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A rapid quantitative method for the separation of free fatty acids from other lipids*†

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SUMMARY

A method for the separation of free fatty acids from other lipids is presented. Silicic acid treated with isopropanol-KOH and washed with ethyl ether serves as the column packing. The sample is introduced in ethyl ether, and neutral lipids are eluted with this solvent. Fatty acids are removed from the column with 50 ml of 2% formic acid in ethyl ether followed by 75 to 100 ml of ethyl ether. Phospholipids are retained on the column. The results of 26 analyses of fatty acids mixed with various other lipids showed an average recovery of 98.3% for free fatty acids.

In studies of lipid metabolism it is often necessary or desirable to separate free fatty acids (FFA) from other lipids. When radioactive tracers are used it is essential to obtain a clear-cut separation. A rapid and simple method for accomplishing this separation has not been available. On a theoretical basis, anion exchange resins appeared to offer the most promise. Our results with both Amberlite IRA-400® and Dowex 3®, were very unsatisfactory, however. Fresh resin charged according to the method of Hornstein *et al.* (1) would remove the FFA from a lipid mixture. Quantitative recovery of these acids from the column was virtually impossible even under drastic conditions of elution. If the same resin was used four or five times, being recharged after each use, quantitative gravimetric recovery of FFA became possible. Gas-liquid chromatographic analyses of the eluted acids showed, however, that fatty acids from previous use were exchanging with the FFA of the sample. This paper describes a rapid, quantitative method for the separation of FFA and other lipids.

MATERIALS AND METHODS

Materials. Reagent grade methanol, acetone, ethyl ether, and formic acid were used. The ethyl ether was distilled in glass. Isopropanol-KOH was prepared according to the method of Keeney (2). The isopropanol-KOH solution should contain approximately 50 mg KOH per ml. Mallinckrodt silicic acid, 100 mesh, labeled "suitable for chromatographic analysis by the method of Ramsey and Patterson" was used.

Method. To improve the flow of solvent through the column, coarser particles of silicic acid were selected by suspending 100 g in 400 ml of methanol and decanting what did not settle in 5 minutes. This was repeated once with methanol and once with 400 ml of acetone. The silicic acid was rinsed with ethyl ether and permitted to dry in air.

Five grams of prepared silicic acid were weighed into a small beaker. To this was added with mixing 10 ml of isopropanol-KOH and 30 ml of ethyl ether. After standing 5 minutes, the silicic acid was slurried into a glass column and washed with 100 ml of ethyl ether. The column used in our laboratory has a diameter of 2 cm and a height of 15 cm; however, the dimensions of the column are not critical. If air bubbles form, they should be removed by stirring with a glass rod. A solvent flow rate of 5 ml per minute is satisfactory.

The sample, dissolved in a small quantity of ethyl ether, was placed on the column and thoroughly washed into the packing by several small portions of ethyl ether. Cholesterol, cholesterol esters, mono-, di-, and triglycerides were eluted in one fraction from the column with 100 to 150 ml of ethyl ether (fraction 1). The FFA retained on the column were removed by 50 ml of 2% formic acid in ethyl ether (v/v), followed by 75 to 100 ml of ethyl ether (fraction 2). The solvent can be easily removed from the samples

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TABLE 1. SEPARATION OF FREE FATTY ACIDS* AND NEUTRAL GLYCERIDES† ON KOH-TREATED SILICIC ACID

	Neutral Glycerides	Free Fatty Acids
No. of separations	15	15
Mean wt added	176.8 mg	105.8 mg
Range wt added	92.9–452.2 mg	75.4–185.0 mg
Mean % recovery	99.9	97.5
Range % recovery	96.4–101.8	96.0–99.6

* Mixture of fatty acids: 12:0, 14:0, 16:0, 18:0, and 18:2.
† Butter oil.

TABLE 2. GLC ANALYSES OF FATTY ACID MIXTURE AS METHYL ESTERS BEFORE AND AFTER SEPARATION FROM NEUTRAL GLYCERIDES ON KOH-TREATED SILICIC ACID

Fatty Acid Methyl Ester	Before	After*
	wt %	*wt %*
12:0	20.2	21.9
14:0	23.4	23.1
16:0	25.6	26.2
18:0	23.3	21.6
18:2	7.6	7.3

* Mean for six columns.

under reduced pressure or on a steam bath under a stream of nitrogen.

If phospholipids are present in the lipid sample they are retained on the column. Phospholipids can be removed from the silicic acid by the use of methanol, following the elution of FFA. Since the methanol fraction also contains inorganic potassium salts, the method is neither quantitative for phospholipids nor desirable for their isolation.

To evaluate the method initially, various proportions of known mixtures of glycerides and FFA were prepared. Butter oil was used as the glyceride mixture while the free fatty acids were a mixture of lauric, myristic, palmitic, stearic, and oleic acids. Gravimetric results from 15 separations were obtained. All fractions were checked for efficiency of separation by infrared spectrophotometry. In addition, the fatty acid mixtures eluted from each of six columns were converted to methyl esters and individually analyzed by gas-liquid chromatography. A Barber-Coleman Model 20 instrument with a 10-foot diethylene glycol succinate column was used as previously described (3). These analyses were compared with the methyl ester analysis of the original fatty acid mixture.

The separation of FFA from other neutral lipids was determined. Palmitic acid was mixed with monostearin, soybean oil diglycerides, cholesterol, and cholesteryl palmitate separately and in combination. Gravimetric results for these analyses were obtained. The efficiency of separation was again checked by infrared spectrophotometry.

To study the ability of the method to separate labeled materials, a known aliquot of C^{14}-labeled neutral lipids or fatty acid was added to glyceride-fatty acid mixtures before separation. After separation on the column, all fractions were assayed for radioactivity in a thin-window gas-flow Geiger-Mueller counter. The activity in these fractions was compared with the activity in an equal aliquot of the original labeled material.

The behavior of phospholipids on the column was determined by adding them separately and in combination with neutral lipids and FFA. Phospholipids for the study were extracted from eggs according to the procedure of Harper *et al.* (4), as modified by Jensen and Morgan (5). The phospholipids were isolated from the extracted fat by the silicic acid method of Hirsch and Ahrens (6). Phosphorus determinations on the various fractions obtained from the KOH-treated silicic acid column were performed by the method of Smith *et al.* (7).

RESULTS AND DISCUSSION

Gravimetric results for 15 analyses of glycerides and fatty acid mixtures are presented in Table 1. Infrared spectrophotometry showed the glyceride fraction to be free of carboxyl absorption while the FFA fractions were free of absorption due to ester linkages. Table 2 reports the composition of the FFA before and after separation on the column. The data presented in these tables demonstrate the efficiency of the method in separating glycerides and FFA.

The separation of other neutral lipids and palmitic acid is reported in Table 3. In each case a known weight of palmitic acid was added to a particular lipid compound before separation. In all instances the neutral lipids were eluted with ethyl ether in the first fraction. Infrared spectrophotometry confirmed the absence of contamination between the neutral lipid fraction and the FFA fraction.

The results obtained with C^{14}-labeled compounds are reported in Table 4. The counts per minute reported in the table represent actual counts corrected for background and dilution.

The question remained whether phospholipids would interfere with the isolation of FFA. The behavior of

TABLE 3. SEPARATION OF PALMITIC ACID AND NEUTRAL LIPID COMPOUNDS ON KOH-TREATED SILICIC ACID

Sample Mixtures	No. Separations	Mean Wt Added	Mean Recovery
		mg	%
Monostearin	4	52.7	102.8
Palmitic acid		96.2	98.1
Diglycerides*	2	128.5	101.9
Palmitic acid		83.6	101.2
Cholesterol	1	109.5	100.4
Palmitic acid		119.8	99.3
Cholesteryl palmitate	1	123.0	101.5
Palmitic acid		104.5	95.5
Combination†	1	512.8	98.7
Palmitic acid		120.1	103.8

* Soybean oil diglycerides, 86%.

† Mixture of monostearin, 64.0 mg; cholesterol, 62.8 mg; cholesteryl palmitate, 57.9 mg; neutral butter oil, 328.1 mg.

TABLE 4. DISTRIBUTION AND RECOVERY OF C^{14}-LABELED LIPIDS AFTER SEPARATION ON KOH-TREATED SILICIC ACID

Labeled Substance Added*	Control Activity†	Activity Recovered	
		Fraction 1	Fraction 2
	cpm	*cpm*	*cpm*
Tripalmitin-1-C^{14}	171,400	178,900	0
Cholesterol-4-C^{14}	369,280	360,720	900
Cholesteryl palmitate-1-C^{14}	657,360	667,920	660
Palmitic acid-1-C^{14}	185,600	0	185,200
Linoleic acid-1-C^{14}	100,490	0	96,270
Butyric acid-1-C^{14}	233,250	0	220,000

* Mixed with glycerides and FFA prior to separation.

† An aliquot equivalent to that separated.

TABLE 5. CHROMATOGRAPHY OF PHOSPHOLIPIDS ALONE OR IN COMBINATION WITH OTHER LIPIDS ON KOH-TREATED SILICIC ACID

Sample Mixture	No. Separations	Mean Wt Added	Mean Wt Recovered	
			Fraction 1	Fraction 2
		mg	*mg*	*mg*
Phospholipids*	2	81.0	0	0
Palmitic acid		114.3	1.9	109.6
Phospholipids	2	54.0		
Palmitic acid		100.0		
Neutral egg lipid	2	347.9	347.5	99.6
Phospholipids		81.0		

* Isolated from egg yolks.

phospholipids on the column was studied either alone or in combination with palmitic acid and neutral lipids. These results are reported in Table 5. When only phospholipids were used, fractions 1 and 2 were free of any lipid material. When mixtures were used, the quantitative recovery of FFA was not affected. Infrared spectrophotometry confirmed the efficiency of separation of the various fractions. Phosphorus analyses showed the first two fractions to be free of phosphorus.

A small quantity of lipid material was recovered in fraction 1 when samples of phospholipid and palmitic acid combinations were separated (Table 5). Infrared spectrophotometry and negative phosphorus analyses suggest that the material was an ester contaminant in the palmitic acid standard. Cerebrosides do not contain phosphorus, but an infrared comparison of this fraction with a known spectra of cerebrosides was not performed. When the same phospholipids were chromatographed separately, however, fraction 1 was free of lipid material.

Phospholipids can be removed from the column with methanol but they are contaminated with potassium salts. If one also desires to recover phospholipids quantitatively from a complex mixture, it is suggested that they first be isolated on untreated silicic acid (6).

The method has been successfully used in this laboratory for the isolation of FFA from biological sources. The performance of the method was demonstrated by the following example. Less than 0.01 mg of palmitic-1-C^{14} acid was mixed with bovine serum lipids containing approximately 2% FFA. Free fatty acids were isolated from the serum lipids and assayed for radioactivity. The results of three analyses demonstrated an average recovery of 98% of the labeled fatty acid.

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