notes on methodology

One-step quantitative extraction of medium-chain and long-chain fatty acids from aqueous samples

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SUMMARY Medium-chain (C_6 and longer) fatty acids, as well as 12-hydroxystearic and long-chain fatty acids, can be quantitatively extracted into toluene and titrated in the toluene phase with tetrabutylammonium hydroxide. The method may be useful in determinations of fecal and serum fatty acids and of the products of lipolysis.

SUPPLEMENTARY KEY WORDS medium-chain triglycerides · free fatty acid · titration · octanoic acid · 12-hydroxystearic acid · feces · serum · lipolysis

WORKERS using medium-chain fatty acids have experienced difficulty in extracting more than 70% from aqueous solutions or emulsions by the usual techniques (1–5). During studies on lipolysis of medium-chain triglycerides by gastric lipase (6), we found that medium-chain fatty acids were extracted quantitatively with toluene or benzene in a single distribution. We report here a simple method for complete extraction and titrimetric determination of medium-chain and long-chain fatty acids in aqueous samples.

Materials and Methods. Lipids were obtained from The Hormel Institute (Austin, Minn.), and their class purity was confirmed as being greater than 99% by thin-layer chromatography. Sodium oleate was obtained from Applied Science Laboratories (State College, Pa.). Commercial 25% tetrabutylammonium hydroxide (TEBAH) titrant in methanol (Distillation Products Industries, Rochester, N.Y.), after dilution with 9 volumes of redistilled methanol, was 0.08 N by titration against 0.10 N HCl. Bromothymol blue indicator (Fisher Scientific Products, Fair Lawn, N.J.) was dissolved in absolute ethanol (0.1 g/100 ml).

15-ml, conical, graduated Pyrex centrifuge tubes with Teflon-lined or polyethylene-lined screw stoppers ("Polyseal," Bel-Art Products, Pequannock, N.J.) were used; phase volumes were read directly.

Abbreviation: TEBAH, tetrabutylammonium hydroxide.

Trioctanoin-^{14}C (New England Nuclear, Boston) was 99% radiopure by zonal scanning (7). Trioctanoin was emulsified in 10 mM sodium taurodeoxycholate and 0.15 M sodium phosphate buffer (pH 6) by sonication; the trioctanoin emulsion had a concentration of 60 mM.

Extraction Procedure. A 2.0 ml sample of solution containing 5–400 μmoles of fatty acid is placed in a 15 ml graduated tube, and 1.0 ml of 1 N H_2SO_4 is added. To the 3 ml acidified aqueous sample is added 9.0 ml of ethanol–toluene 1:2. The tube is stoppered, vigorously shaken for 3 sec, and centrifuged at about 1500 *g* for 5 min. The total volume is 12 ml, and the upper phase measures 6.4 ml. A 2 ml sample of water or saline is treated similarly to serve as a combined sample and reagent blank.

An aliquot of the upper toluene phase (we used 3.0 ml) is placed in a 20 ml glass test-tube containing 0.1 ml of indicator. The solution is titrated to a blue end point with TEBAH from a microburet. Nitrogen is bubbled through the solution for mixing. The blank (3–6 μl of TEBAH, or less than 0.5 μmole) is subtracted from the volume of TEBAH used and the difference is used to calculate the amount of fatty acid present in the sample.

Efficiency of Extraction (Table 1). Stoichiometry of TEBAH titration using bromothymol blue as indicator was established by titrating 0.1- to 2.0-ml aliquots of 200 mM solutions of octanoic and oleic acid in benzene. To test extraction, we adjusted aliquots (containing 5–400 μmoles of fatty acid) of 50 and 200 mM solutions of sodium octanoate and sodium oleate to 2.0 ml with borate buffer (pH 9, 0.15 M in Na^+) and extracted. For long-chain fatty acids, aqueous solutions containing 50, 100, and 150 μmoles were prepared by neutralization of weighed amounts of the fatty acid and heating to give a clear soap solution. The solution or gel obtained on cooling was extracted directly. The method was shown to extract more than 98% of octanoic, decanoic, stearic, oleic, and linoleic acids, as well as 12-hydroxystearic

TABLE 1 PERCENTAGE EXTRACTION OF MEDIUM- AND LONG-CHAIN FATTY ACIDS AND OF 12-HYDROXYSTEARIC ACID

Acid	Mean ± SD	Range (n)
Hexanoic*	81.0 ± 1.2	78.9–81.8 (6)
Octanoic	98.9 ± 1.5	97.6–101.8 (12)
Decanoic	98.6 ± 0.7	97.7–99.3 (6)
Stearic	101.7 ± 1.5	98.8–103.0 (6)
Oleic	99.5 ± 4.3	93.3–104.4 (12)
Linoleic	101.1 ± 1.1	100.3–103.3 (6)
12-Hydroxystearic	101.9 ± 2.2	98.9–105.5 (6)

* Results are given for the standard extraction using 9 ml of ethanol–toluene 1:2 and 3 ml of acidified aqueous sample (ethanol as percentage of nonhydrocarbon solvents, 50%). When 7 ml of ethanol–toluene 1:6 was used (ethanol as percentage of nonhydrocarbon solvents, 25%), 97.6 ± 1.6% of the hexanoic acid was extracted.

TABLE 2 INFLUENCE OF ORIGINAL CONCENTRATION OF OCTANOIC ACID ON THE PERCENTAGE EXTRACTED

Fatty Acid Concentration*	Mean ± SD	Range (n)
mM		
50	100.4 ± 1.2	99.6–100.1 (8)
100	99.1 ± 1.8	97.6–102.8 (8)
200	98.0 ± 1.0	98.9–100.5 (8)

* In the original 2 ml of aqueous solution.

TABLE 3 INFLUENCE OF SOLVENT ON PERCENTAGE OF OCTANOIC ACID EXTRACTED

Solvent	Mean ± SD	Range (n)
Benzene	100.7 ± 1.7	98.4–102.8 (6)
Toluene	98.9 ± 1.5	97.6–101.8 (6)
Hexane	69.0 ± 1.9	68.3–72.9 (6)

acid. Hexanoic acid was incompletely extracted (81.0 ± 1.2%), but extraction could be increased to 97.6 ± 1.6% (SD, n = 6) by reducing the concentration of ethanol in the acidified aqueous sample to 25%.

Efficiency of extraction of fatty acids (along with glycerides) from a mixture of lipolysis products was checked by pipetting 100 μl of an alcoholic solution of 99% pure trioctanoin-^{14}C into four 22-ml counting vials; the alcohol was evaporated in a stream of air. To one vial was added a toluene-based scintillant. To the other three vials, 1 ml of unlabeled trioctanoin emulsion was added. 1 ml of a lipase solution (duodenal or gastric aspirate) was added, the three bottles were capped and incubated for 1 hr, and the sample was acidified and extracted as described. The radioactivity in a 0.5 ml aliquot of the upper phase was determined by scintillation counting and expressed in dpm. The extraction of ^{14}C (shown by zonal scanning of thin-layer chromatograms [7] to be 15–30% fatty acid, with monoglyceride, diglyceride, and triglyceride in various proportions) ranged from 95 to 107%, with a mean of 101.6 ± 1.8% (n = 15).

Influence of Fatty Acid Concentration (*Table 2*). At higher concentrations of fatty acid, the efficiency of extraction decreased. Complete extraction (100.4 ± 1.2%) of 100 μmoles of octanoic acid (in 2 ml) was obtained, but when 400 μmoles (per 2 ml) was present, the apparent recovery decreased to 98.0 ± 1.0% ($P < 0.001$). The volume of the upper phase was unchanged.

Toluene and Benzene vs. Petroleum Hydrocarbon (*Table 3*). Because long-chain fatty acids are extracted from aqueous acidic solutions without difficulty (1–5), variables were studied with octanoic acid only. Toluene and benzene were found to be equally good, but extraction with hexane was incomplete.

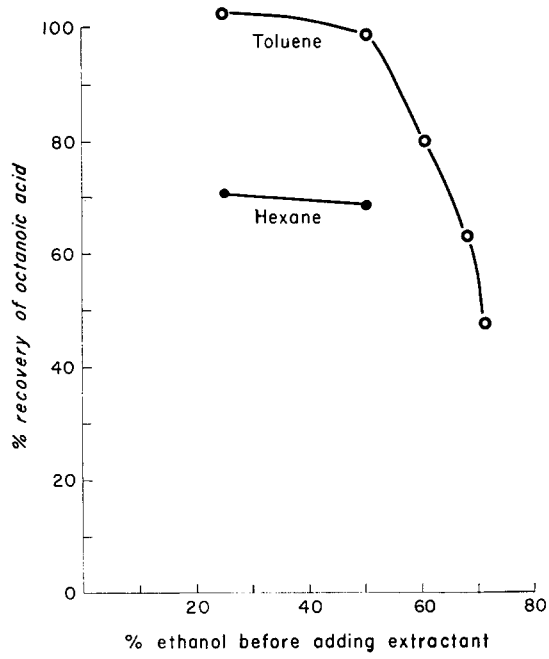

FIG. 1. Effect of ethanol concentration on extraction of octanoic acid into toluene (○) or hexane (●). Concentration of ethanol refers to that present in the acidified aqueous sample before the addition of toluene or hexane (2 volumes per volume of acidified aqueous sample).

Influence of Ethanol (*Fig. 1*). Different volumes of ethanol were added to solutions, containing known amounts of sodium octanoate, which were 0.25–0.3 N in H_2SO_4. Each mixture was extracted with a volume of toluene equal to twice the volume of acidified aqueous sample present before ethanol was added.

Octanoic acid was completely extracted with toluene (or benzene) when the ethanol concentration was 50% or lower, but percentage extraction decreased markedly when the ethanol concentration was increased. Thus, the lower the ethanol concentration the better the extraction. However, decreasing the ethanol concentration to 25% did not significantly improve extraction into hexane.

Influence of Salt (*Table 4*). Various amounts of sodium octanoate and sodium decanoate were diluted to 2 ml with distilled water; 1 N H_2SO_4 containing 0, 0.1, and 1.0 M ammonium sulfate was added. There was no significant difference when 0.1 M salt was present, but there was a statistically significant decrease from 99 to 96% ($P < 0.02$) in extraction when there was 1.0 M ammonium sulfate in the solution.

Discussion. This extraction method with toluene or benzene results in virtually complete extraction of C_8 and C_{10} medium-chain fatty acids, representative long-chain fatty acids, and 12-hydroxystearic acid. Methods in which petroleum hydrocarbon were employed (2, 4, 5) extracted less than 60% of octanoic acid, although Brad-

TABLE 4 INFLUENCE OF SALT CONCENTRATION ON PERCENTAGE OF OCTANOIC ACID EXTRACTED

Concentration of Ammonium Sulfate	Mean ± SD	Range (n)
M		
0	98.6 ± 1.4	96.6–101.3 (6)
0.1	98.9 ± 1.5	96.9–101.3 (6)
1.0	95.9 ± 1.6	94.0–98.9 (6)

Results are given using toluene as extractant; benzene produced similar results. With 1.0 M ammonium sulfate present, there was less ($P < 0.02$) extraction than with no salt. Similar results were obtained with decanoic acid.

dock, Fleischer, and Barbero showed (4) that extraction became complete after removal of the ethanol by distillation from the aqueous phase. However, our results indicate that octanoic acid can be completely extracted from 50% ethanolic solutions with toluene. Furthermore, in many methods (e.g., 4, 8), the fatty acid extracted must be quantitated after an aliquot of the extract has been evaporated and the residue dissolved in a water–ethanol mixture for titration against NaOH. Direct titration of the extract avoids possible losses from evaporation.

Jover and Gordon (8) described a method for extracting fecal fatty acids with toluene, but the extraction was incomplete. Our results indicate that two modifications in their method should allow complete extraction and quantitative titration of fecal fatty acids having chain lengths greater than six carbon atoms. First, water should be added after acidification to reduce the ethanol concentration from 62 to 50%; and the volume of toluene for extraction should be increased to twice the volume of water present in the acidified aqueous sample. Second, direct titration of the toluene phase with TEBAH would eliminate the undesirable evaporation step.

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