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The simultaneous determination of glycerol and fatty acids in glycerides by gas-liquid chromatography*

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SUMMARY

The conversion of glyceryl esters to derivatives suitable for gas-liquid chromatography was accomplished by hydrogenolysis with lithium aluminum hydride followed by direct acetylation of lithium aluminum alcoholates with acetic anhydride. Fatty acids and glycerol were converted quantitatively to their corresponding acetate esters by this procedure. Gas-liquid and thin-layer chromatographic procedures were used to demonstrate quantitative acetylation. Acetate esters of fatty alcohols and glycerol were recovered together and analyzed by gas-liquid chromatography to give a simultaneous estimation of fatty acid composition and the ester—glycerol ratio. Quantitative results were obtained for mono-, di-, and triglycerides.

The quantitative estimation of glycerol and fatty acids in glycerides requires either acid or alkaline hydrolysis and an extraction process to separate the aqueous glycerol and nonpolar fatty acid phases. Glycerol in the aqueous phase is then assayed by paper chromatography (1), periodic acid oxidation (2–4), an enzymatic procedure employing glycerophosphate dehydrogenase (5), or a microbiological determination (6). Fatty acids in the nonaqueous phase are characterized by a number of procedures that generally require the preliminary conversion of fatty acids to their methyl esters. These procedures for the estimation of glycerol and fatty acid composition have several limitations (7). Hydrolysis may be incomplete or the glycerol partially decomposed. Periodic acid oxidation is relatively nonspecific, and the purification of glycerol is often necessary in quantitative studies. Fatty acids in the nonaqueous phase usually require concentration or isolation before esterification. An alternative method, the direct transesterification of glycerides, requires a second glyceride sample for the glycerol determination.

In the present investigation, a method is described for the simultaneous determination of both glycerol and fatty acids. Glycerides are reduced with lithium aluminum hydride yielding the lithium aluminum alcoholates of the fatty alcohols and glycerol (8–10). The lithium aluminum alcoholates are then acetylated by the direct addition of acetic anhydride, a procedure used by Grassetti and Klein (11) for the acetylation of the lithium aluminum alcoholate formed in the reduction of vitamin-A aldehyde.

$$\begin{array}{l} CH_2\text{—}O\text{—}COR_1 \\ | \\ CH\text{—}O\text{—}COR_2 + LiAlH_4 \rightarrow \\ | \\ CH_2\text{—}O\text{—}COR_3 \end{array} \qquad \begin{array}{l} R_1CH_2\text{—}O\text{—}LiAl/4 \quad CH_2\text{—}O\text{—}LiAl_4 \\ + \qquad\qquad\qquad\qquad | \\ R_2CH_2\text{—}O\text{—}LiAl/4 + CH\text{—}O\text{—}LiAl/4 \\ + \qquad\qquad\qquad\qquad | \\ R_3CH_2\text{—}O\text{—}LiAl/4 \quad CH_2\text{—}O\text{—}LiAl/4 \end{array}$$

$$RCH_2\text{—}O\text{—}LiAl/4 + (CH_3CO)_2O \rightarrow RCH_2\text{—}O\text{—}COCH_3 + CH_3COO\text{—}LiAl/4$$

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The acetates are then analyzed by gas-liquid chromatography. This procedure has been employed in determining the glycerol and fatty acid composition of mono-, di-, and triglycerides.

METHODS

Materials. Glyceryl triacetate and acetic anhydride were obtained from Eastman Organic Chemicals (Rochester, New York). Mono-, di-, and triglycerides were kindly supplied by Dr. F. Baur, Procter and Gamble Co. (Cincinnati, Ohio). Their purity was demonstrated by thin-layer chromatography. Lithium aluminum hydride was obtained from Metal Hydrides, Inc. (Beverly, Massachusetts). All solvents were reagent grade. Anhydrous ether was obtained from Mallinckrodt Chemical Co. (St. Louis, Missouri). Fatty acid methyl esters were prepared in this laboratory by transesterification, fractional distillation, and low temperature crystallization. The fatty acid content of glyceride and fatty acid methyl ester preparations showed over 99% purity on gas-liquid chromatographic analysis.

Hydrogenolysis and Acetylation. The procedure of Grassetti and Klein (11) was modified for the hydrogenolysis and acetylation of glyceride samples. The glyceride sample, 100 mg, was dissolved in dry ether and placed in a 100-ml round-bottom flask. Lithium aluminum hydride, 200 mg, was dissolved in 30 ml of dry ether. The residue was allowed to settle and the lithium aluminum hydride solution was transferred by pipette 1 ml at a time to the glyceride solution until boiling stopped. A 50% excess of the lithium aluminum hydride solution was then added and the reaction mixture allowed to stand for 30 minutes. Twenty milliliters of acetic anhydride was added, dropwise at first. This mixture was heated to evaporate ether and then refluxed for 2 to 4 hours at the boiling point of acetic anhydride. Excess acetic anhydride was decomposed by the addition of 30 ml absolute ethanol followed by refluxing for 1 hour. Ethyl acetate and ethanol were removed on a rotary vacuum evaporator. Evaporation at temperatures up to 70° did not remove glyceryl triacetate. The contents of the flask were extracted with ether and the ether was washed with water. This ether solution was filtered through anhydrous sodium sulfate and reduced in volume for gas-liquid chromatography. Samples varying in size from 5 mg to 1 g can be used together with a proportionate decrease or increase in lithium aluminum hydride in the procedure described above. (CAUTION: *glyceride samples should be dry and lithium aluminum hydride handled carefully, especially if large amounts are used.*)

Thin-Layer Chromatography. Thin-layer plates of silica gel were prepared as described by Stahl (12). The developing solvent was chloroform—methanol—glacial acetic acid 96:2:2 (v/v). After drying, the plates were sprayed with 50% sulfuric acid and charred on a hot plate. R_f values were: triglycerides and fatty alcohol acetates, 0.90; diglycerides, 0.80; fatty alcohols, 0.55; and monoglycerides, 0.18.

Gas-Liquid Chromatography. Analyses were obtained with an Aerograph A-90-C gas-liquid chromatograph[1] equipped with a Wheelco 1 mv recorder[2] and a Wheelco Type A electronic integrator.[2] A 5-foot stainless steel column, 0.25 inch I.D., containing 25% G.E. SF-96 silicone grease on 35 to 80 mesh Chromosorb,[1] and 10-foot stainless steel columns, 0.25 inch I.D., containing 25% polyethylene glycol succinate (EGS) on 80 to 100 mesh firebrick and 10% EGS on 60 to 80 mesh Gas Chrom P,[3] were used in chromatographic separations at temperatures ranging from 170° to 210°. Helium was the carrier gas, and the inlet pressure was 20 psi. The chart speed was 30 inches per hour.

Additional qualitative analyses were obtained with a Barber-Colman Model 10 gas-liquid chromatograph[2] equipped with an argon ionization detector. A 7-foot column, 0.25 inch I.D., containing 3% neopentyl glycol sebacate on 80 to 100 mesh Gas Chrom P,[3] and a 100-foot stainless steel capillary column, 0.01 inch I.D., containing Apiezon L,[2] were used in the chromatographic separations.

RESULTS

Retention Time and Relative Response. Hexadecanyl acetate, the reference compound, and tetradecanyl acetate were prepared by the hydrogenolysis and acetylation of methyl palmitate and methyl myristate. Glyceryl triacetate was both purchased and synthesized. Other alcohol acetates were prepared by the hydrogenolysis and acetylation of pure glycerides and corn oil. Relative retention times for these compounds on EGS and silicone grease columns are reported in Table 1. Glyceryl triacetate and octadecanyl acetate had the same retention time on the EGS column at 205°; however, the separation factor was 1.14 when the temperature of the EGS column was lowered to 170°. Since glyceryl triacetate is more polar than fatty alcohol acetates, the retention time of glyceryl

[1] Wilkens Instrument and Research, Inc., Walnut Creek, California.
[2] Barber-Colman Co., Rockford, Illinois.
[3] Applied Science Laboratories, State College, Pennsylvania.

TABLE 1. RELATIVE RETENTION TIME OF ALCOHOL ACETATES ON EGS POLYESTER AND SILICONE GREASE COLUMNS AT 205°

Alcohol Acetate	EGS Polyester*	Silicone Grease†
Tetradecanyl acetate	0.64	0.51
Hexadecanyl acetate	1.00	1.00
Hexadecenyl acetate	1.06	
Glyceryl triacetate	1.38	0.085
Octadecanyl acetate	1.37	1.90
Octadecenyl acetate	1.57	1.75
Octadecadienyl acetate	1.87	1.75

* 10% EGS on 60 to 80 mesh Gas Chrom P in 10-foot stainless steel column, 0.25 inch I.D. At 20 psi helium pressure, the retention time for hexadecanyl acetate was 3.3 minutes.

† 25% G.E. SF-96 silicone grease on 35 to 80 mesh chromosorb in 5-foot stainless steel column, 0.25 inch I.D. At 20 psi helium pressure, the retention time for hexadecanyl acetate was 7.0 minutes.

triacetate is influenced more by the polarity of the liquid phase than the retention times of fatty alcohol acetates. Carbon numbers (13) for glyceryl triacetate with four different liquid phases are presented in Table 2.

Quantitative gas-liquid chromatography with a thermal conductivity detector requires the measurement of relative response factors (14). Known mixtures of glyceryl triacetate and methyl palmitate were therefore chromatographed to determine relative molar response (Table 3). The mean relative response factor for glyceryl triacetate was 95 ± 7. Since methyl esters and alcohol acetates have similar functional groups, relative response factors for alcohol acetates are similar to these factors for methyl esters with the same number of carbon atoms. The relative response factor for hexadecanyl acetate was calculated as 103.3 from the equation obtained for relative response in the homologous methyl ester series (14). Glyceryl triacetate and hexadecanyl acetate were then normalized as 92 and 100 respectively and used together with relative molar response factors calculated for other fatty alcohol acetates (Table 4) in subsequent quantitative analyses.

Hydrogenolysis and Acetylation. Preliminary experiments with methyl myristate and glyceryl triacetate were used to demonstrate that hydrogenolysis and acetylation were quantitative reactions. Methyl myristate was reduced, acetylated, and chromatographed on silicone grease and EGS columns. Analyses showed one peak with the retention time of tetradecanyl acetate. Methyl myristate and tetradecanol were absent. When either glycerol or glyceryl triacetate was reduced, acetylated, and chromatographed on silicone grease columns, only glyceryl triacetate was obtained. In several experiments, ether solutions of the glyceryl triacetate acetylation product were chromatographed before their extraction with water. These analyses yielded one peak and showed no evidence of partially acetylated or unreacted glycerol.[4] Glyceryl mono- and diacetates are partially soluble in ether and were detected as products when other reaction conditions were used. A glyceryl triacetate—methyl palmitate mixture in ether solution was subjected to gas-liquid chromatography before and after extraction with water in order to determine the recovery of glyceryl triacetate. The mixture contained 34.6 ± 1.5 mole % glyceryl triacetate before extraction and 34.2 ± 3.3 mole % glyceryl triacetate after extraction.

Quantitative acetylation of fatty alcohols and glycerol was confirmed by thin-layer chromatography. In one experiment, 1.0 g of hexadecanol was dissolved in dry ether and reacted with excess lithium aluminum

TABLE 2. CARBON NUMBERS OF GLYCERYL TRIACETATE ON VARIOUS LIQUID PHASES

Liquid Phase	Carbon Number	Temperature
Apiezon L	8.1	175°
SF-96 silicone	8.6	205°
Neopentyl glycol sebacate	11.4	184°
Ethylene glycol succinate	17.7	205°

TABLE 3. CHROMATOGRAPHIC ANALYSIS OF KNOWN MIXTURES OF GLYCERYL TRIACETATE AND METHYL PALMITATE ON A SILICONE GREASE COLUMN

Mixture	n*	Glyceryl Triacetate in Mole Percent Known	Found†	Relative Response†
A	7	28.2	27.1 ± 1.7	96.0 ± 6
B	6	55.3	51.7 ± 4.3	93.5 ± 8
Mean	13			95 ± 7

* Number of determinations.

† Mean ± standard deviation of observation.

[4] Partially acetylated glycerol derivatives are separated from glyceryl triacetate on EGS and silicone grease columns.

TABLE 4. RELATIVE MOLAR RESPONSE OF ALCOHOL ACETATES IN THE THERMAL CONDUCTIVITY DETECTOR

Alcohol Acetate	Relative Molar Response
Tetradecanyl acetate	92.8
Hexadecanyl acetate	100.0
Glyceryl triacetate	92.0
Octadecanyl acetate	106.0
Octadecenyl acetate	104.0
Octadecadienyl acetate	101.0

TABLE 5. Ester—Glycerol Ratios Determined by Gas-Liquid Chromatography with EGS Polyester Columns

Sample	n*	Ester/Glycerol†
Monopalmitin-A	6	1.05 ± 0.05
Monopalmitin-B	4	1.01 ± 0.12
Monostearin	9	0.99 ± 0.10
Monolein	6	1.14 ± 0.17
Dimyristin-A	3	2.19 ± 0.10
Dimyristin-B	3	2.10 ± 0.03
Olive Oil-A	5	3.10 ± 0.10
Olive Oil-B	4	2.96 ± 0.20
Cottonseed Oil	5	3.22 ± 0.19

* Number of determinations.
† Mean ± standard deviation of observation.

hydride. Acetic anhydride, 50 ml, was then added, the ether was evaporated, and the mixture refluxed. After 2 hours, 50 ml of absolute ethanol was added and this mixture refluxed an additional hour. Fifty-microliter samples were withdrawn at various times for thin-layer chromatography. Large aliquots were chromatographed in several experiments to insure detection of unreacted material. Chromatography showed that acetylation was complete in 1 hour and unaffected by the addition of ethanol. When glyceryl triacetate was reacted with lithium aluminum hydride and acetylated, thin-layer chromatography showed that the acetylated product before ether extraction and a large aliquot from the final ether extract contained only traces of partially acetylated glycerol. Although the lithium aluminum alcoholate of vitamin-A alcohol is acetylated by refluxing with acetic anhydride in ether (11), quantitative acetolysis of lithium aluminum alcoholates requires ether evaporation and refluxing in boiling acetic anhydride. When hexadecanol and glycerol lithium aluminum alcoholates were acetylated with acetic anhydride by refluxing in ether, appreciable quantities of unreacted and partially acetylated alcohols were demonstrated by thin-layer chromatography throughout a 60-minute acetylation period.

Quantitative Estimation of Glycerol and Fatty Acid Composition. Mono-, di-, and triglycerides were reduced, acetylated, and chromatographed. Ester—glycerol ratios obtained from these analyses are presented in Table 5. Methyl esters of olive oil and cottonseed oil were prepared by refluxing with boron trifluoride—methanol (15). A comparison of fatty acid composition data obtained with fatty alcohol acetates and fatty acid methyl esters (Table 6) indicates that there is no loss of polyunsaturated compounds.

TABLE 6. Fatty Acid Composition (in Mole %) of Olive Oil and Cottonseed Oil Analyzed as Alcohol Acetates and as Methyl Esters by Gas-Liquid Chromatography with an EGS Polyester Column

Fatty Acid	Olive Oil		Cottonseed Oil	
	Acetate*	Methyl Ester*	Acetate*	Methyl Ester*
14:0	0	0	0	1.4 ± 0.6
16:0	14.8 ± 0.8	13.1 ± 0.6	25.3 ± 2.0	23.9 ± 0.7
18:0	0	<1	0	1.1 ± 0.2
18:1	80.9 ± 1.6	82.3 ± 1.3	17.2 ± 1.1	16.7 ± 0.5
18:2	5.2 ± 0.4	4.1 ± 0.9	57.5 ± 1.3	56.8 ± 0.7

* Mean ± standard deviation of observation.

DISCUSSION

The experimental results indicate that hydrogenolysis–acetylation is a simple and quantitative procedure. Fatty acid esters are reduced to their alcoholates by lithium aluminum hydride. Lithium aluminum alcoholates are acetylated by direct reflux with acetic anhydride. This reaction is probably catalyzed by the lithium and aluminum acetates present in the mixture. Excess acetic anhydride is decomposed by refluxing with ethanol. An ether solution is then washed with water to remove ethanol without extracting fatty alcohol or glycerol acetates. The ether solution is concentrated and acetates are readily separated by gas-liquid chromatography. Reaction products are obtained in the expected proportions and are not contaminated with unreacted starting materials. Thin-layer chromatography demonstrates that the acetylation reaction is complete in 60 minutes. However, the yield of glyceryl triacetate is sometimes low, and care must be taken to obtain the correct ester—glycerol ratio.

Hydrogenolysis—acetylation has several advantages. Analyses are rapid and may be completed within 8 hours. Hydrogenolysis avoids losses during the hydrolysis of glycerides that are often troublesome in the quantitative analysis of glycerol (4, 7). Since glycerol is converted to glyceryl triacetate, the recovery of glycerol is not affected by its volatility (3). A positive identification of glycerol is obtained from the retention time of glyceryl triacetate. Polyhydroxy compounds, with the possible exception of lactic acid, would not interfere in the procedure. However, this study has been limited to pure glycerides, olive oil, and cottonseed oil. Glyceride purification may be required before complex biological materials are analyzed. Esterification or interesterification reactions are not required for the preparation of fatty acid derivatives suitable for gas-liquid chromatography. Furthermore, both glyceryl triacetate and alcohol acetates can be collected as pure

compounds and analyzed for their specific radioactivities. Since low molecular weight acetates may be soluble in water, this procedure would require modification for the analysis of materials such as butter fat.

The quantity of sample necessary for a complete analysis depends only on the sensitivity of the detector. In this study, as little as 5 mg of glyceride was sufficient for a complete analysis. Thermal conductivity detectors are much less sensitive than argon or flame ionization detectors. Ionization detectors should facilitate the analysis of smaller lipid samples. However, a thorough calibration of the particular detector will be necessary before quantitative results are obtained.

Quantitative studies are further limited by the separation efficiency of the column employed. Since glyceryl triacetate has a relatively high polarity, its retention time varies considerably with the polarity of the liquid phase. Glyceryl triacetate is readily separated from most fatty alcohol acetates on low polarity columns such as silicone grease, Apiezon L, or neopentyl glycol sebacate. Acetic anhydride and glyceryl triacetate have similar retention times on low polarity columns. Thus, acetic anhydride must be removed for quantitative analyses with these columns. Ethylene glycol succinate columns gave excellent results for the mixtures analyzed. If fatty alcohol acetates such as heptadecenyl acetate overlap the glyceryl triacetate, then a column of different polarity can be used or a conventional ester preparation employed to determine the heptadecenoate present in the original sample.

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