Journal of Chromatography, 339 (1985) 35—44
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2479

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARDIOLIPIN

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(Received September 19th, 1984)

SUMMARY

Resolution of freshly prepared and of commercially available (degraded) samples of cardiolipin into 15—30 components has been accomplished by reversed-phase high-performance liquid chromatography using a 3-μm particulate Microsorb C_{18} column irrigated with linear gradients of acetonitrile—methanol—10 m*M* phosphate buffer pH 7.4. Selected resolved components were crystallized and characterized by infrared absorption spectra. Saponification of other components and identification of component fatty acids by reversed-phase high-performance liquid chromatography demonstrated the presence of ten fatty acids (14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:4), with linoleic acid (18:2) identified in all resolved components. From fatty acid composition data it appears that several resolved fractions consist of single cardiolipin molecular species.

INTRODUCTION

For continuing studies of autoxidation of unsaturated lipids we required uniform preparations of cardiolipin (1,3-diphosphatidylglycerol), a highly autoxidizable class of anionic phospholipids (pK_a 1.05) localized in the inner mitochondrial membrane and implicated in various intracellular activities [1, 2]. As many as seventeen fatty acids have been identified in cardiolipin from different sources, with linoleic acid (18:2) predominant. Thus the number of possible cardiolipin molecular species is immense, and little has been achieved in the resolution of these complex mixtures into individual molecular species. Generally only groups of cardiolipin species have been resolved by high-performance liquid chromatography (HPLC) [3—5]. However, reversed-phase HPLC has not been adequately examined for cardiolipin preparations despite its successful application to other classes of phospholipids [6—8]. We describe herein an application of reversed-phase HPLC for resolution of cardiolipin from

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various sources into components, some of which appear to be individual molecular species.

EXPERIMENTAL

Solvents for extractions and thin-layer chromatography (TLC) were redistilled reagent grade of several manufacturers. Solvents methanol, acetonitrile, and tetrahydrofuran for HPLC were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water for HPLC buffer was first deionized and then redistilled. Buffers were prepared from water boiled to purge carbon dioxide and air, using only potassium salts (buffers from sodium salts tended to precipitate from acetonitrile solutions). All solvents were filtered prior to use through 0.45-μm membrane filters (Gelman Sciences, Ann Arbor, MI, U.S.A.). Fatty acid reference samples were the highest quality available from Supelco (Bellefonte, PA, U.S.A.), Applied Science Labs. (State College, PA, U.S.A.), Calbiochem-Behring (San Diego, CA, U.S.A.), and Sigma (St. Louis, MO, U.S.A.).

Three commercially available bovine heart mitochondrial cardiolipin samples were examined: CL-1 (chloroform solution) as free acid in sealed ampule from Applied Science Labs., and CL-2 and CL-3 as sodium salts in ethanol solutions from Sigma and Chemical Dynamics (South Plainfield, NJ, U.S.A.), respectively. Additionally, cardiolipin samples were prepared from brain, heart, kidney, and liver from five male Sprague—Dawley rats (400—500 g) by the method of Neilsen [9]. Rat mitochondrial cardiolipin (free acid) from silica gel columns irrigated with chloroform—methanol (19:1) was rechromatographed by TLC, eluted with chloroform—methanol (3:1), evaporated under vacuum, and stored at —20°C until use. For further work, cardiolipin free acid forms were redissolved in chloroform—methanol (4:1), cardiolipin sodium salts in heptane—isopropyl alcohol—water (20:25:8) [3].

Preparative TLC was conducted with 1 mm thick layers of Silica Gel 60 F-254 (E. Merck, Darmstadt, F.R.G.) on 20 × 20 cm chromatoplates. Analytical TLC was conducted on 0.25 mm thick 20 × 20 cm chromatoplates coated with Silica Gel 60 F-254 or with Sil G-25 (without gypsum) (Macherey, Nagel & Co., Düren, F.R.G.). Multiple ascending irrigations chiefly with chloroform—methanol—water (65:25:4) but also in the proportions 70:30:5 and 80:25:4 were used. Purity of cardiolipin fractions was also assessed by two-dimensional TLC irrigated in one dimension with chloroform—methanol—water—28% ammonium hydroxide (260:140:16:1) and in the second dimension with chloroform—methanol—acetone—glacial acetic acid—water (10:2:4:2:1). Components were detected in spraying with N,N,-dimethyl-*p*-phenylenediamine to detect peroxides [10] and then with 50% aqueous sulfuric acid (with charring) to detect all components.

Reversed-phase HPLC was conducted using two Waters Assoc. (Milford, MA, U.S.A.) Model M6000A pumps controlled by Waters Assoc. Model 720 Systems Control unit. Samples (180—200 μg) were injected through a Waters Assoc. Model U6K injector onto a 100 × 4.6 mm column of 3-μm particulate Microsorb C_{18} (Rainin Instrument, Woburn, MA, U.S.A.), and chromatographed at a flow-rate of 2.0 ml/min, with effluent monitoring conducted with

a Perkin-Elmer Model LC-55 variable-wavelength spectrophotometric detector set at 208 nm. Four ternary mixtures of acetonitrile—methanol—10 m*M* phosphate buffer 7.4 in the proportions 60:30:10 (solvent A), 60:38:2 (solvent B), 60:28:2 (solvent C), and 60:36:4 (solvent D) were used in two linear gradient systems: sytem I, 100% solvent A changing to 100% solvent B in 90 min, and system II, solvent C—solvent D (75:25) changing to solvent C—solvent D (30:70) in 30 min followed by change from solvent C—solvent D (30:70) to 100% solvent D in 40 min.

The resolutions obtained were reproducible repeatedly with a given cardiolipin preparation, but as the Microsorb C_{18} column aged through use, retention times for individual components decreased slowly.

Individually collected components defined by elution curves were evaporated under vacuum and the aqueous residue acidified and extracted with diethyl ether. Dried ether extracts were evaporated under vacuum, and the purified cardiolipin (free acid) was crystallized from diethyl ether—acetone (ca. 1:9) and characterized by melting point (Kofler block) and infrared absorption spectra over the range 400—4000 cm^{-1} recorded on 0.1-mm diameter potassium bromide disks incorporating the sample, using a Perkin-Elmer Model 337 infrared spectrophotometer equipped with a beam condenser.

Fatty acid composition of cardiolipin components was determined by HPLC following alkaline hydrolysis. Each HPLC fraction was evaporated under vacuum (at room temperature) to remove organic solvent, and the aqueous residue was transferred to a reaction vial, to which were then added 3 ml of 2% methanolic potassium hydroxide [11]. After incubation at 37°C for 12 h the hydrolysates were acidified with 1.0 ml of 0.1 *M* hydrochloric acid, extracted three times with diethyl ether, and the ether phase was separated, dried, and analyzed for fatty acid composition by chromatography. TLC of fatty acids was conducted on silica gel G-25 chromatoplates and on silica gel 60 F-254 for fatty acyl hydroperoxides, using hexane—diethyl ether—glacial acetic acid (80:20:1) for multiple ascending irrigations and with N,N-dimethyl-*p*-phenylenediamine and 50% aqueous sulfuric acid for visualization. Fatty acids HPLC was conducted with a 30 cm × 4 mm proprietary (μBondapak family) reversed-phase (10 μm particulate) fatty acid analysis column from Waters Assoc., using isocratic elution with tetrahydrofuran—acetonitrile—water—glacial acetic acid (250:350:490:1) at a flow-rate of 2.0 ml/min [12]. Effluent was monitored by refractive index changes measured with a Waters Assoc. differential refractometer R401. Baseline resolution was achieved for all the relevant fatty acids except for the two pairs 16:1/18:3 and 16:0/20:4, and these pairs were resolved by TLC readily. Resolved fatty acids were identfied from retention data in comparison with authentic reference fatty acids; amounts of fatty acid were estimated from peak heights of components on the elution curves in comparison with measured responses of graded amounts of reference fatty acids.

RESULTS

Freshly prepared cardiolipin from rat brain, heart, kidney, and liver migrated on thin-layer chromatograms as a single component free of peroxides, with

the same mobility as the commercially available samples CL-1, CL-2, and CL-3. However, TLC of these three samples evinced the unique composition of each. The free acid cardiolipin CL-1 was extensively peroxidized. Moreover, the sodium salts CL-2 and CL-3 had different compositions, as CL-2 appeared as two distinct spots (Fig. 1). Rechromatography of the major non-peroxide components of each sample gave the purified peroxide-free samples for HPLC studies.

Reversed-phase HPLC of the cardiolipin preparations gave good results using linear gradients of the ternary solvent mixture acetonitrile—methanol—phosphate buffer. Both the number of components resolved and component band widths and retention times were dependent on buffer pH and strength. At slightly acid pH (pH 6.5) a satisfactory chromatogram was obtained, but at lower pH resolution deteriorated even though retention times of major components were unaffected. Above pH 6.5 retention times were pH-dependent, increasing sharply between pH 6.5 and 6.9, less so thereafter. Optimal resolution and peak shape were obtained with slightly alkaline (pH 7.4) buffer. Furthermore, over the buffer concentration range of 2.5—10 m*M* resolution was improved and retention times increased with increasing buffer strength. However, at buffer strength above 10 m*M* increased resistance to

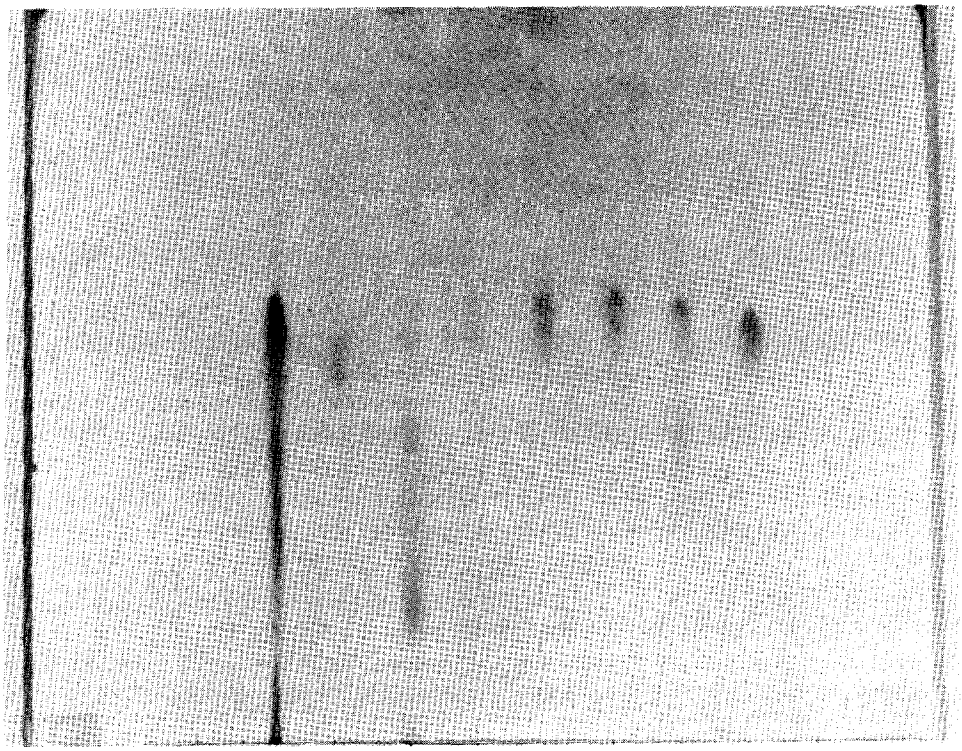

Fig. 1. TLC of commercially available bovine heart mitochondria cardiolipin on Sil G-25 irrigated with chloroform—methanol—water (65:25:1). Left to right: highly degraded and partially purified preparation therefrom; sample CL-1 and purified CL-1; CL-2 and purified CL-2; CL-3 and purified CL-3.

solvent pumping occurred. Accordingly, optimum HPLC conditions were judged to be obtained with 10 m*M* phosphate buffer pH 7.4, which was used throughout in subsequent studies.

Two linear gradient conditions were found useful in resolving cardiolipin components for our purposes. In Fig. 2 cardiolipin from several rat tissues is resolved by system I into distinct patterns of up to fifteen components each. Clearly, each tissue cardiolipin is unique, but with brain cardiolipin being composed of the most resolved components. In Fig. 3 the three commercially available cardiolipin samples were similarly resolved in system I. The HPLC elution curves of Fig. 3 thus confirm the composition differences indicated by TLC (Fig. 1).

In the case of the commercially available cardiolipin samples the presence of

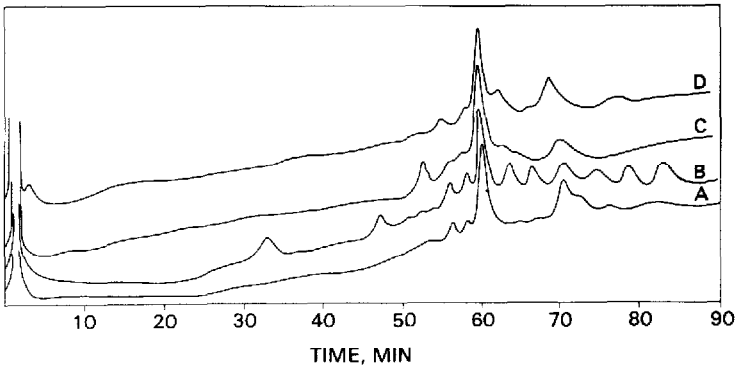

Fig. 2. HPLC in system I of rat tissue cardiolipin. (A) Kidney; (B) brain; (C) heart; (D) liver.

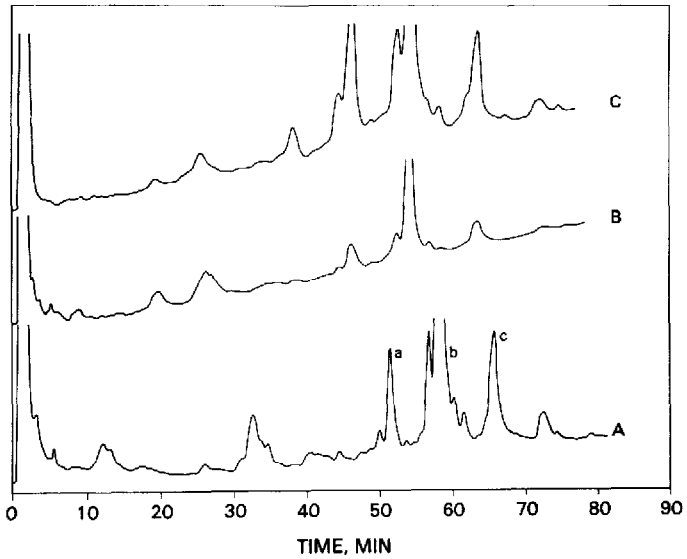

Fig. 3. HPLC of three commercially available bovine mitochondria cardiolipin samples in system I. (A) Sample CL-1; (B) sample CL-2; (C) sample CL-3. Components of curve A marked a, b and c were recovered, crystallized, and characterized by melting point and infrared absorption spectra.

more mobile components not so much in evidence for freshly prepared rat tissue cardiolipin suggests peroxidation of native cardiolipin, notorious for its ease of autoxidation in air. The linear gradient system clearly serves for resolution of both cardiolipin and peroxidized cardiolipin. However, we have not attempted to identify these more mobile, putatively peroxidized components.

The three major components (a, b and c) of sample CL-1 (Fig. 3A) were recovered and characterized for identification purposes by additional TLC and HPLC, melting behavior, and infrared absorption spectra in comparison with the unfractionated parent sample CL-1. Components a, b and c had essentially identical TLC properties and exhibited melting behavior: component a, m.p. 184—190°C; component b, m.p. 180—188°C; component c, m.p. 190—193°C; sample CL-1, m.p. 175—190°C, thus like that of synthetic cardiolipin sodium salts containing only 14:0 (m.p. 186—188°C), only 16:0 (m.p. 199—201°C), only 18:0 (m.p. 201—202°C), or equal amounts of 18:0 and 18:1 (m.p. 202—204°C) [13, 14]. Bovine heart cardiolipin free acid has m.p. 206—208°C [15, 16].

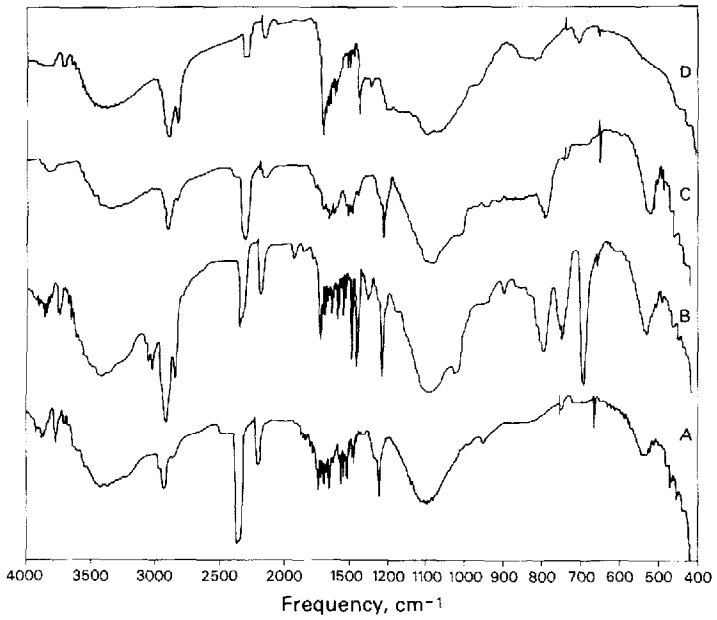

Fig. 4. Infrared absorption spectra of three major resolved components a, b and c (Fig. 3A) from HPLC in system I of sample CL-1. (A) Component a; (B) component b; (C) component c; (D) whole (unresolved) sample CL-1.

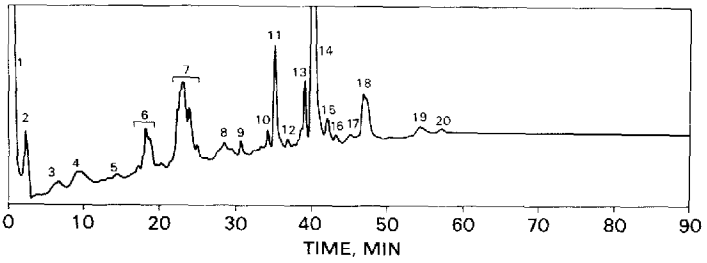

Fig. 5. HPLC of sample CL-2 showing improvements obtained with system II.

Furthermore, unique infrared absorption spectra of sample CL-1 and of the three resolved components a, b and c (Fig. 4) resembled spectra of cardiolipin previously recorded [13, 15–19]. Moreover, specific bands at 3380–3450 cm^{-1} (broad, OH), 2860–2940 cm^{-1} (C–H), 2330–2370 cm^{-1} (two bands, P–OH), 1740–1745 cm^{-1} (ester C=O), 1560–1730 cm^{-1} (at least five bands each, C=C), 1240–1275 cm^{-1} (P=O), and 1085–1110 cm^{-1} (broad, P–OR) strongly support the identity of these resolved components as 1,3-diphosphatidylglycerol derivatives.

Improved component resolution was achieved using solvents C and D in the two-step gradient conditions of system II. Following a relatively rapid linear gradient to solvent C–solvent D (30:70) a slower gradient to 100% solvent D was imposed, thereby resolving sample CL-2 into approximately thirty components (Fig. 5), of which twenty were individually collected. TLC of individual fractions did not resolve components from one another but did show that the first eight contained peroxidized material. The twelve later eluted fractions were not peroxidized.

The fatty acids compositions of each of the twenty fractions of sample CL-2 are presented in Table I. Fatty acids from 14:0 to 20:4 were identified, but linoleic acid was the only one found in all resolved components and was the predominent one in fourteen. Our data also evince the presence of eicosanoic acid (20:0) in four fractions, this fatty acid not having been previously identified as occurring in bovine heart mitochondrial cardiolipin.

DISCUSSION

Our work provides means of improved resolution of cardiolipin preparations, whether degraded or not, and evinces in detail the vast complexity of naturally occurring cardiolipin. The identity of resolved components as 1,3-diphosphatidylglycerols is supported by their chromatographic properties, by melting point and infrared spectral characterization of three major components, and by fatty acids composition. However, examination of all individual components by more sophisticated means such as chemical-ionization or field-desorption mass spectrometry [20] has not been attempted.

The chromatographic behavior of resolved cardiolipin fractions was typical of that anticipated from reversed-phase HPLC. Thus, more rapidly eluted fractions were relatively enriched in shorter-length fatty acyl moieties with greater unsaturation (Table I) and included more polar peroxidized material. More slowly eluted fractions contained relatively greater amounts of longer-chain fatty acids with less unsaturation. However, chromatographic behavior may depend on more than hydrophobic interactions between cardiolipin and bonded reversed-phase support but also on ionic character of the analytes. The superior resolution of components at pH 7.4 in comparison with neutral or acidic pH and features common to elution curves of both cardiolipin free acid and sodium salts suggest that all resolved components are acidic phospholipids migrating uniformly as ionic species, most probably as potassium salts in the 10 m*M* buffer used. In this matter, the effect of buffer pH is just the opposite of that encountered in reversed-phase HPLC of fatty acids where an acidic medium providing ion suppression is beneficial.

TABLE I

FATTY ACID COMPOSITION OF CARDIOLIPIN SAMPLE CL-2

Component No.	Fatty acid composition* (nmol)									
	14:1	14:0	16:1**	18:3**	18:2	16:0**	20:4**	18:1	18:0	20:0
1	—	118 (36)	—	—	51 (16)	156 (48)	—	—	—	—
2	—	73 (18)	50 (12)	—	50 (12)	234 (58)	—	—	—	—
3	—	26 (20)	30 (24)	—	55 (43)	—	—	17 (13)	—	—
4	45 (16)	—	—	28 (10)	183 (63)	—	—	33 (11)	—	—
5	77 (18)	75 (17)	—	—	217 (50)	—	—	28 (7)	32 (8)	—
6	75 (13)	76 (13)	117 (21)	—	286 (51)	—	—	10 (2)	—	—
7	—	35 (13)	70 (25)	24 (9)	95 (35)	31 (11)	20 (7)	—	—	—
8	48 (10)	75 (16)	—	40 (9)	230 (49)	—	35 (7)	—	42 (9)	—
9	—	73 (18)	96 (23)	—	193 (47)	—	—	50 (12)	—	—
10	—	—	47 (12)	44 (11)	233 (57)	—	—	—	—	80 (20)
11	—	—	—	56 (15)	257 (67)	—	—	71 (18)	—	—
12	—	26 (5)	30 (6)	66 (13)	207 (42)	—	—	32 (7)	65 (13)	69 (14)
13	—	—	65 (24)	—	200 (73)	—	—	9 (3)	—	—
14	—	—	—	70 (21)	225 (69)	—	—	18 (6)	12 (4)	—
15	—	—	—	130 (52)	58 (24)	—	—	—	60 (24)	—
16	—	—	80 (21)	108 (28)	90 (23)	22 (6)	—	40 (10)	45 (12)	—
17	—	—	—	53 (14)	112 (28)	—	25 (6)	—	212 (52)	—
18	—	—	—	152 (48)	67 (20)	35 (10)	—	—	—	70 (20)
19	—	—	—	125 (27)	244 (54)	—	45 (10)	—	40 (9)	—
20	—	—	—	68 (20)	205 (60)	—	24 (7)	—	—	45 (13)

*Nmol composition data can be compared only within each sample and not between samples. Values between parentheses are compositions in percent. Order of fatty acids listed is that of elution from fatty acid analysis column.

**Unresolved pairs 16:1/18:3 and 16:0/20:4 were resolved by TLC.

Data in Table I also establish that no two resolved fractions have the same fatty acid composition, thereby ruling out the possibility that different salt forms of the same compound contribute to the observed elution patterns [19]. These data also suggest that in several instances individual cardiolipin molecular species have been resolved for the first time. For example, minor component No. 15 (Fig. 5) composed of 18:3, 18:2, and 18:0 in stoichiometric proportions 2:1:1 appears to be a dilinolenoyl linoleoyl oleoyl diphosphatidylglycerol, thus a single molecular species (disregarding regioisomerism). However, equimolecular mixtures of tetralinolenoyl diphosphatidylglycerol and dilinoleoyl dioleoyl diphosphatidylglycerol, though deemed improbable, could also account for the observed fatty acid composition.

Moreover, by ignoring low levels of 18:0 and 18:1 found, the 1:3 proportions of the two fatty acids found in components No. 13 (16:1 and 18:2) and No. 14 (18:3 and 18:2) indicate that each be single cardiolipin molecular species (again disregarding regioisomerism), thus trilinoleoyl palmitoleoyl diphosphatidylglycerol and linolenoyl trilinoleoyl diphosphatidylglycerol, respectively, putatively contaminated with traces of congeners. As identified reference compounds are undescribed and unavailable and the ultimate resolving power of the HPLC systems is now known, it remains uncertain whether these resolved components represent more than one cardiolipin molecular species.

Although several other resolved components are composed of but three or four fatty acids, the stoichiometry found establishes these as mixtures, a matter clear from the elution curve in some cases (Fig. 5). Increasing diversity of fatty acid composition for other components attests their nature as mixtures.

ACKNOWLEDGEMENT

This study was supported financially by the U.S. Public Health Service (research Grant ES-02394).

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