Faster and Easier Methods for Quantitative Lipid Extraction and Fractionation from Miniature Samples of Animal Tissues

Kathryn Elmer-Frohlich* and **Paul A. Lachance**
Department of Food Science, Cook College, Rutgers University, New Brunswick, NJ 08903

A less time-consuming dry-column method for total lipid extraction from tissue samples was scaled down and modified to permit the evaluation of 1-g samples of liver and muscle tissues from rats and from obese (ob/ob) and lean mice. Lipid yields obtained by the new dry-column method compared well with those obtained by the widely accepted traditional chloroform/methanol method. For subsequent separation of neutral and polar lipid classes, a solid-phase fractionation method was developed. Its performance was verified by thin-layer chromatography. Both column chromatographic methods were found to be especially convenient when running multiple samples simultaneously.

KEY WORDS: Lipid class separation, liver and muscle tissues, total lipid extraction.

The methods generally used for the extraction of lipids from biological tissue are based on the widely accepted traditional chloroform/methanol methods of Folch *et al.* (1), and Bligh and Dyer (2). In recent years, Marmer and Maxwell (3) developed a dry-column chromatographic method for isocratic elution of total lipids that is less time-consuming than the traditional methods.

Prior to the present study the alternate quantitative lipid extraction procedure (3) had not been used for muscle and liver samples of sample weight as low as 1 g; furthermore, it had never been used for liver in general or for mouse or rat tissue of any kind. In this study, a scaled-down and modified version of the method of Marmer and Maxwell (3) was developed.

A validation and yield study was performed on 1-g samples of rat liver and hindlimb skeletal muscle tissues, and the total lipid yields from the modified and scaled-down dry-column method were compared to those from the traditional method. Female Sprague Dawley rats used in this study had been bred and cared for by nutrition graduate student researchers at Douglass College, Rutgers University, and maintained on a stock rodent chow diet, Purina no. 5001. (Ancestors were purchased from Charles River Laboratories, Wilmington, MA.) Following sacrifice at ten weeks of age, their tissues were minced and pooled to eliminate between-animal variation in tissue lipid content, then divided into 1-g samples, and frozen at −80°C until time of extraction. Similar studies also were carried out with tissues from chow-fed obese (ob/ob) and lean mice.

Although the commercially available solid-phase extraction (SPE) tube generally is intended for use in sample clean-up, with the appropriate series of solvent elutions it was found to be convenient and effective for chromatographic separation of neutral and polar lipids. The selective elution of each lipid class was verified by thin-layer chromatography.

*To whom correspondence should be addressed at: Opta Food Ingredients, Inc., University Park at MIT, Cambridge, MA 02139.

METHODS

Sample preparation and charging of dry column. Crystalline butylated hydroxytoluene (BHT) was obtained from Sigma Chemical Company (St. Louis, MO; cat. no. B-1378); glass-distilled dichloromethane (CH_2Cl_2) was obtained from EM Industries, Inc. (Cherry Hill, NJ); granular anhydrous sodium sulfate (Na_2SO_4) was obtained from J.T. Baker Inc. (Phillipsburg, NJ). The N_2-flushed vial containing the tissue sample was removed from the freezer and allowed to warm at room temperature in the dark for about 10 min before opening in order to avoid condensation onto the sample. The 1-g sample, with weight recorded to the nearest 0.01 g, was put into an ice-chilled mortar. Then, 0.1 mL (0.0204 mg BHT/mL CH_2Cl_2) and anhydrous Na_2SO_4 (4 g) were added.

The sample was ground with the pestle for 30 sec. A glass rod was used to scrape the sample free from the porcelain, if necessary, to aid in obtaining a uniform ground mixture. Celite 545 (3 g), not acid-washed (Fisher Scientific Company, Pittsburg, PA, cat. no. C212-500), was added and the mixture was ground for an additional 30 sec for liver, or 30–50 sec for muscle to create a homogenized powder.

Powder from the mortar and pestle was freed with a glass rod, the mortar was tilted, and the powder was scraped and poured through a glass powder funnel into a 16 mm ID × 30 cm glass chromatography column already packed with glass wool at its tip and a trap of 1:9 $CaHPO_4 \cdot 2H_2O$/Celite 545 (2 g), above the 8 mm diameter tip. Calcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) was purchased from Fisher Scientific, cat. no. C-123. The powder funnel on top of the column was tapped uniformly with a plastic rod to allow quantitative transfer of the sample powder from the funnel into the column. A glass tamping rod was used to pack down the powder to the proper bed height, 4.6 cm above the trap of height 2.1 cm. Tamping down was accomplished by several partial compressions, while rotating the column to assure uniform packing and the absence of trapped air. To account for any potential lipid residue still left adhering to the mortar, pestle, and glass rod, 15 mL of CH_2Cl_2/MeOH (90:10) was added to the mortar (glass-distilled methanol was obtained from Fisher Scientific). The pestle and glass rod were stirred in the solution, left immersed for about 1 min, and the rinsings then were transferred into the column as described below.

Isolation of total lipids by isocratic elution. A tared 25 mm × 150 mm glass test tube with a Teflon-lined screw cap containing 0.1 mL BHT solution, as already described, was positioned under the column tip to collect eluate. A pasteur pipet was used to add all 15 mL of the 90:10 solvent mixture from the mortar to the column. The solvent traveled down the inner walls of the column so as not to disturb the packing.

After approximately 5 to 7 minutes, when the first drops of eluate appeared, the starting time was recorded, and 90:10 solvent mixture was added along the inner walls of

the column, consuming the column volume. A total of 50 mL was added in addition to the initial 15 mL rinse from the mortar. Once elution was complete, the elution time was recorded, and the volumetric flow rate was calculated (approx. 0.6 mL/min). This information served as a check on the reproducibility of chromatographic conditions from column to column. Multiple columns were run simultaneously and left unattended until elution was nearly complete.

The dry weight of the total lipid extracted was achieved by N_2-flushing the eluate at 40°C to dryness and calculating weight by difference from the tube and cap tare weight. The dry total lipid was redissolved in chloroform (10 mg total lipid/mL $CHCl_3$). Glass-distilled chloroform with 1% ethanol was obtained from Burdick and Jackson (Mukegon, MI; cat. no. 048-4). After vortexing, 2.0 mL was removed into a 16 mm × 150 mm glass tube with a Teflon-lined screw cap for the next procedure. Both tubes were N_2-flushed and refrozen at −80°C until the next procedure.

Separation of lipid classes by solid phase fractionation. Some of the total lipid extracts were separated into their component polar and neutral lipid fractions in 3-mL Supelclean Solid-Phase Extraction (SPE) Tubes packed with silica gel (LC-Si #5-7010, Supelco, Inc., Bellefonte, PA). Multiple columns were set up and run simultaneously.

For each sample, a volume of 2 mL total lipid extract (10 mg lipid/mL $CHCl_3$) was brought from −80°C to equilibrium at room temperature in the dark. The SPE tube was supported with a stand and clamp over a test tube rack containing tubes to collect the three eluates per sample—the hexane conditioner, the neutral fraction in chloroform and acetone, and polar fraction in methanol. Throughout the chromatographic steps the top surface of the column bedding always remained wet with solvent so that the void space would never be occupied by air, which would cause channeling. Furthermore, as a simple check on the reproducibility of chromatographic conditions from column to column, volumetric flow rates were recorded (approximately 0.2 mL/min). After conditioning the SPE tube with 2 mL hexane, the total lipid extract was added to the column and eluted. Quantitative transfer of the lipid extract was assured by rinsing the emptied lipid sample vessel and the used transfer pipet with aliquots of the 5 mL of acetone, following the elution of the 2 mL of total lipid extract. Hexane and acetone were high-performance liquid chromatography (HPLC)-grade, purchased from Fisher Scientific. The nonpolarity of the chloroform and acetone allowed the neutral (nonpolar) lipids to elute selectively, leaving the polar lipids in the column bed. Last, the polar fraction was eluted with 10 mL methanol.

The resulting neutral and polar fractions of the mouse tissues were each dried by a gentle N_2 stream in a 35°C block heater, weighed, redissolved in 1 mL chloroform, N_2-flushed, and held at −80°C until the next procedure, *i.e.*, their transesterification to methyl esters for gas chromatographic analysis of component fatty acids (4).

Verification of neutral and polar lipid separation by thin-layer chromatography. Thin-layer chromatography (TLC) data from liver and muscle extracts of rats and mice verified the complete separation of neutral and polar lipids by the employed lipid fractionation method. Figure 1 shows that there were no polar contaminants in the neutral lipids and no neutral contaminants in the polar lipids. The procedure for verification was as follows: TLC plates (Fisher Scientific, Silica Gel G, cat. no. 06-600A) were activated according to Fisher Scientific Catalogue

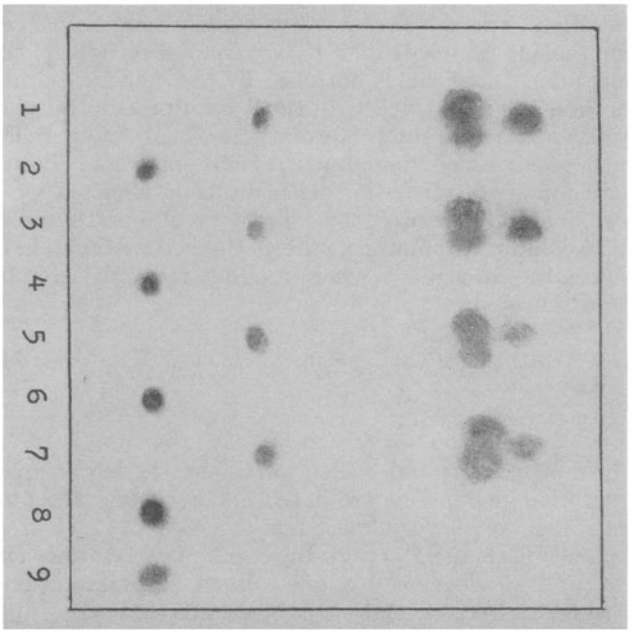

FIG. 1. Confirmation of neutral (N) and polar (P) lipid separation by thin-layer chromatography. Specimens 1, 3, 5, and 7—liver neutral lipids of lean and obese mice; 2, 4, 6, and 8—liver polar lipids of lean and obese mice; and 9—polar standard.

TABLE 1

Comparative Study of Traditional[a] *vs.* Dry-Column[b] Methods of Total Lipid Extraction from Rat[c] Liver and Hindlimb Skeletal Muscle Tissues

Method	Tissue	Total lipid, g/g tissue	Mean ± S.D.
Traditional	Liver	0.0425 0.0448 0.0590 0.0518	0.050 ± 0.007
Dry-column	Liver	0.0419 0.0613 0.0580 0.0546	0.054 ± 0.008
Traditional	Muscle	0.0331 0.0456 0.0269 0.0470	0.038 ± 0.010
Dry-column	Muscle	0.0308 0.0467 0.0410 0.0324	0.038 ± 0.007

[a]Traditional method involving $CHCl_3$/MeOH extraction (1).

[b]Scaled-down modification of isocratic elution method of Marmer and Maxwell (3).

[c]Female Sprague Dawley rats, fed stock rodent chow diet and sacrificed at ten weeks of age.

instructions. A disposable 10-λ glass micro-pipet was used to apply each of the standards and samples—trimyristin (triglyceride) and lecithin (phospholipid) standards (Supelco, Inc., cat. no. 4-4300 and 4-6012, respectively) and neutral and polar fractions from each of the liver and muscle extracts. The plates were held in a filter paper-lined tank, saturated with the vapor of 100 mL of a solvent system of petroleum ether/ethyl ether/acetic acid/methanol (85:15:2.5:1, by volume). Petroleum ether and ethyl ether were of ACS grade from Fisher Scientific, cat. no. E120-4 and E138-1, respectively; glacial acetic acid (CH_3COOH) was of analytical grade from Mallinckrodt Chemical Works (St. Louis, MO).

Next, the plates were air-dried for 10 min under a fume hood, sprayed with cerium (IV) sulfate tetrahydrate [99+% $Ce(SO_4)_2 \cdot 4H_2O$; Aldrich Chemical Company, Inc., Milwaukee, WI, cat. no. 21, 158-3], and baked at 100°C for 10 min to visualize. With this solvent system, the polar lipids, which are primarily phospholipids, remained at the baseline, while the nonpolar lipids, triacyl- and any trace amounts of diacyl- and monoacylglycerols, traveled.

TABLE 2

Comparative Study of Traditional[a] *vs.* Dry-Column[b] Methods of Total Lipid Extraction from Liver and Skeletal Muscle Tissue Samples of Obese (ob/ob) and Lean Mice[c]

Method	Tissue	Total lipid, g/g tissue	Mean ± S.D.
ob/ob Mouse			
Traditional	Liver	0.2940	0.314 ± 0.028
		0.3347	
		0.2869	
		0.3411	
Dry-column	Liver	0.3139	0.319 ± 0.021
		0.3460	
		0.3208	
		0.2957	
Traditional	Muscle	0.2520	0.264 ± 0.010
		0.2659	
		0.2761	
		0.2603	
Dry-column	Muscle	0.3019	0.271 ± 0.038
		0.2460	
		0.3058	
		0.2304	
Lean mouse			
Traditional	Liver	0.1247	0.116 ± 0.017
		0.0913	
		0.1210	
		0.1289	
Dry-column	Liver	0.1140	0.121 ± 0.010
		0.1151	
		0.1178	
		0.1359	
Traditional	Muscle	0.0391	0.049 ± 0.009
		0.0469	
		0.0615	
		0.0476	
Dry-column	Muscle	0.0550	0.046 ± 0.007
		0.0402	
		0.0491	
		0.0410	

[a,b]As in Table 1.

[c]Male obese mice C57BL/6J (ob/ob) and their lean siblings obtained from Jackson Laboratories (Bar Harbor, ME), sacrificed at twenty-two weeks of age.

RESULTS AND DISCUSSION

Results of the isocratic elutions by the dry-column method were compared with the traditional extraction method for rat liver and muscle. Table 1 shows that consistent yields between methods for each tissue were obtained. The total lipid contents were in agreement with accepted values in the literature. Numerous lipid determinations also were made of skeletal muscle and liver tissues of obese (ob/ob) and lean mice by the modified Marmer and Maxwell procedure. The results shown in Table 2 also agree well with lipid extraction data obtained by the traditional chloroform/methanol method of quantitative extraction.

Consequently, we conclude that the new dry-column method, which is less tedious and time-consuming than the traditional one, is an acceptable method for isolation of total lipids from 1-g tissue samples, lending itself well to multiple extractions such as are required in series experiments on rat or mouse tissues. Furthermore, subsequent class separation of lipids by the described solid-phase fractionation method was found to be both effective and convenient.

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