notes on methodology

Rapid separation of lipid classes in high yield and purity using bonded phase columns

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Summary A method utilizing aminopropyl bonded phase (Bond Elut) columns has been developed to separate lipid mixtures into individual classes in high yield and purity. Up to ten lipid mixtures can be processed in 1 hr and the columns are reusable after suitable washing. Although the method was developed with standard lipid mixtures, it was shown that it is also applicable to biological extracts. Due to the rapidity and high yields (>95%) of this procedure, it is superior to preparative HPLC or TLC, or other chromatographic methods for the separation of lipid mixtures for subsequent analysis.—**Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps.** Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* 1985. **26:** 135–140.

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The separation and isolation of neutral and polar lipid classes for subsequent use or analysis has been the subject of many reports in the literature. In addition, a number of excellent reference books have been published (1–3). In general, lipid isolations have been accomplished by preparative TLC, adsorbent chromatography on silicic acid, florisil, or alumina, partition chromatography, and more recently by HPLC methodologies. There are, however, problems inherent in all these procedures. Preparative TLC is bulky and cumbersome, sensitive to sample load, and can be quite expensive in both time and materials. Silicic acid or alumina column chromatography usually involves a gradient elution procedure in which sample peaks may overlap resulting in less than desired purity, and require considerable amounts of solvent for larger amounts of sample (4, 5). HPLC isolation of lipids requires expensive equipment, copious amounts of solvent, and can be time-consuming. Thus, a rapid method for lipid isolation in high yield and purity would be very useful.

We have taken a somewhat novel approach to this problem using chromatographic mode sequencing. This technique involves creating selectivity in the isolation of compounds by serially altering either the solid phase support, the solvent, or both. In a biphasic solid/support solvent system, a unique interaction exists between the compound to be isolated and the functional group of the solid phase. Compounds that are very diverse in chemical structure may differ greatly in their interactions with the solid phase surface moieties, whereas compounds that are similar in chemical nature may show only subtle but exploitable differences. Thus, by varying the solvent environment (pH, polarity, etc.) around the solid phase, or by changing the solid phase itself, compounds (in this case lipids) can be selectively isolated with a high degree of purity and recovery.

The work detailed in this report outlines procedures for the use of disposable primary aminopropyl bonded phase columns, under pressure, to isolate and purify individual polar and neutral lipid classes. Up to ten lipid mixtures can be processed in 1–1½ hr. We present evidence that the lipid classes can be isolated essentially to homogeneity with greater than 95% recovery. Data utilizing both standard lipid mixtures and lipid extracts from bovine adipose tissue are presented and the approximate load limits of the columns are determined.

MATERIALS AND METHODS

Materials

Neutral lipid standards (triolein, cholesteryl oleate, diolein, diheptadecanoin, oleic acid, monostearin, and monononadecanoin) were obtained in 99% purity from Nu-chek Prep Inc., Elysian, MN. Phospholipid standards (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and phosphatidylinositol) were from Serdary Research Laboratories, London, Ontario, Canada. Radioactive lipid standards were obtained as follows and used without further purification. 1-Palmitoyl-2 [9,10-^{3}H]palmitoyl phosphatidylcholine (sp act 60 Ci/mmol), cholesteryl [1-^{14}C]oleate (sp act 56.6 mCi/mmol), [1-^{14}C]oleic acid (sp act 59 mCi/mmol), [1,2-^{3}H(N)]cholesterol (sp act 40.7 Ci/mmol), and [9,10-^{3}H(N)]triolein (sp act 111.1 Ci/mmol) were obtained from New England Nuclear, Boston, MA; dioleoyl phosphatidyl-L-[U-^{14}C]serine (sp act 60 mCi/mmol), phosphatidyl [U-^{14}C]inositol (sp act 270 mCi/mmol), [4-^{14}C]cholesterol (sp act 58.4 mCi/

Abbreviations: TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; PL, phospholipid; FA, fatty acids (unesterified); CE, cholesteryl ester; TG, triglyceride; CHOL, cholesterol; DG, diglyceride; MG, monoglyceride; GLC, gas–liquid chromatography.

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mmol), and dioleoylphosphatidyl [2-^{14}C]ethanolamine were from Amersham Corp., Arlington Heights, IL. Standard lipid solutions containing all compounds were made to a concentration of 1 mg/ml and contained 1 μCi each labeled lipid/ml (except diolein and monostearin). Solvents were reagent grade, glass-distilled (from Burdick and Jackson, Muskegon, MI). Bond Elut aminopropyl disposable columns (500 mg),[3] a Vac Elut vacuum elution apparatus with adaptors, and Vac Elut sample collector racks were obtained from Analytichem International, Harbor City, CA. TLC plates (Silica Gel G, 250 μg) were from Analtech Inc., Newark, DE, and HPTLC EM 60 plates were supplied by V.W.R. Scientific, Chicago, IL.

Chromatographic procedure

Routine visual checks of the chromatographic purity of eluate fractions was done using EM 60 HPTLC plates. Samples were spotted using a Camag 200 nl (~1 mm spot diameter) capillary and a Nanomat Auto Spotter supplied by Applied Analytical Industries, Inc., Howard, MA. At least 0.5 μg of lipid was spotted versus known standards, and in some cases the plates were deliberately overloaded (>2 μg per spot) to assess contamination of each fraction with other lipids. Plates were developed vertically in a solvent system of hexane–diethyl ether–acetic acid 70:30:1 (v/v/v). Visualization was by charring with 10% cupric sulfate in 8% phosphoric acid (6) as follows: the HPTLC plates were dipped in the visualization reagent for 3 sec, air dried for 10 min, and heated in a GC oven programmed from 30–180°C at 8°C/min with a final hold of 8 min. The absolute purity and percent recovery of each fraction were assessed by TLC and radioactivity counting as follows: an aliquot of each fraction (along with known standards in a separate lane) was applied to an Analtech 250-μm silica gel G TLC plate using an AIS TLC multispotter. The plates were developed as above and lipid areas were visualized with iodine vapor. Areas corresponding to PL, FA, CHOL, MG, DG, TG, and CE were scraped (after dissipation of iodine) into scintillation vials and counted in 10 ml of ACS counting solution (Amersham Corporation), 1 ml of methanol, and 1 ml of 0.2% sodium dodecylsulfate in a Packard 3255 Tri Carb Liquid Scintillation Counter. Aliquots of each fraction were also counted prior to application to the Bond Elut columns and from each fraction prior to TLC. Recovery of each lipid was calculated by counts recovered in each fraction/counts applied to the column, and purity was calculated by the number of counts of contaminating lipid in each fraction (with known specific radio-activity).

Mono- and diglycerides were quantitated by GLC, after elution from the Bond Elut columns, utilizing monononadecanoin and diheptadecanoin, respectively, as internal standards. The mono- and diglycerides were reacted with 14% BF_3 in methanol in boiling water for 1.5 hr to produce fatty acid methyl esters. These were then analyzed by GLC on a Varian 6000 gas chromatography equipped with a Vista 401 data system for peak integrations. Separations were achieved on a 30-meter DB-1 capillary column (0.242 mm i.d., volume 5.52 ml) programmed from 125°-220°C at 8°C/min with no initial hold. Other column conditions were as follows: injector temperature, 300°C; detector temperature, 320°C; splitter flow, 92.3 ml/min; splitter ratio, 13.37; hydrogen flow, 6.97 ml/min.

Column procedures

Lipid mixtures were eluted from the bonded phase aminopropyl columns using the apparatus shown in **Fig. 1** according to the procedures outlined below. All solvents used in the elution procedure are shown in **Table 1** with their relative strengths, and a schematic representation of the elution procedures is shown in **Fig. 2.** The solvent mixtures used in the elution scheme shown should be of the same composition in Table 1 since even a small variation (1–2%) in solution composition can drastically change the character of the column and separations.

Standard lipid mixtures were evaporated to dryness under nitrogen and taken up in a minimal volume of chloroform (<0.5 ml). Aminopropyl columns (500 mg) were placed in the Vac Elut apparatus and washed twice under vacuum (~10 kPa) with 2-ml portions of hexane. A collection rack with receiving tubes was then placed in the Vac Elut (Fig. 1). The vacuum was released immediately after the second hexane wash to prevent the columns from becoming completely dry. Lipid mixtures in chloroform were applied to the column under vacuum, and the chloroform was pulled through. This left the entire lipid mixture on the column. Next, the column was eluted with 4 ml of Solvent A. This eluate (I, neutral lipids) was saved and new collection tubes were placed in the collecting racks. The column was then eluted with 4 ml of Solvent B, the eluate (II, fatty acids) was saved, and new collecting tubes were placed in the rack. This column was then eluted with 4 ml of Solvent C, and the eluate (III, phospholipids) was saved.

Eluate I from above was dried under N_2 and reconstituted in 0.2 ml of hexane. A new column was placed in the Vac Elut apparatus and eluate I (in hexane) was loaded on the column as described previously. Solvent D (4 ml) was then passed through the column and the eluate (IV, cholesteryl esters) was saved. A new amino-

[3]When ordering, it is important to specify columns having stainless steel frits. Earlier columns were fitted with soft polyethylene frits which contain a plasticizer elutable with organic solvents.

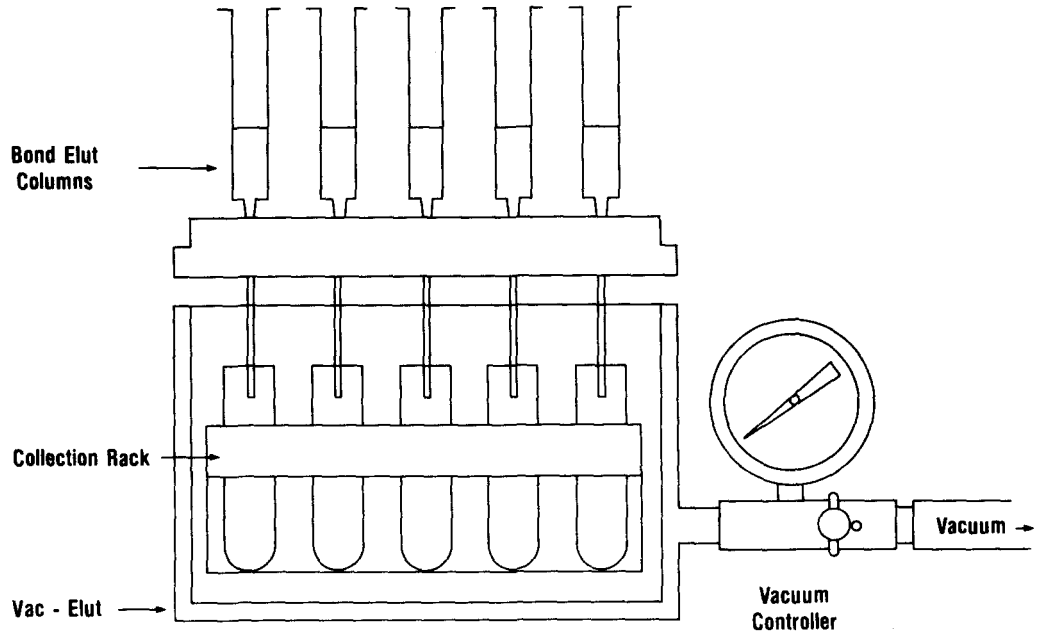

Fig. 1 Vac Elut apparatus used to hold Bond Elut columns for isolation of lipid classes. The collection rack holding receiving tubes is shown in the 3-dimensional drawing.

propyl column was then piggy-backed (positioned below) with the existing column, using the column adaptor provided by Analytichem. Six ml of Solvent E was passed through both columns and the eluate (V, triglycerides) was saved. The stacked columns were then eluted with 12 ml of Solvent F; the eluate (VI, cholesterol) was saved and the columns were separated. The upper columns were then eluted successively with 4 ml of Solvent G followed by 4 ml of Solvent H to give eluates (VII, diglycerides and VIII, monoglycerides) which were saved. Eluates (II–VIII) were taken to dryness under N_2, taken up in a known volume of chloroform, counted for radioactivity, and chromatographed by the procedures described earlier. These procedures were repeated using lipid extracts from bovine adipose tissue which had been spiked with 1 μCi of each labeled lipid and compared with results obtained by preparative TLC after development in hexane–diethyl ether–acetic acid 70:30:1 (v/v/v).

RESULTS

The recoveries and relative purities of standard lipid mixtures separated by the "Bond Elut" procedure are shown in **Table 2.** A visual verification of the purity of each lipid fraction is shown in **Fig. 3** (standard mixture) and **Fig. 4** (adipose tissue extract). As the data

TABLE 1. Solvents used in the Bond Elut isolation and purification of lipid classes

Name	Solvents	P'	Amount Equal Wt. Std. Mix	Fatty Adipose Tissue Ext.	Lipid Eluted
A	Chloroform–2-propanol 2:1	4.07	4 ml	4 ml	All neutral lipids
B	2% Acetic acid in diethyl ether	2.86	4 ml	4 ml	FA
C	Methanol	5.1	4 ml	4 ml	All PL
D	Hexane	0.01	4 ml		CE
E	1% Diethyl ether, 10% methylene chloride in hexane	0.437	6 ml	8 ml	TG
F	5% Ethyl acetate in hexane	0.315	8 ml + 4 ml	16 ml + 4 ml	C
G	15% Ethyl acetate in hexane	0.616	4 ml	8 ml	DG
H	Chloroform–methanol 2:1	4.43	4 ml	4 ml	MG

Lipid extracts were processed by the procedures described in Methods. Solvent strengths (P') are taken directly or calculated proportionally from the Burdick and Jackson Solvent Handbook (1980). The volumes represent the amounts of each to elute the particular lipid, and are dependent upon sample load at high sample concentrations.

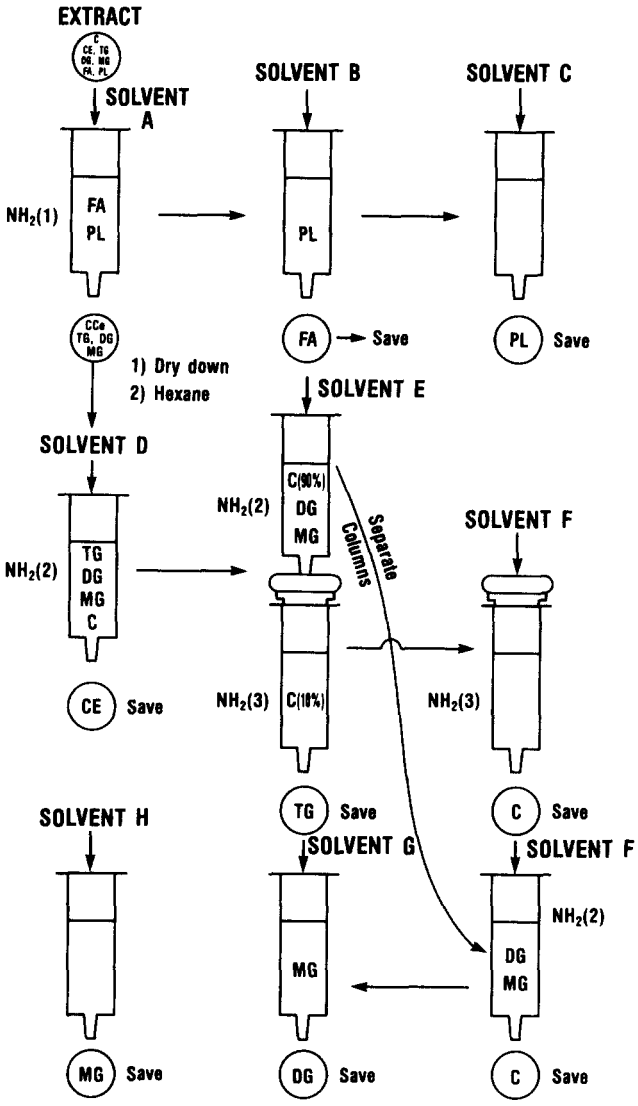

Fig. 2 Diagrammatic representation of the elution scheme used for the Bond Elut separation and isolation of lipid classes from mixtures on aminopropyl columns. Solvents are tabulated in Table 1 and a discussion of the procedures appears in the text.

clearly indicate, each lipid class was separated to near homogeneity with >95% recovery.

Although standard lipid mixtures containing equal amounts (by weight) of each lipid class were used to establish the elution scheme for best recovery and purity, it was necessary to verify the applicability of this technique to biological lipid extracts. Bovine adipose tissue, in which triglycerides and unesterified fatty acids predominate as the major lipid species, proved to be an appropriate model system. As shown in **Table 3**, the Bond Elut procedure proved superior to preparative TLC in the recovery and purification of lipid classes from both standard mixtures and bovine adipose tissue extracts. In this comparison, recoveries by the Bond Elut procedure were 100% for both the standard mixture and bovine adipose tissue extract, as compared with approximately 80% by preparative TLC. In addition, the distribution of radioactivity in each lipid class recovered by Bond Elut closely paralleled the distribution in the original lipid mixture. The elution volumes given in Table 1 had to be slightly modified for the adipose tissue extract to accommodate increased amounts of triglyceride and fatty acid relative to other classes.

The elution scheme for optimal separation is shown in Table 1 and Fig. 2. Phospholipids and fatty acids were retained on the first column after eluting neutral lipids with Solvent A. It was necessary to use this solvent mixture (chloroform–propanol) instead of the more common chloroform–methanol, because of the tendency of phosphatidylcholine to elute in the latter solvent mixture. It must be reemphasized that the relative compositions of the solvent mixtures shown in Table 1 should not be changed, since the physical environment of the columns may be altered such that separations, recoveries, and degrees of purity become less than optimal. For example, we found that the eluate from Solvent A (neutral lipids) must be thoroughly dried and redissolved in 100% hexane, since the presence of any more polar solvent (e.g., chloroform) may result in some TG elution in the cholesteryl ester fraction when the column is subsequently eluted with pure hexane (Fig. 2, eluate from Solvent D).

As is evident from the scheme in Fig. 2, neutral lipids elute from the aminopropyl columns in order of increasing polarity. It was necessary to piggy-back the columns (Fig. 2) after the elution of CE, since approxi-

TABLE 2. Recoveries and purities of lipid classes isolated by the Bond Elut procedure

Solvent	Lipid	% Recovery	n	% Contamination	n
D	Cholesteryl ester	98.9 ± 4.7	20	1.16 ± 0.92	5
E	Triglyceride	98.4 ± 5.3	16	2.03 ± 1.78	4
G	Diglyceride	96.7 ± 4.9	4	Trace	4
H	Monoglyceride	96.7 ± 2.5	7	Trace	7
F	Cholesterol	100.0 ± 4.1	17	1.13 ± 0.47	4
B	Free fatty acid	101.4 ± 7.8	12	1.96	2
C	Phospholipids	97.3 ± 5.3	8	1.15	2

Lipid classes were isolated and purified on primary isopropyl columns, and were assessed as described in Methods. Recoveries and purities are those obtained after eluting the columns with the given solvents.

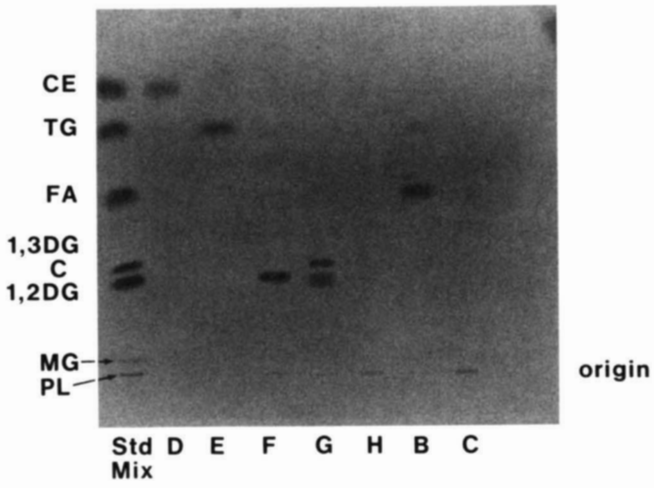

Fig. 3 Visual verification of the purity of lipid classes isolated by Bond Elut from a standard mixture. Lipid classes were purified on primary aminopropyl columns as described in methods. Aliquots of each purified fraction were chromatographed and the plate was visualized as in Methods, except plates were charred with 50% sulfuric acid. The letters under each lane represent the solvent used to elute the fraction chromatographed in that lane and the standard mixture is described in Methods. Standard C = standard cholesterol.

mately 10% of the cholesterol passes through when TG is eluted. However, cholesterol from both columns was then easily eluted by Solvent F. If quantitative recovery of phospholipids is not required, the elution procedure can be modified such that the lipid mixture is taken up in hexane and fatty acids and neutral lipids recovered according to Fig. 2, eliminating the phospholipid elution step (since phospholipids have low solubility in hexane). The load limit of the 500-mg aminopropyl columns was found to be approximately 10 mg of total lipid. The results indicate that the Bond Elut method for lipid separation can be adapted to accommodate different lipid distributions from a variety of sources.

DISCUSSION

We have developed a rapid economical method for the preparative scale isolation and separation of major lipid classes. By the inclusion of appropriate internal standards in the lipid extract prior to separation, the subsequent eluates can be quantitated by various analytical methods. In addition, radiolabeled lipids can be rapidly isolated and purified by this method for subsequent scintillation counting. Since the columns can be regenerated by washing in a reverse elution order, the method is much more economical and rapid than current procedures, such as HPLC, preparative scale TLC, and gradient elution from silicic acid or alumina columns. The high degree of purity of lipid classes purified by Bond Elut makes the method superior to silicic acid or alumina gradient elution procedures in which peak fractions tend to overlap. Up to ten lipid extracts can be processed in 1 hr by the Bond Elut method. Alternatively, Analytichem supplies the column material for packing by the user; thus columns may be packed with large amounts of adsorbent for larger sample sizes. We are routinely using this technique for the separation of blood plasma lipid classes prior to analysis, and are quite confident that it is adaptable to separations of lipids from a variety of other sources.

The separations and recoveries that were obtained (Table 3) by the Bond Elut procedure were a function not only of lipid polarity, but also of solvent strength and polarity. Polar lipids such as FA or PL, or those having a polar group (such as MG, DG, and CHOL), are likely to interact more strongly with the aminopropyl group on the Bond Elut columns through hydrogen bonding to the primary amine group. Thus, the most difficult separation to achieve was that between cholesterol and diglyceride, both having one free hydroxyl group. This separation was eventually accomplished by using varying amounts of ethyl acetate to break up these hydrophobic interactions. The presence of a large amount of one particular lipid class in the extract did not interfere with either the recovery or purity of other lipid fractions. Separation of the lipids from bovine adipose tissue, which contains copious amounts of triglycerides and fatty acids, was accomplished with recoveries and purities comparable to those for the standard lipid mixture used to develop the separation methodologies. Thus, the method of lipid separation and isolation can be adapted to lipid mixtures from various sources. In summary, this report describes a new method for preparative scale isolation

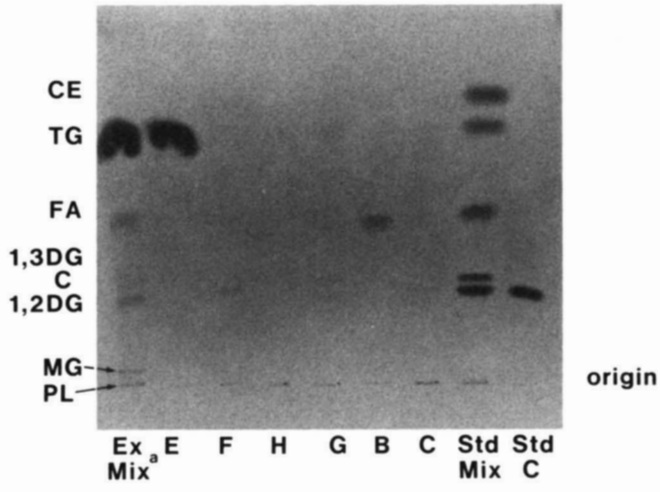

Fig. 4 Visual verification of the purity of lipid classes isolated by Bond Elut from a bovine adipose tissue extract. See legend, Fig. 3.

TABLE 3. Comparison of Bond Elut and TLC methods for the separation and recovery of lipid classes[a]

Class	Standard Mixture			Fatty Adipose Tissue Extract		
	TLC	Bond Elut	Standard	TLC	Bond Elut	Standard
CE	11.8	17.1	16.3	>35.6	34.1	35.5
TG	17.4	19.5	19.1			
C	19.6	22.7	21.5	>26.5	26.0	27.0
DG	0.38	0.4				
FA	19.0	21.5	22.3	20.2	26.7	27.3
PL	31.6	18.8	20.9	16.0	12.0	10.1
Other areas				1.7		
Recovery of CPM	77.7%	100.6%		80.7%	104%	

[a]Lipid classes were separated and recovered from standard lipid mixtures and adipose tissue extracts by the procedures described in Methods. Numbers represent the percentage of added counts recovered in each fraction. The percentages for the standard mixtures are the distributions of added radioactivity.

of lipid classes. The method is more economical (in terms of material and time) and rapid than currently available procedures and results in nearly total recovery and purity of the individual lipid classes. We are currently extending these techniques to the separation of individual phospholipids from lipid mixtures.

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