oil, in spite of the fact that this refining procedure does not result in any reduction of the cyclopropenoid content. It was established that certain phosphatidal constituents are substantially responsible (3), and that numerous amines and other nitrogenous compounds (3,17) exert a similar enhancing and stabilizing effect. These observations have led to the development of two modified Halphen tests for quantitative application.

In the method of Deutschman and Klaus (8), 1 ml of oil accurately weighed, 5 ml of pyridine, and 5 ml of a sulfur-saturated CS_2 solution are reacted for 1 hr at 48C, followed by an additional 45-min period at 100C. The colored reaction product is then quantitatively diluted to a vol of 10 ml. After a 2-hr holding period for color stabilization the absorbance is read spectrophotometrically at 505 mμ against a corn oil blank. The cyclopropenoid content is then read from a standard absorbance curve based upon known concns of pure sterculic acid in corn oil. The method has a reported accuracy of ±10% with a 95% confidence at levels of 0.03–0.04% sterculic acid, the mid-range of the standard absorbance curve. The method is considerably less accurate at lower concns, and higher concns must be brought within this mid-range by dilution.

A modification of the Halphen test developed by Bailey et al. (3,4) features spectrophotometric measurement in the 495–500 mμ region and color stabilization. The color response is calibrated in terms of reproducible standards as established by hydrogen bromide titration values obtained at 55C for various cyclopropenoid concns.

While none of these modified Halphen methods have the inherent facility or precision of the hydrohalogenation methods, nor applicability to high concns unless a dilution principle is employed, they may be very useful in those instances either where (a) nonremovable hydrohalogen reactive noncyclopropenoid components give spurious results, or (b) the cyclopropenoid level is less than 0.01% sterculic acid, and the greater sensitivity of this test can be utilized with advantage over a more precise but less sensitive method.

Hydrohalogenation Methods

As early as 1907, Kühn and Bengen (28) observed that cottonseed oil no longer gives a positive Halphen test after shaking for 1 hr with an equal vol of concd hydrochloric acid.

On the basis of experimental evidence, primarily iodine values, Bailey et al. (2) concluded that concd HCl (sp gr 1.19) was specific for the cyclopropene group in the presence of other olefinic groups commonly found in oils. This apparent specificity was the basis of a method of cyclopropenoid analysis (33) involving the determination of the increase in chlorine content of the sample caused by hydrohalogenation, which is stoichiometrically related to the cyclopropenoid content of the original. Although epoxides, hydroperoxides, and possibly other autoxidation products interfere and can introduce an error into the analysis they can be eliminated by pretreating the samples in accordance with one or more of the accepted methods, such as lithium aluminum hydride reduction (42), mild acetolysis (43) or treatment with activated alumina (6,16,21,22). In the absence of interfering substances the method has an average precision of ± 0.37% over a concn range of 0-50% sterculic acid, which is based upon a precision of ± 0.025% in the chlorine analysis. The numerous manipulations required and the extreme precision necessary in the tedious chlorine analyses combine to make the method cumbersome, and less reliable than the more precise and rapid HBr titration method of Harris et al. (20,21).

The basis of the HBr-acetic acid titration method is the reported observation of Smith et al. (42) that an anhydrous HBr-glacial acetic acid reagent, 0.1*N* in HBr, will sluggishly but stoichiometrically titrate cyclopropenoid fatty acids, mole per mole, at room temp. Although cyclopropanyl, olefinic, or conjugated olefinic groups do not give a titration with this reagent, epoxides, peroxides, and conjugated hydroxydiolefins do (10,42), and may, therefore, give rise to spurious cyclopropenoid analyses.

Either lithium aluminum hydride reduction (42) or mild acetolysis (46) have been suggested and used to eliminate epoxides. Both of these elimination approaches are, however, predicated upon the assumption of a known reactant-to-product mol wt ratio. Since, however, the reductive efficiency of lithium aluminum hydride with esters and glycerides is reported to vary markedly (18) and is seldom if ever 100%, there is considerable uncertainty as to this ratio. A similar condition results from acetolysis, where this ratio would depend on the initial epoxide level as well as the degree of acetylation, neither of which is known. It is apparent, therefore, that the assumptions of the method are not strictly valid. Consequently, considerable error may be involved in the analysis unless the entire sample subjected to reduction or acetolysis is

recovered for analysis, and the cyclopropenoid content calculated on an original sample-wt basis. Finally there is some question as to the certainty of the titration value itself due to the indefiniteness of the end point resulting from the extreme sluggishness of the reaction at room temp.

Recently a technique employing a stepwise titration at 3 and 55C with the HBr-acetic acid reagent has been reported (20), which allows a more rapid titration to a sharper end point. This method also permits a quantitative differentiation of epoxides from cyclopropenoids in glycerides and esters. In this procedure the epoxides are first titrated selectively at 3C. The temp is then raised to 55C and a second titration on the same sample obtained for the cyclopropenoids. For mixtures this method has a reported accuracy of ± 0.17% for epoxides, which is better than can be achieved through acetolysis (20), and ± 0.15% for cyclopropenoids when these are the only two reactive components present. This accuracy is unattainable when significant amounts of interfering components, such as peroxides or conjugated hydroxydienes, are present.

Interfering substances present a major problem at low cyclopropenoid concns, particularly in the light of observations by Harris et al. (21) that all oils as a rule tend to develop oxidation products which give a 55C titration. This spurious cyclopropenoid titration, though small, acquires a major significance at low cyclopropenoid levels since it is of a magnitude comparable to the true cyclopropenoid titration. On the basis of data obtained on peanut oil-*Sterculia foetida* oil mixtures low in cyclopropenoid content these investigators developed a method (21), applicable to refined, rancid, and crude cottonseed oils, which overcomes this problem by pretreating the oil samples with activated alumina before the analysis. For refined oils this entails percolation of the oil through an activated alumina column in a hexane system, while crudes require dual columns involving both the AOCS neutral-oil method treatment and the alumina-hexane treatment (21), in the order named. Rancid oils must be converted to methyl esters which are then treated like crude oils (21). The cyclopropenoid content of refined, rancid, crude, as well as known, deliberately adulterated cottonseed oils can be determined with an accuracy of ± 0.01% by these methods (21). Even as much as 3% of interfering substances such as epoxides or conjugated hydroxydienes (22) can be accommodated by this procedure without impairing its efficiency or accuracy.

At high cyclopropenoid levels, however, the alumina column pretreatment can not be employed to remove even minor amounts of interfering substances with the same confidence because the fractionating effect of the alumina upon glycerides (44) through selective adsorption may cause an enhancement of the cyclopropenoid concn (22). Though the percentage error would of course be the same at both high and low cyclopropenoid levels, the absolute error would be much greater for high cyclopropenoid concns. Obviously the removal of major amts of interfering substances would also result in an altered cyclopropenoid concn irrespective of any such fractionation and the measured concn as determined by titration would be higher than that originally present.

The question, therefore, arises as to just how the analysis of high cyclopropenoid concns can be managed. It appears on the basis of the available information that one of these two tentative approaches might be followed. One is to accept the 55C HBr stepwise titration as truly representative of the cyclopropenoid concn on the basis of observations of Harris et al. (21,22) which show that this is a reasonable assumption if the 3C titration is absent or insignificant. Alternatively, if there is a significant 3C titration, the stepwise titration method might be employed following the lithium aluminum hydride pretreatment of Smith et al. (42), making appropriate adjustment of mol wt ratios as indicated by saponification equivalents of the material before and after lithium aluminum hydride reduction. Neither of these approaches would resolve or eliminate any conjugated hydroxydiene interference.

Under very restricted conditions, e.g., when the contaminating substances are saturated, there is a possibility that advantage might be taken of the claim (15a) that bromine reacts stoichiometrically with sterculic acid. However, the general reactivity of this reagent with olefins, which can also be expected to be present, and therefore its nonspecificity for cyclopropenoids makes its general applicability to cyclopropenoid analyses questionable.

The only chemical method available for cyclopropanoids is that of Kartha and Ojha (27) resulting from work on Δ^3-carene and chrysanthemum mono- and dicarboxylic acids. These investigators have described a tentative method, employing a mercuric acetate-iodine monobromide reagent, which appears to quantitatively rupture the cyclopropane ring with a concurrent addition of iodine. The difference in iodine addition by the above method and that obtained by the conventional Hanus method is stated to be a probable measure of the cyclopropanoid content. This method is still in a developmental stage. Additional work by these authors on other pure cyclopropanoids is reportedly in progress. Cyclopropenoids would interfere with the analysis unless eliminated prior to the iodinizations.

Instrumental Methods

Infrared Absorption

This is one approach which is applicable at least theoretically to both cyclopropane and cyclopropene derivatives, since these structures are reported to give characteristic absorption bands at 9.8 μ (7,24), and 9.9 μ (46), respectively.

Varma et al. (45) determined the specific extinction coefficient for a sample of reputedly pure sterculic acid at 9.92 μ and developed, on the basis of this value, a method for determining the sterculic acid content of *Sterculia foetida* oils. The method has a reported precision of ± 0.25% at concns of 71-72% sterculic acid. This approach is not invalidated by the assignment of an erroneous cyclopropanoid structure to sterculic acid by these investigators.

Although cyclopropanoids such as dihydrosterculic acid, exhibit a band of comparable intensity at 9.8 μ, there has been no recorded attempt to utilize this band as a basis of a quantitative method. Both lack of interest and the unavailability of a reasonably pure cyclopropanoid necessary to validate a method are undoubtedly responsible. Although the development of such a method may present some difficulties with methyl esters, because this ester band also occurs at 9.8 μ, it should be more feasible for other cyclopropanoid compounds.

IR absorption has also been used for the indirect determination of cyclopropenoid concn by measuring the absorptivity of a cyclopropenoid-derived reaction product. Magne et al. (32) have developed such a method for the estimation of the sterculic acid con-

tent of mixtures which utilizes the band at 11.0-11.1 μ, characteristic of unsymmetrically substituted olefins which comprise a specific proportion of the reaction products of cyclopropenoids with aqueous hydrogen chloride (2). The method was standardized with a number of corn oil *Sterculia foetida* oil compositions of known cyclopropenoid content. It has a reported precision of ± 0.43% cyclopropenoid expressed as sterculic acid when applied to glycerides.

The accuracy of IR methods is in many instances likely to be considerably less than the indicated precision because of the inherent dependency of such methods upon the presumed purity of the calibrating standard. Since it is extremely doubtful that pure cyclopropenoid fatty-acid standards or derivatives were ever available, those methods calibrated on this presumption will almost invariably yield fictitiously high analyses. Confidence in IR methods is also, as a general rule, limited to analyses on the same species employed in the calibration, because any departure in mol wt from that of the calibrating standard will introduce an error of uncertain magnitude and also because the background correction method of the calibration may not be applicable to the unknown.

Gas-Liquid Chromatography

GLC has been used by numerous investigators to establish the compositional characteristics of various cyclopropenoid-containing oils. Smith et al. (43) and Wilson et al. (46) used this tool to determine the cyclopropenoid content of *Sterculia foetida, Hibiscus syriacus,* and *Lavatera trimestris* oils, and Cornelius et al. (5) similarily determined the cyclopropenoid composition of *Bombax oleagineum.* Nordby et al. (35) claims to have quantitatively characterized a number of reputedly highly pure derivatives of sterculic acid by means of this same technique. In general, GLC results have been supplemented with an analysis by at least one other method, such as the Halphen test (35), HBr equivalent (43,46), or hydrogenation followed by GLC (5,43,46) to establish a higher level of confidence in the results. A comparison of the respective data shows almost perfect agreement, less than 1% difference, between the supporting and GLC analyses in some instances, and poor agreement, 4 to 14% difference, in others.

However, none of these reports carries any details concerning procedures followed, conditions employed, or any descriptions, or interpretations of the GLC curves obtained. Any analysis on this class of substances is greatly dependent upon such interpretations because, as was shown by Masson (29) with methyl sterculate, these compounds are readily isomerizable on the GLC column to conjugated dienes. The analytical calculation resulting from these curves would, therefore, be based upon, or at least highly colored by artifacts. In the absence of a standardization with an authentic cyclopropenoid standard, the quantitative reproducibility and ultimate effect of these artifacts upon the cyclopropenoid analyses are unknown and unpredictable. The information reported in the literature at best merely indicates that GLC may be a more useful quantitative tool in this area of analysis than anticipated, but hardly a proven one, and draws attention to the need for additional investigation and experimental substantiation.

GLC has been used by Smith (43) and Wilson (46) to estimate the cyclopropanoid composition of the methyl esters of the component fatty acids of *Sterculia foetida, Hibiscus syriacus,* and *Lavatera trimestris* oils. These investigators also determined the cyclopropanoid content of these oils after hydrogenation as did Cornelius et al. (5) with hydrogenated *Bombax oleagineum.* The precision achieved by these investigators is not reported.

Although the superior thermal stability of cyclopropanoids should make them more amenable to this method of analysis, the isolated nonsystematic data currently found in the literature can scarcely be cited as validating evidence of the quantitative applicability of GLC to these substances. Until details of procedures, concns investigated, and precision achieved are known, GLC will continue to remain an unproven but potentially very useful analytical tool for cyclopropanoid analysis.

Nuclear Magnetic Resonance

While no published methods of direct cyclopropenoid or cyclopropanoid analysis utilizing nuclear magnetic resonance NMR were to be found, there is little doubt that it is potentially applicable. It has been employed by Rinehart et al. (39) to roughly estimate the proportions of isomeric pairs present in the acetolysis products of sterculic acid. This approach, although indirect, might conversely be the basis of a method for measuring the original sterculic acid content of selected compositions rich in sterculic acid, and could conceivably be expanded to encompass other cyclopropenoids provided the acetolysis product ratio is constant for all cyclopropenoids.

Preliminary investigations at this laboratory on the applicability of NMR to the analysis of cyclopropenoids (1) have followed two approaches. The first, a direct approach, involved a measurement of the methylene proton signal of the cyclopropenyl ring to establish the cyclopropenoid concn. The other, an indirect approach, was based upon the measurement of detectable impurities present, and thus would establish the cyclopropenoid concn by difference. When these were applied to a methyl sterculate conc (80% by HBr titration) the direct method failed because of the inability to cleanly resolve the ring methylene proton signal from that of the terminal methyl group. The indirect method, however, gave an analysis by difference, which agreed within ± 5% of that obtained by the stepwise HBr titration. The detectable impurity, characterized by an isolated olefinic group signal, was assumed to be a monoene having the same mol wt as the cyclopropenoid. This agreement, which is of about the same order of magnitude as the attainable instrumental precision of measurement, substantially confirms the claim that the HBr.HAc reagent titrates cyclopropenoids stoichiometrically.

There have been no attempts to quantitatively apply NMR to long-chain cyclopropanoids. However, since the methylene proton signal of the cyclopropanyl group occurs at a higher field strength (24a), it is readily distinguishable from that originating from a cyclopropenyl group. In addition, the direction of this shift, away from the terminal methyl signal, should reduce the perturbation problem and facilitate quantitative measurements. This, however, remains to be verified experimentally.

Discussion

The methods available for the quantitative analysis of cyclopropenoids characteristically fall into two basic groups; (a) primary methods, of known stoichiometry, such as the hydrohalogenation methods, whose accuracies are independent of any cyclopropenoid calibrating standard, and (b) secondary methods, such as the Halphen test, IR, and GLC, which are

dependent for accuracy upon cyclopropenoid calibration standards of known purity.

Whenever applicable, primary methods are usually preferred. However, the analyst must choose judiciously from among the available methods, selecting the particular one apparently most applicable to his problem in the light of anticipated interferences and complications. Confirmatory analyses by two independent methods are indicated for even minimal confidence in results on unknown compositions, which might contain unpredictable interfering substances. The stepwise HBr titration method, because of its stoichiometric characteristics and consequent freedom from calibration artifacts, is the most precise and accurate method and is clearly the choice whenever applicable, particularly in those instances where ultrasensitivity is not required and interference is not an insurmountable problem. This method can, in addition, serve as an admirable secondary standard for calibrating others, such as the Halphen test and IR methods, since it permits a reproducible standardization in terms of a uniformly definable cyclopropenyl value or HBr equivalent, and eliminates the calibration dependency of these methods upon so-called primary cyclopropenoid standards of questionable purity or stability.

The Halphen method would be preferred at extremely low concns, where high sensitivity is required, and either the Halphen or IR method might be preferable in those instances where severe or special interference problems exist. Where distinction between cyclopropenoids of different structures or mol wts is required only GLC has potential utility.

Little attention has apparently been given to the development of methods for the analysis of cyclopropanoids. Only GLC has received any attention and that primarily as a side issue to cyclopropenoid analysis. IR spectroscopy and NMR have potential utility in cyclopropanoid methodology.

ACKNOWLEDGMENT

Manuscript assistance by E. L. Skau.

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[Received October 30, 1964—Accepted December 22, 1964]