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Rapid separation and quantitation of combined neutral and polar lipid classes by high-performance liquid chromatography and evaporative light-scattering mass detection

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Abstract

Modifications are described for an innovative and widely used high-performance liquid chromatography technique that resolves a very broad spectrum of lipids for quantitation by evaporative light-scattering detection. Substitution of acetone for 2-propanol in a portion of the solvent gradient program yields consistent resolution of diacylglycerol and cholesterol without sacrificing baseline resolution of the remaining major lipid classes. Moreover, previously noted instabilities in triacylglycerol retention time are eliminated. The introduction of acetone also enables a 20% reduction in flow-rate without an increase in total run time. As a further modification of the mobile phase composition, acetic acid and ethanolamine are substituted for the serine–ethylamine combination that was originally shown to improve column performance. The combination of acetic acid and ethanolamine yields the same result but the increased volatility of these solutes over serine results in decreased baseline noise. Finally, 1,2-hexadecanediol is introduced as an internal standard that is well suited for this method. The chromatographic performance obtained with these modifications is demonstrated in compositional analyses of lipid extracts from rat liver, heart, kidney and brain. © 1998 Elsevier Science B.V.

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1. Introduction

High-performance liquid chromatographic (HPLC) separation and quantitation of the lipids contained in samples of biological origin can reveal detailed metabolic information about the source of those samples. A major advance in lipid analysis was brought about by the introduction of commercially available mass detectors and the publication of a novel paper by Christie [1], presenting a comprehensive and efficient chromatographic method to be used in conjunction with mass detectors for lipid analysis. The major advance introduced by Christie was the ability to achieve the resolution and detection of most of the major lipid classes in a single chromatographic run. This has been the impetus for a growing number of laboratories to adapt this method for the compositional analysis of complex lipid mixtures isolated from a variety of sources including tissues [1–4] and cultured cells [5]. In the process, several improvements to the original method have been described. These include addition of salts to the

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aqueous portion of the mobile phase to improve phospholipid detection and extend column lifetime [6], modification of the elution gradient to achieve more reliable separation of cholesteryl ester and triacylglycerol [7], and the introduction of a suitable internal standard [3]. In adapting Christie's method for use in our laboratory we have discovered further modifications that yield additional performance gains for the chromatographic method.

2. Experimental

2.1. Materials

Cholesteryl oleate, trioleoylglycerol, 1,2-dioleoyl-*sn*-glycerol, 1-monooleoyl-*rac*-glycerol, cholesterol, galactocerebrosides (type I, type II) and ethanolamine were purchased from Sigma Chemical Company (St. Louis, MO, USA). Galactocerebrosides (bovine brain) were obtained from Matreya (Pleasant Gap, PA, USA). Avanti Polar Lipids (Alabaster, AL, USA) was the source of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, phosphatidylinositol (bovine liver), cardiolipin (bovine heart) and sphingomyelin (egg yolk). 1,2-Hexadecanediol was obtained from Aldrich Chemical Company (Milwaukee, WI, USA) and recrystallized from hexane before use. Solvents (HPLC grade) were purchased from common chemical suppliers.

2.2. Chromatographic equipment

The HPLC system components were from Thermo Separation Products (Fremont, CA, USA) and consisted of an AS3000 autosampler, a P4000 quaternary pump and a SP4510 data interface module. All components were controlled by SpectraSYSTEM software. A 10 cm×4.6 mm column containing Spherisorb S5W silica (Phase Separations, Norwalk, CT, USA) and equipped with a 0.2-μm column prefilter was heated to 45°C in an Eppendorf CH-30 column heater (Baxter, McGaw Park, IL, USA). Lipids were detected with a SEDEX 55 evaporative light-scattering detector (Richard Scientific, Novato, CA, USA) set to 67% of maximum gain, a drift tube temperature of 45°C and 2.2 bar nitrogen pressure. The detector analog output was sent to the SP4510 data module.

2.3. Chromatographic method

Lipid elution was obtained with the mobile phase solvent program shown in Table 1. The mobile phase was created by combinations of three solvent mixtures. The first mixture (A) consisted of isooctane–tetrahydrofuran (99:1, v/v), the second (B) contained acetone–dichloromethane (2:1, v/v), and the third (C) contained 2-propanol–water (7.5 m*M* acetic acid and 7.5 m*M* ethanolamine) (85:15, v/v).

Calibration curves for detector response versus mass of lipid injected were obtained by injecting 10-μl aliquots of lipid standard mixtures dissolved in isooctane–tetrahydrofuran (9:1, v/v) at a series of dilutions to yield 0.05–10 μg of each lipid per injection. The nonlinear response of the detector to lipid mass, which is characteristic of mass detection by light scattering [3], was well fit by the quadratic equation with the constant term equal to zero. The nonlinear regression utility supplied with SigmaPlot (Jandel Corporation, San Rafael, CA, USA) was used to obtain the best fit of each lipid calibration data set to the quadratic equation (Fig. 1). The mass of each lipid in tissue lipid extracts was obtained from the corresponding fitted calibration curves.

Table 1
Ternary gradient mobile phase composition

Time (min)	Percent solvent[a]			Flow-rate (ml/min)
	A	B	C	
0	100	–	–	1.6
4	90	10	–	1.6
7	70	30	–	1.6
7.1	40	50	10	1.6
20.4	39	–	61	1.6
20.5	40	–	60	2
22.5	40	–	60	2
22.6	–	100	–	2
25	–	100	–	2
25.1	100	–	–	2
30	100	–	–	2

[a]A, isooctane–tetrahydrofuran (99:1, v/v); B, acetone–methylene chloride (2:1, v/v); C, 2-propanol–water (7.5 m*M* acetic acid, 7.5 m*M* ethanolamine) (85:15, v/v).

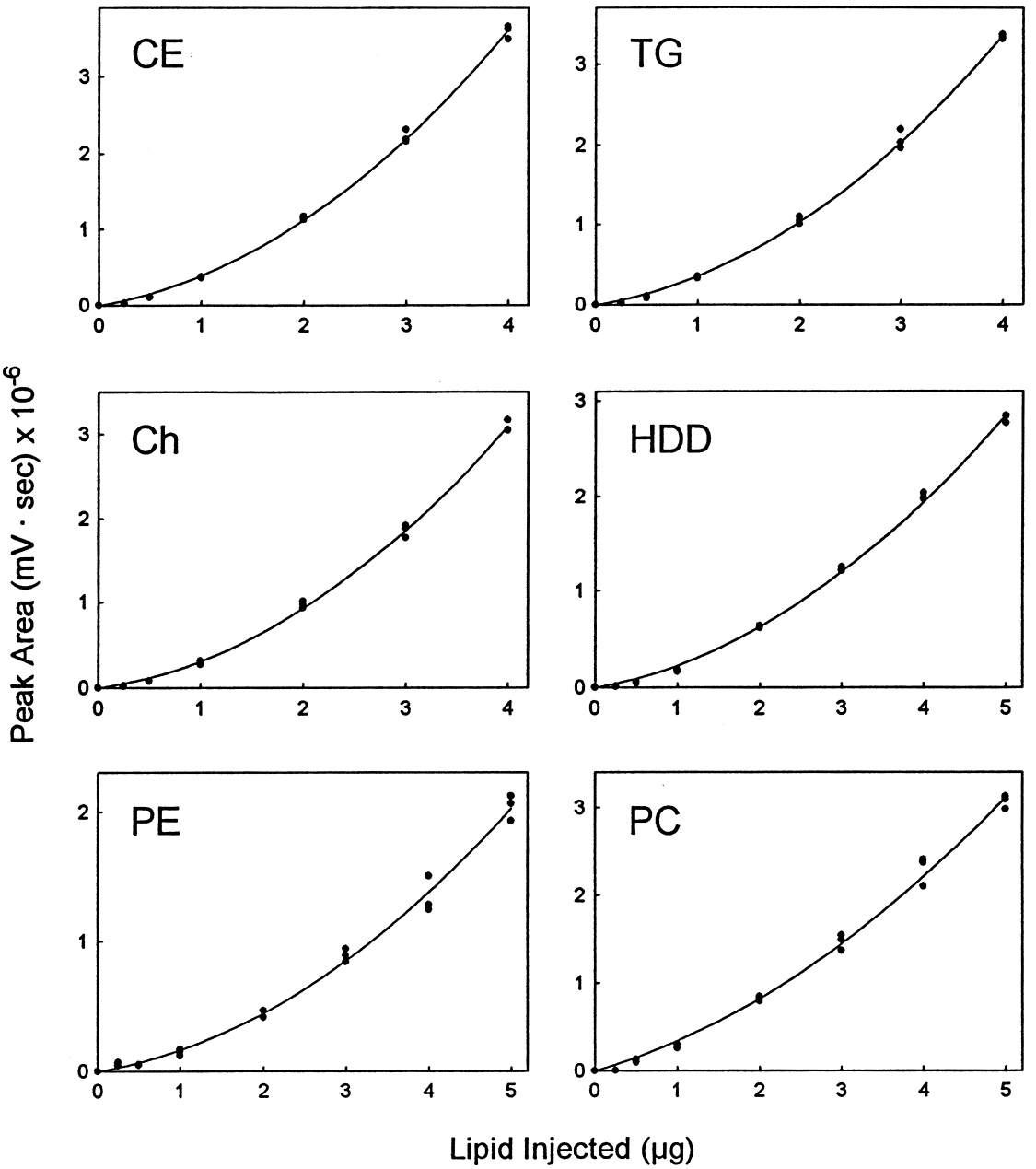

Fig. 1. Calibration curves for lipid standards. A series of dilutions of a solution containing a mixture of lipid standards were injected (10 μl) in triplicate. The resultant peak areas detected for each lipid in each injection at each dilution are plotted (filled circles). The lines represent the best fit of the data to a quadratic equation.

2.4. Sample preparation

Liver, heart, kidney and brain tissue were obtained from a male Sprague-Dawley rat. One part (wt.) of each tissue was homogenized in six parts (vol.) of 150 m*M* NaCl, 5 m*M* 3-(*N*-morpholino)propanesulfonic acid, 1 m*M* ethylenediaminetetraacetic acid, 0.01% (w/v) phenylmethylsulfonyl fluoride, pH 7.4. The homogenates were diluted 2-fold in the same buffer (without phenylmethylsulfonyl fluoride) for

lipid extraction. Lipids were extracted by the method of Slayback et al. [8] with ethyl acetate–acetone (2:1) containing 0.01% 2,6-di-*tert*.-butyl-4-methylphenol as an antioxidant. A selected volume of a 2 mg/ml solution of 1,2-hexadecanediol in ethyl acetate–acetone (2:1) was added to the extraction mixture to serve as the internal standard. The solvent extracts were dried under streams of nitrogen followed by vacuum desiccation. The dried lipids were redissolved in isooctane–tetrahydrofuran (9:1, v/v) for HPLC analysis.

3. Results and discussion

The original chromatographic method presented by Christie [1] operated with 2-propanol in the second solvent reservoir. We found that substitution of acetone for 2-propanol in this solvent mixture, as well as changes in the proportions of solvents in the second and third reservoirs and a modified mobile phase program (Table 1), yielded significant enhancements in chromatographic performance over the original method. As a minor modification, methylene chloride was substituted for chloroform in solvent mixture B to reduce the potential for solvent toxicity. One outcome of these changes was consistent resolution of diacylglycerol and cholesterol, which we found unattainable employing the original method. A possible explanation for this may be that the column we used was slightly smaller (4.6 vs. 5 mm diam.) and packed with 5-μm silica rather than the 3-μm silica used by Christie. Nevertheless, in switching to acetone we discovered additional advantages as well. The mobile phase modifications allowed a 20% reduction in mobile phase flow-rate during the lipid elution portion of the run cycle which resulted in increased detection sensitivity as evidenced by a 20–50% increase in peak areas (data not shown). The reduction in flow was obtained without any increase in total run-time, unlike other adaptations of Christie's method which lengthened the total run time [3,7]. A further outcome of these modifications was the elimination of the sensitivity of TG retention time to extended column re-equilibration which Christie noted was a concern in the original method [1] and was later confirmed by others [7].

Finally, since it was previously demonstrated that addition of polar solutes to the aqueous portion of solvent mixture C improved column performance [6], we added 7.5 m*M* each of acetic acid and ethanolamine to the water in solvent C. These solutes were selected because they produced significantly less baseline noise with the SEDEX detector than the serine and ethylamine combination originally described [6]. In selecting acetic acid and ethanolamine we also found a noticeable improvement in the detection sensitivity for unesterified fatty acids.

An example of the chromatographic results ob-

Table 2
Replicability of detector response and lipid retention times for 20 μl of lipid standards (2 μg each) injected 10 times

Lipid[a]	Peak area $\times 10^{-4}$			Retention time (min)		
	Mean[b]	S.E.M.	R.S.D.%	Mean	S.E.M.	R.S.D.%
CE	144.63	0.60	1.3	1.07	0.001	0.3
TG	101.98	3.17	9.9	4.43	0.03	2.1
Ch	116.75	2.49	6.8	6.23	0.03	1.6
HDD	51.64	1.59	9.7	8.63	0.01	0.3
CL	13.49	0.83	19.5	13.40	0.05	1.1
PE	45.80	0.74	5.1	14.96	0.04	0.8
PI	30.75	0.48	5.0	15.34	0.05	1.1
PS	32.31	0.65	6.3	16.69	0.01	0.2
PC	90.77	2.93	10.2	17.48	0.03	0.5
Sph	91.04	1.15	4.0	19.59	0.05	0.7

[a]CE, cholesteryl ester; TG, triacylglycerol; Ch, cholesterol; HDD, 1,2-hexadecanediol; CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; Sph, sphingomyelin.
[b]$n=10$.

tained with the current chromatographic method is shown in Fig. 2. The mass of each lipid in the injection that produced this chromatogram was 5 μg. The neutral lipids produced the largest detector response per unit of mass, as noted by others previously [3]. The detection limit (peak/noise>3) for neutral lipids at maximum detector gain was approximately 50 ng. The replicability of lipid retention times and detector responses (Table 2) indicate this method is sufficiently stable.

The lipids detected in rat tissue lipid extracts with the current method are shown in Fig. 3. The tissue lipid compositions calculated from the chromatographic data are presented in Table 3. Conversion of the data to relative lipid composition values shows that these data compare quite favorably with those from other studies (Table 4). One notable variation was in the percent composition obtained for sphingomyelin with the current method. In all tissues, the current method yielded the lowest sphingomyelin value by a factor of approximately 2–3-fold smaller. These variations may result from differences between the tissue donors or analytical methods. That a similar variation is evident among previously published values indicates analysis of sphingomyelin content needs further evaluation. Overall, the current results demonstrate that the method presented in this report yields a simple, sensitive, stable, and comprehensive quantitation of all the major lipid classes contained in a biological sample.

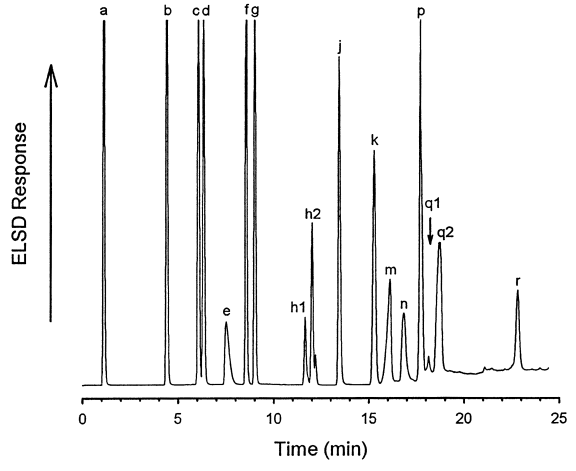

Fig. 2. Separation and mass (light-scattering) detection of lipid standards. A 10-μl volume of isooctane–tetrahydrofuran (9:1, v/v) containing 5 μg each of cholesteryl ester (a), triacylglycerol (b), cholesterol (c), diacylglycerol (d), oleic acid (e), 1,2-hexadecanediol (f), monoacylglycerol (g), cerebrosides (h1, type II; h2, type I), cardiolipin (j), phosphatidylethanolamine (k), phosphatidylinositol (m), phosphatidylserine (n), phosphatidylcholine (p), sphingomyelin (q1, q2) and lysophosphatidylcholine (r) was chromatographed as described in the text.

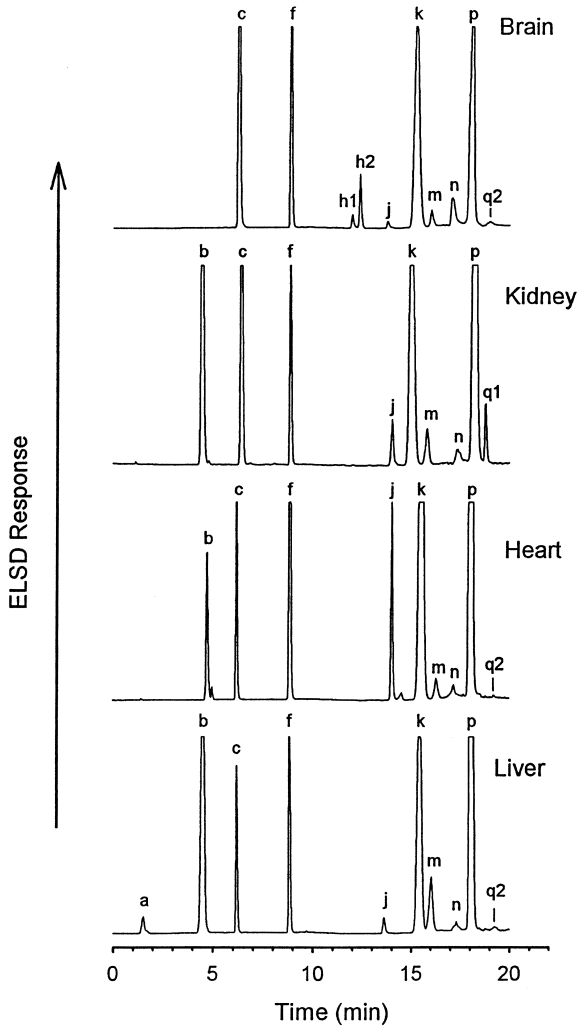

Fig. 3. Separation and mass (light-scattering) detection of lipids extracted from rat tissues. Total lipid mass in each injection was 30–40 μg. The peak identities are given in Fig. 1. The tissue lipid compositions derived from such chromatograms are presented in Table 3.

Table 3
Lipid content of rat tissues determined by HPLC and mass (light-scattering) detection

Lipid class	Tissue lipids (μg/mg protein)[a]			
	Liver	Heart	Kidney	Brain
Cholesteryl ester	1.5±0.02	0.04±0.00	0.1±0.00	
Triacylglycerol	21.0±0.69	4.1±0.11	13.5±0.38	
Cholesterol	6.2±0.19	5.1±0.10	16.8±0.15	17.6±0.44
Cerebrosides				10.1±0.20
Cardiolipin	3.2±0.06	8.4±0.07	5.5±0.07	1.0±0.03
Phosphatidylethanolamine	23.5±0.36	24.0±0.33	24.5±0.48	18.4±0.40
Phosphatidylinositol	10.2±0.29	3.3±0.12	5.8±0.16	2.6±0.14
Phosphatidylserine	4.3±0.28	3.6±0.33	9.4±0.16	7.6±0.16
Phosphatidylcholine	46.9±0.42	27.9±0.55	34.2±0.49	18.5±0.59
Sphingomyelin	1.4±0.04	0.5±0.12	3.4±0.21	0.9±0.09

[a]Mean±S.E.M. (n=9).

Table 4
Comparison of relative lipid compositions of rat tissues obtained in the current report with values from other studies[a]

Rat tissue	Analysis	Neutral lipids (%)[b]				Phospholipids (%)[c]					
		CE	TG	Ch	Cer	CL	PE	PI	PS	PC	Sph
Liver	Current	5.3	73.3	21.4		3.6	26.3	11.4	4.8	52.4	1.6
	Ref. [1]	10.9	49.2	39.9		5.3	23.1	5.1		64.1	2.4
	Ref. [3]	23.1	54.0	23.0		4.6	14.4	9.6	3.7	60.3	7.4
	Ref. [2]					5.6	23.0	8.9	2.6	55.3	3.1
	Ref. [9]					9.1	24.1	10.1	3.7	49.0	4.1
Heart	Current	0.5	44.3	55.2		12.4	35.5	4.9	5.3	41.2	0.7
	Ref. [1]	2.7	46.8	50.4		13.7	37.3	4.1		43.0	2.0
	Ref. [3]	18.0	50.8	31.3		11.7	30.9	3.7	3.2	45.2	5.3
	Ref. [2]					10.8	37.0	4.8	2.5	41.9	1.6
	Ref. [10]					22.4	30.1	4.5	3.4	36.5	3.0
Brain	Current			63.5	36.5	2.0	37.6	5.3	15.6	37.6	1.9
	Ref. [3]	0.4	0.4	63.6	35.6	2.5	33.3	3.8	15.3	38.1	7.1
	Ref. [11]					4.0	36.2	4.1	13.1	37.4	5.1
Kidney	Current	0.3	44.4	55.3		6.6	29.6	7.0	11.4	41.3	4.1
	Ref. [10]					12.7	26.8	6.9	7.6	34.0	12.0

[a]Lipid compositional data expressed in molar units were converted to mass values using average molecular weights of 651 for CE, 880 for TG, 387 for CH, 700 for Cer, 760 for PC and PE, 730 for Sph, 810 for PS, 909 for PI and 1493 for CL.
[b]Percent of total neutral lipid mass for cholesteryl ester (CE), triacylglycerol (TG), cholesterol (Ch), cerebrosides (Cer).
[c]Percent of total phospholipid mass for cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (Sph).

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