Quantitative Estimation of Esters by Thin-Layer Chromatography[1,2]

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A method for the analysis of fatty esters employing separation by thin-layer chromatography and quantitative determination *via* their hydroxamic acids has been developed. Esters of different types are separated on silica gel plates, the spots or zones are scraped from the plates, the esters are extracted from the silica gel, and the iron hydroxamic acid complexes are formed. The latter are then measured by colorimetry.

The method is suitable for analysis of mixtures of ordinary, epoxy, monohydroxy, and dihydroxy fatty esters as well as for mixtures of mono-, di-, and triglycerides.

Used in conjunction with gas chromatography this method permits the fatty acid composition of seed oils containing oxygenated fatty acids to be measured in about 3 hours. The amount required for the total analysis is 1 to 10 mg. per determination, depending upon composition.

Introduction

MANY SEED OILS contain monohydroxy, dihydroxy, epoxy, or keto acids in addition to the more common nonoxygenated acids. Oxidized oils and fats also contain various oxygenated fatty acids, including hydroperoxides. No satisfactory method of analysis of such seed oils or oxidized oils has heretofore been developed. Recently however chromatographic procedures have been described which, if used in conjunction with gas-liquid chromatography, show promise of giving better analyses of such oils than older methods (1,2). A detailed study of reversed phase partition chromatography systems by Gunstone and Sykes (3) has resulted in a complete, if somewhat tedious, procedure for the analysis of the mixed acids from oils, including those with oxygenated components (4).

Of the older methods distillation procedures are, in general, not suitable for ester mixtures containing oxygenated components even when these are acetylated (5). Separation by crystallization (6), urea complex formation (7), or liquid-liquid partition (8) are not quantitative. Although the proportion of hydroxy acids in a mixture can be determined fairly accurately by acetyl value determination, the presence of these acids may interfere with the iodine value determination (9) and alkali isomerization (10) methods for the estimation of the nonoxygenated components. None of the analytical methods based on these procedures is accurate for acid or ester mixtures containing oxygenated components. Certain assumptions must always be made and these frequently are not true. In addition such methods require rather large samples.

Physical measurements of optical rotation (11) or infrared absorption (12) have proved applicable in instances when the structure of the hydroxy constituent is known, but these are not suitable for general application. Recently near-infrared methods for the determination of epoxy and vicinally unsaturated hydroxy components have been described (13). However for a complete analysis of mixtures containing oxygenated constituents, the oxy acids or esters must first be separated quantitatively from nonoxygenated esters to avoid any interference in the determination of the latter. This must be carried out by countercurrent distribution or by some chromatographic techniques and these procedures have already been used to a limited extent.

We have developed a procedure to separate and analyze classes of fatty esters according to functional group, which is applicable to very small samples. Separation into classes is accomplished by thin-layer chromatography (14). The separated components are located by staining and are scraped from the plate and transformed to their hydroxamic acid derivatives which are subsequently converted to colored complexes with ferric perchlorate. The amount of each ester class is then determined colorimetrically. This reaction was introduced by Feigl (15) as a qualitative test for esters and it has been applied by several workers to their quantitative determinations (16,17). Being a general reaction for esters this procedure may also be used to analyze mixtures of mono-, di-, and triglycerides.

The quantitative measurement of the several classes of esters in a mixture can be carried out in about 3 hr. and requires only a few mg. of sample. Gas-liquid chromatography of the nonoxygenated esters permits the measurement of individual esters of this class. GLC analyses of the nonoxygenated esters can be performed concurrently with the colorimetric measurement of the several classes, thereby reducing the total time involved.

Experimental

Nonoxygenated esters were obtained from the Hormel Foundation. All the esters were checked for purity either by thin-layer and/or gas-liquid chromatography and all were pure by these criteria.

Reagents. Sodium hydroxide and hydroxylamine hydrochloride solutions were each 2.5% in 95% ethanol. Stock ferric perchlorate solution (18) contained 5.0 g. of ferric perchlorate (nonyellow) in 10 ml. of 70% perchloric acid plus 10 ml. distilled water diluted to 100 ml. with cold absolute ethanol. Four ml. of this solution and 3 ml. of 70% perchloric acid were diluted to 100 ml. with chilled absolute ethanol daily prior to use as reagent. All solutions were refrigerated.

Chromatography. The plates were prepared according to Stahl (14) using silica gel G (Merck, Darmstadt) as adsorbent. The esters were separated on the plate developing with diethyl ether in hexane in proportions suitable for the types of esters to be separated. This ranged from 10 to 30% diethyl ether.

The sample, dissolved in chloroform, was applied along the bottom of the plate in several spots at a distance of 0.5 cm. from each other in sufficient amount to obtain a total of 1.0 to 3.0 microequivalents of each component. A single plate (20 x 20 cm.) may be divided in three parts by drawing straight lines along the development direction so that three determinations can be carried out simultaneously on the same plate. When the proportions of some of the esters

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TABLE I

	Actual value (mole %)	Found (mole %)
Mixture A		
Methyl palmitate	25.0	25.7
Methyl 2-hydroxystearate	14.8	14.8
Methyl 14-hydroxystearate	14.5	16.3
Methyl 6-hydroxystearate	9.3	10.2
Methyl 9:10-dihydroxystearate	36.4	33.0
Mixture B		
Methyl elaidate	44.0	44.7
Methyl epoxy oleate	26.9	25.2
Methyl ricinoleate	29.1	30.1
Mixture C		
Tripalmitin	38.1	36.8
Dipalmitin	28.8	28.7
Monolaurin	33.1	34.5

were small, the spots of minor components on the chromatograms were combined for color development. The spots of the major components were taken separately. A preferred procedure which allows three determinations of all components requires that enough sample be put on the plate to provide the correct amount of minor components for color development. Small aliquots of the major components are then used for their measurement.

Detection and Elution of the Spots. After the development of the plate, the solvent was allowed to evaporate from it, the spots were then made visible in iodine vapor, and the rows of spots were delineated. Fig. 1 shows an example in which the ordinary esters (top) are separated from three different monohydroxy esters (middle) and one dihydroxy ester (bottom) using 15% diethyl ether in hexane as the solvent system. This example is an instance in which members of one class of oxygenated esters are measurable separately. Separations of individuals within an oxygenated ester class sometimes occur, but this is not always possible (19).

Each row of spots was scraped off the plate with a spatula into a large test tube. One ml. of diethyl ether was added to each, and the slurry was stirred with a glass rod to break up lumps. Approximately 14 ml. of diethyl ether was then added, washing the glass rod. The tubes were shaken for 1 min. and filtered into test tubes, washing the original tubes and the filter papers with 2-ml. portions of diethyl ether.

If an extract is to be diluted before reaction, or is to be used for GLC, it must be made to a known volume from which an aliquot is taken for color development. The final volume of all tubes should be made equal with ether before addition of the hydroxylamine hydrochloride and sodium hydroxide reagents.

Use of iodine vapor to make spots visible has been found to give low values for unsaturated esters measured by GLC. When analysis of these esters is to be performed it is advisable to use 2′,7′-dichlorofluorescein (20) to make spots visible. The color of this reagent interferes with the colorimetry, so only the edges of the plates should be sprayed with this reagent, and the center portion taken for analysis.

Recovery of esters from known samples applied to plates was measured colorimetrically and found to be better than 95% in all cases.

Color Reaction. Each test tube with extract was treated as follows: 0.1 ml. of each of the sodium hydroxide and hydroxylamine hydrochloride solutions was added from burettes. The sodium chloride which precipitated prevented bumping during the boiling of the diethyl ether in the next step. The solutions were evaporated to dryness in a water bath (65–70°C.) and the last traces of solvent were eliminated with nitrogen or air. The tubes were then cooled under tap water, 5 ml. of the ferric perchlorate reagent was added, and the color obtained was measured after 30 min. at 520 mμ in a Beckman Spectrophotometer against ethanol using a slit of 0.02 mm.

Results

Standardization of the Method. Solutions containing graded known amounts of methyl palmitate were treated for color development as described above. The relationship of color to amount of ester was linear up to 3 microequivalents. The yield of color per mole of ester is equal for all esters tested. Ketones form oximes with hydroxyl amine, but this does not interfere with the color reaction for measurement of esters, for under our conditions methyl 12-keto-9-octadecenoate gave the same absorbance per microequivalent as did methyl palmitate. To determine the reproducibility of the method a mixture of three components was prepared and analyzed ten times. Composition of the mixture in mole % was methyl palmitate 58.8%, methyl 6-hydroxy stearate 19.0%, and methyl 9:10-dihydroxy stearate 22.2%. The composition found by analysis was 59.0 ±2.5%, 18.6 ±1.4%, and 22.4 ±1.5%, respectively.

Examples of analyses of some model mixtures of methyl esters and glycerides are shown in Table I. The results indicate the method is valid for a wide variety of fatty acids in ester linkage.

Before applying this technique to some seed oils two model mixtures were analyzed, both of which

FIG. 1. Thin-layer chromatogram of a standard mixture of fatty acid methyl esters. The chromatogram was developed with 15% diethyl ether in hexane. The spots were located by charring after spraying with 50% sulfuric acid, and the reproduction was obtained by direct photocopy of the plate.

From bottom to top, the rows of spots represent 1) methyl 9,10-dihydroxystearate, 2) methyl 6-hydroxystearate, 3) methyl 14-hydroxystearate, 4) methyl 2-hydroxystearate, and 5) methyl myristate, palmitate, and stearate.

contained seven fatty acid methyl esters. The determinations of nonoxygenated methyl esters in these samples were carried out by gas-liquid chromatography at 180°C. using an 8-ft. column of ethylene glycol succinate (15%) on Gas Chrom P (60–80 mesh) and a β-ionization detector. The results obtained with these two mixtures, D and E, are shown in Table II. These analyses, involving both thin layer and gas-liquid chromatography, demonstrate that the measurement of individual nonoxygenated esters is feasible. The mixture D is the sample shown in Figure 1.

TABLE II

	Mixture D		Mixture E	
	Actual (mole %)	Found (mole %)	Actual (mole %)	Found (mole %)
Methyl myristate	31.2	32.2[a]	.....	.....
Methyl palmitate	12.5	11.3[a]	18.1	18.3[a]
Methyl stearate	4.6	5.5[a]	16.1	16.4[a]
Methyl oleate	.....	.....	16.5	16.8[a]
Methyl linoleate	.....	.....	16.6	17.2[a]
Methyl 2-hydroxystearate	23.5	21.3	.....	.....
Methyl 14-hydroxystearate	7.2	8.0	12.1	11.3
Methyl 6-hydroxystearate	8.5	9.5	9.2	9.7
Methyl 9:10-dihydroxystearate	12.5	12.2	11.4	10.2

[a] Obtained by gas-liquid chromatography.

Analysis of Seed Oils Containing Oxygenated Fatty Acids. Vernonia anthelmintica, Artemisia absinthium, and castor oils were saponified by standing over night at room temperature with 10% ethanolic KOH. The fatty acids were extracted after acidifying the soaps with the calculated amount of 10% HCl to avoid the destruction of epoxy acids present in both *Vernonia* and *Artemisia* oils. All the operations were carried out under nitrogen. The acids were methylated with diazomethane, and solutions of the methyl esters in chloroform were spotted on the plates and analyzed as indicated above.

Fig. 2 shows the chromatograms of the *Vernonia anthelmintica* (A) and castor oil methyl esters (B). The *Vernonia* methyl esters exhibited four spots. These were 1) dihydroxy esters, 2) monohydroxy esters (?), 3) epoxy esters, and 4) nonoxygenated esters. The castor oil esters exhibited five spots. These were 1 and 2) dihydroxy esters (?), 3) monohydroxy ester (ricinoleate), 4) unknown, and 5) nonoxygenated esters. The quantitative analyses of these samples and of *Artemisia* seed oil esters are given in Table III. The analysis of the *Vernonia* oil was performed on the same sample analyzed by Gunstone (21), and our analysis agrees well with his.

Discussion

The method for the determination of fatty acid methyl esters described here is suitable when very small samples of lipids are available because only a few mg. are needed for the complete determination of the fatty acid composition. Using thin-layer chromatography the separation of the oxygenated and the nonoxygenated fatty acid methyl esters is complete, so that no assumptions need be made concerning the composition of overlapping fractions such as were necessary in many older methods for these substances.

Although the method is more convenient when applied to mixtures containing no extreme proportions of fatty acids, it has been shown to give good results with oils having a large amount of one component. This method will have applicability in a wide variety of problems in fat chemistry and lipid biochemistry which have heretofore been inaccessible for lack of suitable micromethods. Kinetic studies of lipid reactions involving a change in polarity such as glyceride hydrolysis, oxidation, or reduction can be easily followed by the proposed method. The analysis of any lipid in biological matter which can be made to migrate on a thin layer plate, and which has an ester linkage, should be possible by this means.

TABLE III

	Vernonia oil (mole %)	Artemisia[b] (mole %)	Castor oil[c] (mole %)
Dihydroxy acid methyl esters	.....	.....	1.9
Hydroxy acid methyl esters	7.8	38.2	.....
Epoxy acid methyl esters	72.8	7.9	.....
Methyl ricinoleate	.....	.....	89.8
Methyl myristate[a]	.....	.....	trace
Methyl palmitate[a]	3.9	13.5	0.6
Methyl stearate[a]	1.5	2.6	0.4
Methyl oleate[a]	4.6	4.3	2.5
Methyl linoleate[a]	9.5	33.5	3.8
Methyl linolenate[a]	.....	.....	0.8

[a] Obtained by gas-liquid chromatography.
[b] Partially autoxidized.
[c] A nonidentified fatty acid methyl ester (carbon number 21.2 amounting to 0.2% of the total methyl esters) was found in the gas-liquid chromatography analysis of the nonoxygenated methyl esters.

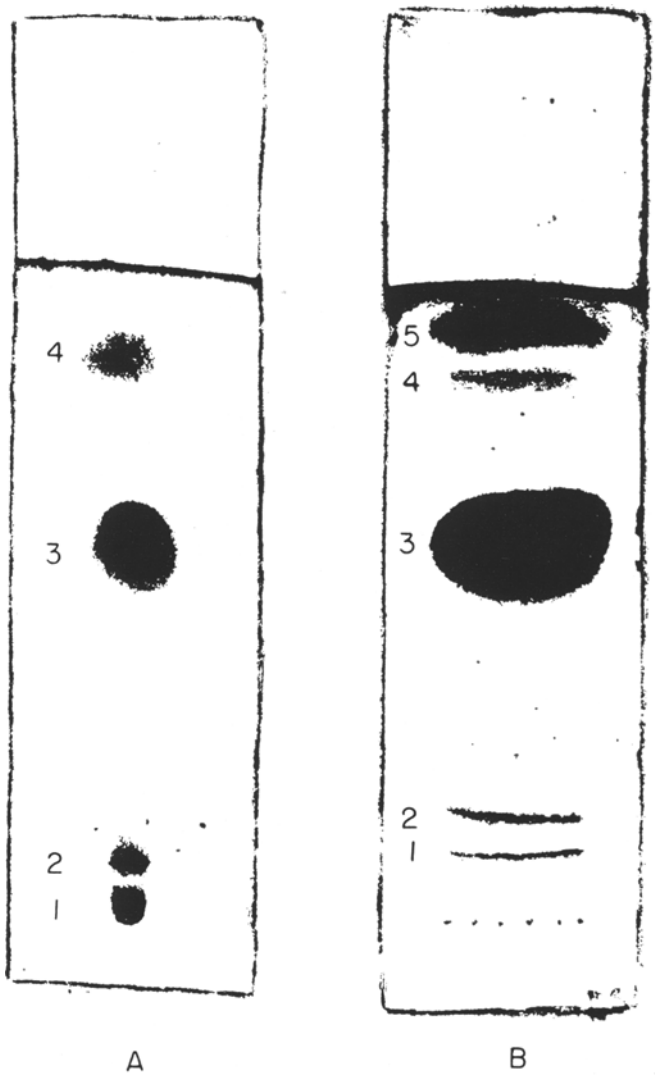

FIG. 2. Thin-layer chromatograms of *Vernonia anthelmintica* methyl esters (A) and castor oil methyl esters (B). The chromatogram (A) was developed with 15% diethyl ether in hexane and the chromatogram (B) with 30% diethyl ether in hexane. The spots were located by charring after spraying with 50% sulfuric acid, and the reproductions were obtained by direct photocopy of the plates.

Preliminary results in this laboratory on the analysis of blood lipids have been encouraging.

The analyses as performed here do not permit measurement of individuals of all classes of fatty esters. If measurement of single components is desired, paper chromatography of the separated classes can be performed. Gas chromatography provides this function in the analysis of nonoxygenated esters, and may be extended to other classes of esters.

The method may also have some application to glyceride analysis in those oils which contain fatty acids of different polarity. For example, triglycerides containing one, two, or three epoxy fatty acids should be separable. Waxes, cholesteryl esters, and glycerides have been separated by thin-layer chromatography (20), and mixtures containing these can be analyzed by the proposed method. Thin-layer chromatography has proved to be a powerful tool for qualitative separations, and this and other reports (22,23) indicate that it is equally promising for quantitative measurements.

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The Adsorption and Chromatography of Alkyl Benzene Sulfonates on Charcoal

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A chromatographic procedure for the separation and characterization of sodium alkyl benzene sulfonates is outlined. It is based on properly sized activated charcoal and uses methanol with varying amounts of benzene and a small content of ammonia and water. Milligram quantities of surfactants are used and the fractions analyzed colorimetrically. The rationale of the method is established by adsorption measurements which indicate that in this system the surfactant is adsorbed by both van der Waals and ionic forces with strong dependence upon both molecular weight and structure.

THE DESIRABILITY of a simple, rapid, and accurate method for the identification of individual surfactants and the separation of their mixtures continues to be obvious. The present paper reports some progress in developing a chromatographic method for the identification and separation of alkyl benzene sulfonates which, however, is still far from being simple and rapid, and also discusses the principles underlying this separation.

Charcoal was originally chosen as the adsorbent in the hope that adsorption would involve only the hydrocarbon part of the surfactant and thus be highly selective to the structure of this moiety. Further work showed, however, that while the premise was incorrect, the desired selectivity was nevertheless obtained. The adsorptive power of charcoal proved also to be impractically strong in either water or alcohol, but by following the experience of the Analytical Methods Subcommittee of the AASGP (1), could be controlled over a wide range by addition of benzene and ammonia. In this way a workable chromatographic method could be devised capable of separating alkyl benzene sulfonates of same molecular weight and different structure or of same structure and different molecular weights. Branching affects adsorption, however, in the same way as a lower molecular weight so that complete resolution of more complex mixtures cannot always be obtained.

Experimental

Analytical Method. Used throughout was a methylene blue spectrophotometric method based on the work of Mukerjee (2). Since alcohol interferes with this method and was normally present in the solutions, all samples were first evaporated to dryness in a test tube placed in an oven at 80°C. The residue was typically dissolved in 20 ml. of water and aliquot portions made up to 20 ml. in a 125-ml. Erlenmeyer flask to which 2 or 3 drops of 0.1% aqueous methylene blue solution and 20 ml. of $CHCl_3$ were added. The flask then was shaken vigorously for a few minutes and allowed to settle for about 2 hr. A part of the bottom layer was then carefully pipetted out and its optical density at 655 mμ was determined in a Beckman DU spectrophotometer using covered 1-cm. cells. A separate blank was used for each series of measurements and was always quite small. Standard solutions of the detergents used or of freshly dissolved pure sodium lauryl sulfate were used for calibration.

Materials. The surfactants of known structure were