notes on methodology

Determination of fatty acid content and composition in ultramicro lipid samples by gas–liquid chromatography

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SUMMARY Simplified quantitative manipulations of very small amounts (30 μg) of lipids for determination of fatty acid content and composition by gas–liquid chromatography after (*a*) methanolysis (*b*) reduction and acetylation are described.

KEY WORDS determination · ultramicro · fatty acids · methyl esters · alcohol acetates · glycerol · gas–liquid chromatography · liver lipids

THE CONVENTIONAL PREPARATION of fatty acid esters from lipid material for GLC involves purification by washing, chromatography, distillation, or other means (1) to avoid interference from materials other than esters and the production of artifacts which affect the results and reduce the life of the chromatographic columns. The purification steps make it difficult to achieve a satisfactory material recovery from lipid samples of less than a milligram. This communication describes methods of analyzing 10–30 μg of fatty acids without the usual purification procedures. The methods have been successfully applied to rat liver lipids.

Quantitatively reliable results were obtained with a sample prepared in less than 4 hr. The chromatographic column lasted only 2–4 weeks; this was accepted as a reasonable price for the simplicity of the method.

Reagents. Diethyl ether, acetone, and methanol were purchased from Merck & Company, Inc., Rahway, N.J.; hexane from Matheson Co., Inc., E. Rutherford, N.J.; acetyl chloride from Eastman Kodak Company, Rochester, N.Y.; lithium aluminum hydride from Metal Hydrides Inc., Beverly, Mass.; hexamethyl disilane and trimethyl chlorosilane from Peninsular Chemical Research, Gainesville, Fla.; methyl palmitate, butyl stearate, and arachidic acid from Applied Science, Inc., State College, Pa.; nonadecanoic acid from Aldrich Chemical Company, Inc., Milwaukee, Wis.; chromatographic column support and liquid phase from Analabs, Hamden, Conn. Fatty acid ester mixture standard F was supplied by the National Heart Institute, Bethesda, Md. Solvents were distilled before use through a 4 inch column packed with beads.

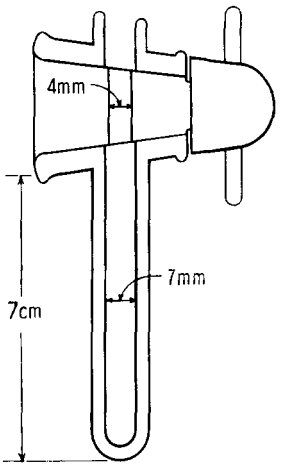

FIG. 1. Ampule for reactions with submicro samples.

Apparatus. An ampule for the chemical reaction was made by sealing off one arm of a stopcock, which had a 1:10 taper spring-loaded Teflon plug, and shortening its other arm (Fig. 1). The ampule was heated by placing the lower quarter in a controlled temperature bath.

An apparatus for recovering and concentrating the reaction product (Fig. 2) was made by sealing a 2 mm capillary tube to the center of a 50 ml round bottom flask. The recovery apparatus included a support with tubing manifold for evaporation with nitrogen.

The reflux trap shown in Fig. 3 was used in the preparation of dry ethyl ether, which was distilled through it from lithium aluminum hydride.

The gas chromatograph was a Barber Coleman Model 25-C with a 6 ft x 3 mm helical glass coil packed with 20% diethylene glycol succinate polyester on 70–80 mesh Chromosorb ABS. The column was operated at 175°C with argon at 30 psi and a radium detector. Samples were injected with a 10 μl Hamilton syringe.

Esterification. Methanolysis with methanol and H_2SO_4 was tried under various conditions described by others

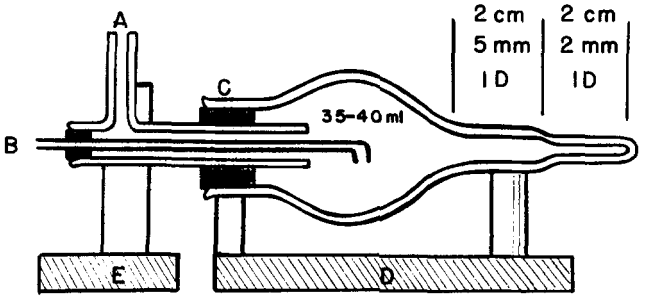

FIG. 2. Concentrating device. *A*, glass tee (5 mm ID); *B*, 10 cm of $^1/_{16}$ inch tubing in rubber stopper; *C*, rubber stopper; *D* and *E*, wooden racks. Each unit is connected via a manifold distributor to nitrogen at *B* and to exhaust at *A*.

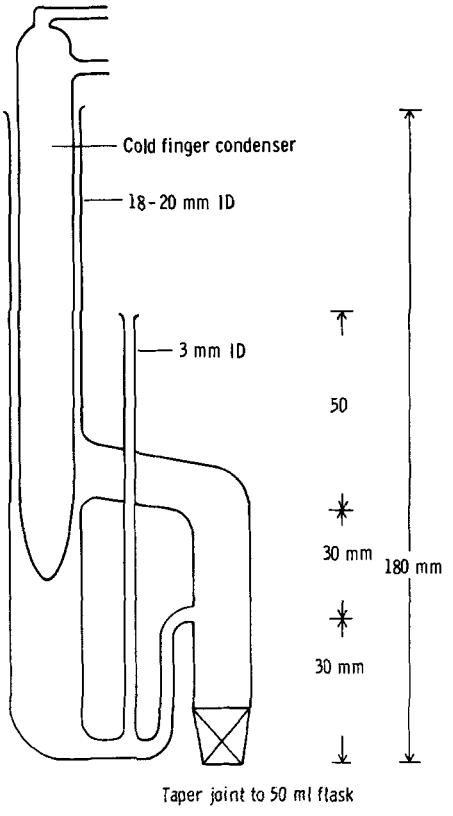

FIG. 3. Reflux trap for preparing fresh dry solvent.

(2–4); but it was found that methyl ester recovery was higher if the sample was first saponified and then esterified.

When methanolysis was used, a portion of sample containing about 10–20 μg of fatty acids was placed in the ampule and 5 μg of the internal standard fatty acid was added. The solvent was removed under nitrogen, and 0.2 ml of methanol–H_2SO_4 (2–20% v/v), (made by adding concd. sulfuric acid slowly to methanol cooled in ice) was added. Air was displaced with nitrogen and the ampule was closed and placed in the thermostat. After the specified time (see Table 1) the ampule was cooled and 1.0 ml of hexane and 0.1 ml of water were added. The sample was agitated for 1 min and the hexane was then aspirated by Pasteur pipette into a horizontally positioned concentrator. Two further extractions by 1.0 ml of hexane were made. The solvent was removed under a stream of dry nitrogen led in through *B* (Fig. 2). The dried residue was rinsed with about 0.2 ml of hexane down into the capillary by placing the concentrator in a vertical position, thereby leaving acid or other residue on the side of the concentrator. This hexane was evaporated in the capillary by a slow jet of nitrogen through a No. 20 hypodermic needle directed into the mouth of the capillary. The residue was dissolved in 5–10 μl of solvent added by means of a Hamilton 10 μl syringe and a similar syringe was used to inject a sample into the gas chromatograph.

Alternatively, lipid in the ampule was first saponified by adding to it 0.2 ml of 18% KOH in methanol and heating at 80°C for 1.5 hr. The ampule was cooled, 0.2 ml of 20% v/v H_2SO_4 in methanol was added, and the mixture was heated at 80°C for another hour. During the subsequent extraction of the ester a short piece of glass rod was agitated in the ampule to break up the potassium sulfate crystals. The method continued as described above.

Acetylation. Fatty acids have been chromatographed as the acetates of their corresponding alcohols by Holla, Horrocks, and Cornwell (5). We adapted their method to the ultramicro scale by using acetyl chloride instead of acetic anhydride. The lipid, well dried under nitrogen, was reduced with 0.1 ml of a one-fifth saturated solution of $LiAlH_4$ in ether for 1.5 hr at room temperature. The $LiAlH_4$ solution was made by crushing a lump about 4 mm long in a test tube with 3 ml of dry ether and allowing the solid material to settle until the supernatant layer was clear. Of this saturated solution 0.5 ml was added to 2 ml of dry ether. Glass surfaces used in this operation were flushed with dry ether before use and the reagent was used immediately.

The solution of fatty alcohol was evaporated under nitrogen, the residue was moistened with two drops of hexane, and 0.2 ml of acetyl chloride containing 3% of acetic anhydride was added. Air was displaced with nitrogen. The ampule was closed, heated at 100°C for 1.5 hr, and cooled. The contents were rapidly transferred, in 1.5 ml of dry ether, to a vacuum-dried concentrator. The residue, after evaporation of the ether, was eluted into the capillary with acetone–hexane 1:1, care being taken not to displace any salt particles. Acetone was used to recover the sample from the capillary for gas chromatography as described under *Apparatus.*

Results and Discussion. Table 1 shows the results of methanolysis of rat liver lipid under several different conditions. In these experiments arachidic acid was the internal standard because rat liver lipid contained less than 0.3% of this acid. Blank runs on the reagents alone showed artifacts of about 0.5% at 16:0 and 18:0 and 0.1–0.3% at 15:0 and 18:1. These percentages are based on a 20 μg total fatty acid sample. No artifact was found at 20:4 as was reported by Johnston and Roots (6). Table 1 shows partial destruction of 20:4 ester with 20% methanol–H_2SO_4 beyond 1 hr at 60°C.

The increase in total fatty acids recovered as esters from 61% of total lipid to 71% by saponification before esterification is not completely understood. Therefore saponification was not used in obtaining the data in Table 3.

TABLE 1 FATTY ACID ANALYSES OF WHOLE RAT LIVER LIPID AFTER METHANOLYSIS OR SAPONIFICATION AND ESTERIFICATION

H_2SO_4 in Methanol (v/v)	Time at 60°C	Yield of Fatty Acids					
		16:0	18:0	18:1	18:2	20:4	Total
%				%			
2	20 min	10.6	10.5	1.5	0	0	22.6
2	3 hr	10.6	11.9	6.2	5.3	5.9	39.9
2	16 hr	11.7	13.2	7.9	13.6	14.6	61.0
5	16 hr	14.6	13.1	8.3	14.3	12.5	62.8
20	10 min	14.5	14.4	6.9	10.9	10.3	57.0
20	1 hr	13.4	13.3	8.0	13.2	13.3	61.2
20	16 hr	13.8	13.1	4.5	5.2	6.1	42.7
Esterification after saponification*		17.2	15.3	9.4	16.1	13.3	71.3

* Saponified with 18% KOH at 80°C for $1^1/_2$ hr followed by esterification with an equal volume of 20% v/v H_2SO_4 in methanol.

TABLE 2 FATTY ACID ANALYSES OF KNOWN TRIGLYCERIDE MIXTURES BY $LiAlH_4$ REDUCTION AND ACETYLATION

	Found Composition				Known Composition	Deviation
	I	II	III	Average		
				weight per cent		
Myristic	12.5	9.8	10.9	11.1	9.2	−1.9
Palmitic	23.2	23.5	25.0	23.9	22.2	−1.7
Stearic	37.8	39.5	33.7	37.0	39.2	+2.2
Oleic	26.5	27.2	30.4	28.0	29.4	+1.4
Glycerol recovery*	92	93	103	96	—	−4%
Glycerol recovery†	109	99	107	105	—	+5%

* With methyl palmitate as standard.
† With butyl stearate as standard.

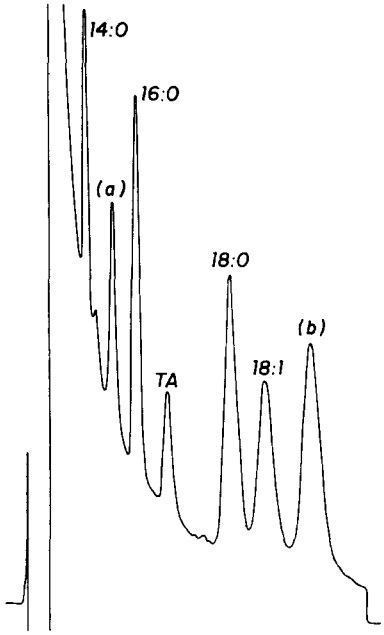

FIG. 4. GLC of fatty alcohol acetates from a known mixture of triglycerides showing triacetin (*TA*) and standards (*a*) and (*b*). Conditions: Column 6 ft × 4 mm I.D., 20% diethylene glycol succinate polyester on Chromosorb ABS. Column temperature 175°C. Argon flow 60 ml/min.

Table 2 shows the results of analyses of 10-μg samples of a known mixture of triglycerides (trimyristin, tripalmitin, tristearin, and triolein) by the acetylation described; Fig. 4 shows a chromatographic chart of acetates obtained from this mixture. The triacetin was estimated by peak height against internal standards added to the ampule just before transfer to the concentrator. Both methyl palmitate and butyl stearate were used as standards and a calibration curve versus triacetin was made separately from the pure materials. The amounts of fatty acid shown are by normalization of triangulated areas.

Table 3 compares fatty acid determinations on a single preparation of rat liver lipid by methanolysis versus acetylation. The last column gives results of methanolysis after hydrogenation. Hydrogenation was performed in methanol solution in the ampule by adding 5 mg of palladium black and placing the ampule in a hydrogen atmosphere at 10 psi. The sample was agitated for 1 hr at room temperature, evaporated to dryness, and methanolyzed as described above. The close similarity of values for methyl esters and for acetyl esters shows that aldehydes and alcohols can be present only in amounts less than 1%, since they would be reduced and appear

TABLE 3 COMPARISON OF FATTY ACID COMPOSITIONS OF TOTAL RAT LIVER LIPID EXTRACTS OBTAINED BY METHANOLYSIS AND ACETYLATION PROCEDURES

	Percentages Based on Normalized Peak Areas		
	Methanolysis*	Reduction and Acetylation	Methanolysis* after Hydrogenation
Myristic	0.1	0.6	—
Palmitic	22.2	22.5	20.3
Stearic	19.8	20.8	58.6
Oleic	16.9	17.2	0.0
Linoleic	20.2	19.6	0.0
Arachidic	0.0	0.0	16.0
Arachidonic	20.8	19.3	5.1
Stearic + oleic + linoleic	56.9	57.6	58.6

* Methanolysis with 20% H_2SO_4 methanol (v/v) for 1 hr.

TABLE 4 CHANGES IN RELATIVE RESPONSE AFTER REPEATED TREATMENT OF COLUMN WITH SILANE DERIVATIVES

Methyl Esters	New Column	Aged Column
Palmitate	1.09	1.20
Stearate	1.00	1.10
Arachidate	1.00	1.00
Behenate	0.995	0.97

Referred to NHI Fatty Acid Ester Standard F.

as the acetyl esters after the reduction and acetylation procedure.

For the determination of glycerol in natural triglycerides or lower glycerides, it is desirable to hydrogenate these glycerides before acetylation to eliminate the interference of any palmitoleate ester with the triacetin peak.

The methods described expose the chromatographic column to considerable amounts of material other than esters. This material eventually "bleeds out" and affects the detector response. The use of silane derivatives to eliminate this difficulty has been described by Atkinson and Tuey (7). We found that injecting 1 μl of hexamethyl disilane followed by 1 μl of trimethyl chlorosilane at 175°C restored the base line and improved the detector response. However, repeated treatments had a marked effect on the relative response of the detector to various esters, as shown in Table 4. Fatty acid determination by esterification, although simpler, gave more rapid contamination of the column than did the acetylation method.

The addition of 3% of acetic anhydride to the acetyl chloride reagent was found to be necessary to avoid charring the sample. The sample was wetted before adding this reagent for the same reason.

Aluminum chloride, present in the acetylation product, should not be injected to the column. The dilution of acetone with hexane for eluting the ester from the reaction residue reduced the solubility of aluminum chloride; 50% acetone is necessary, however, to dissolve the triacetin.

An attempt to remove material other than esters by passing methyl esters through a small silica gel column was not successful with 30-μg samples because the relatively large volume of eluting solvent needed for quantitative recovery of the higher esters gave excessive amounts of solvent artifacts.

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