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Short Communication

Fractionation of egg and soybean phosphatidylcholines by silver resin chromatography

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ABSTRACT

Samples of egg and soybean phosphatidylcholines were fractionated by total number of double bonds on silver ion-saturated resin columns using solvent programming (acetonitrile in methanol). Stainless-steel columns were packed with 15-μm resin which had been isolated by air elutriation. Methods of column packing, silver ion incorporation, solvent–resin interactions and resin regeneration are also discussed.

INTRODUCTION

Silver-loaded, macroreticular, divinylbenzene copolymer sulfonic acid resins have been useful in the separation of unsaturated compounds, particularly by number or configuration of the double bonds [1–5]. This concept has been extended to the separation of polyunsaturated compounds by use of mixed solvent systems [6–8]. Our studies in the metabolism of configurational and positional fatty acids in humans required preparation of highly unsaturated phospholipid standards. Earlier thin-layer chromatography (TLC) work [9–11] using silver nitrate-saturated silica indicated such fractionation of phospholipids was possible. Further fractionation by total number of carbon atoms can, if required, be accomplished by reversed-phase high-performance liquid chromatography (HPLC) [12,13]. The development of an analytical silver resin column for fractionation of 0.1 to 10 mg phospholipid samples is therefore reported.

EXPERIMENTAL[a]

Materials

Rohm & Haas XN1010 sulfonic acid resin (16–50 mesh) was obtained from

[a] The mention of firm names or trade products does not imply that they are endorsed or recommended over other firms or similar products not mentioned.

Aldrich (Milwaukee, WI, U.S.A.). Egg and soybean phosphatidylcholines (PCs, 99% pure; 100 mg/ml chloroform) were obtained from Sigma (St. Louis, MO, U.S.A.). All chromatography solvents were HPLC grade; all other chemicals were used as received.

Methods

Methods for grinding and sieving of the resin [14] and for use of mixed solvent systems [6–8] have been described previously. The wet-sieved (through 400 mesh) resin was washed with acetone and dried in a vacuum oven (60 mmHg/50°C) for 14 h. The dried resin was then fractionated by air-elutriation by the method of Ekman *et al.* [15]. An 8 l/min fraction was isolated and the resin size determined on a Hitachi H-500 electron microscope. The size of the irregular particles (15 $\pm$ 6 μm) was calculated as (length + width)/2. The resin was neutralized with 0.5 *M* sodium hydroxide, slurry packed into a stainless-steel (SS) column and flushed with a 50% excess of 0.5 *M* aqueous silver nitrate. After the resin was washed with water and then acetone, the column was unpacked and the resin was dried in a vacuum oven as described above. The dried resin was packed into a 25 cm $\times$ 4.6 mm SS column in small increments with tapping, then the column was flushed with methanol. Column selectivity was determined by the separation of methyl elaidate and methyl oleate.

The HPLC system consisted of a Spectra-Physics Model 8700 solvent delivery system, a Rheodyne 7125 injector (10 μl sample loop) and an Isco Model 1840 ultraviolet detector. Samples (10-μl) of PCs (10 mg/ml chloroform) were injected and detected by UV at 206 nm. The phospholipid fractions were converted to fatty acid methyl esters (FAMEs) by HCl–methanol as described previously [16].

The FAMEs were analyzed in a Packard Model 428 gas chromatograph equipped with a 100 m $\times$ 0.25 mm (0.2 μm coating) SP 2560 fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.). Helium carrier gas and a flame ionization detector were used. The oven temperature was programmed from 200–220°C at 10°C/min after an initial hold of 15 min.

RESULTS AND DISCUSSION

The PC were fractionated with a mobile phase consisting of varying amounts of acetonitrile in methanol. Soybean PC (10 μl of 10 mg PC/ml in chloroform) was fractionated in solvent-programmed (1.0 ml/min) runs in which, after an initial hold of 5 min, the acetonitrile concentration was increased from 0 to 25% in methanol over 40 min as shown in Fig. 1; the FAME composition of the eluted fractions is tabulated in Table I. The fractionation pattern for egg PCs (same sample size and solvent conditions as used for the fractionation of soybean PCs) is shown in Fig. 2 and the FAME composition in Table II. Several authors [17–19] have described the fatty acid (FA) composition and distribution in a variety of both plant and animal phospholipids. They found that saturated FAs predominated in the 1-position while unsaturated FAs were primarily located in the 2-position. Comparison of the FA distributions of the eluted fractions with this data allowed us to assign structures for the primary phospholipid in each fraction. (See Tables I and II, bottom). The PC species were fractionated based on the number of double bonds in their FA components. The FA patterns differed significantly for soybean and egg PCs. While

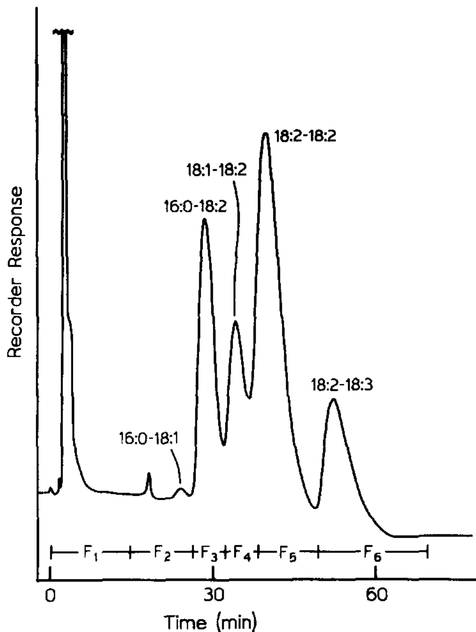

Fig. 1. Fractionation of soybean PCs. Sample size = 10 μl of 10 mg PC/ml chloroform solution. Flow-rate = 1.0 ml/min programmed, after a 5-min hold, from 100% methanol to acetonitrile–methanol (25:75) over 40 min, followed by a hold till the end of the run. F_1–F_6 represent the fractions collected.

soybean PC was composed primarily of 18:2–18:2 PC, fresh egg PC contained mostly sat-18:2 and sat-20:4. Sat-18:2 is defined as a saturated FA (16:0, 18:0 etc.) that is located in the number 1 position of the PC and 18:2 is located at the 2-position. The number 3 position is considered to be occupied by the phosphorus-containing moiety.

During preparation of the resin columns, several points of interest were noted. (1) Column diameter as well as resin particle size are important. Methyl elaidate and

TABLE I

COMPOSITION OF SOYBEAN PHOSPHATIDYLCHOLINE FRACTIONS OBTAINED BY HPLC AND ANALYZED BY GAS CHROMATOGRAPHY

FAME	Composition (%)					
	Standard	F_2	F_3	F_4	F_5	F_6
16:0	14.6	41.5	34.0	6.5	3.0	–
18:0	5.5	12.8	12.4	2.5	1.1	–
18:1ω9	10.5	38.7	7.1	32.8	2.8	–
18:1ω7	2.1	2.0	1.4	7.6	0.5	–
18:2	62.7	5.1	45.1	44.5	89.5	56.6
18:3	5.2	–	–	6.2	4.2	43.4
Primary component(s)		18:0–18:1	16:0–18:2	18:1–18:2	18:2–18:2	18:2–18:3
		16:0–18:1	18:0–18:2	16:0–18:3	18:1–18:3	–

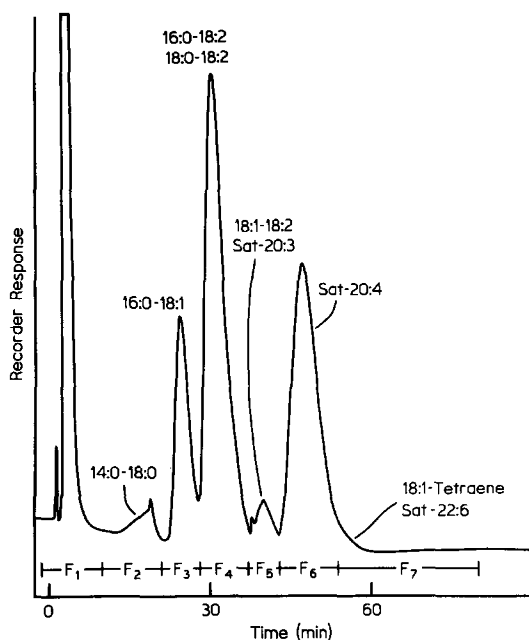

Fig. 2. Fractionation of egg PCs. Sample size and conditions as in Fig. 1.

methyl oleate were poorly separated on a 25 cm × 2.3 mm I.D., silvered resin column (dry-packed) as compared to one of 4.6 mm I.D. Also, if resin particles of < 15 μm were used poor resolution resulted. Apparently the macroreticular structure of the resin was destroyed by very fine grinding. (2) Slurry packing always yielded poorer

TABLE II

COMPOSITION OF EGG PHOSPHATIDYLCHOLINE FRACTIONS OBTAINED BY HPLC AND ANALYZED BY GAS CHROMATOGRAPHY

FAME	Composition (%)					
	Standard	F_3	F_4	F_5	F_6	F_7
14:0	0.4	0.6	0.5	3.1	0.7	1.6
16:0	33.6	40.1	30.8	12.4	17.4	17.1
16:1	1.1	0.8	1.5	3.6	3.1	2.2
18:0	12.5	10.3	12.2	6.6	22.2	5.9
18:1	29.8	46.9	15.2	10.6	5.4	18.2
18:2	15.5	–	36.9	17.3	7.4	3.2
20:2	0.2	–	0.4	–	–	–
20:4ω6	3.6	–	–	–	37.5	28.3
24:1	0.3	–	–	–	2.6	–
22:4	1.0	–	–	–	1.5	7.1
22:6ω3	4.0	–	–	–	–	–
Primary component(s)		16:0–18:1	16:0–18:2 18:0–18:2	18:1–18:2	16:0–20:4 18:0–20:4	18:1–20:4 16:0–22:6

results than dry packing. (3) An attempt to prepare a silver resin column from a dry-packed resin column (unsilvered) by changing the solvents and reagents *in situ* led to the creation of voids and poor resolution. The voids were created because XN1010 resin volume changes with solvent polarity. This problem is more pronounced in the very finely ground resin. (4) A gradual increase of column pressure was noted over a several week period when solvent programming was used. This was apparently caused by solvent-induced fracturing of the resin and the resultant release of fines. This problem was alleviated by reversing the column (no loss of resolution noticed) and/or cleaning the frits periodically. No significant loss of silver ions was noted. (5) Continuous solvent programming and/or "dirty" samples eventually (6–8 months) led to loss of resolution. However, the resin could be regenerated as described previously [8].

Samples which had been eluted through the column were analyzed by gas chromatography (for the presence of FAMEs)to determine if any transesterification had occurred, *e.g.*, catalyzed by residual sulfonic acid groups [20]. No evidence of this reaction was found.

This procedure allowed us to prepare analytical standards of highly unsaturated PCs of known and reproducible composition. Due to the strong affinity of the silver ions to the sulfonic acid groups of the XN1010 resin, no loss of silver ions was noted, even when such polar solvents as methanol and acetonitrile were used. Under these conditons, silver ion loss is a problem with silver nitrate–silica gel columns. Due to the high silver loading capacity of silver-ion saturated XN1010 resin (*ca.* 36%, w/w, for a 100% Ag^+ resin), semi-preparative separations are possible. Currently, a system composed of two 60 cm × 7.5 mm SS columns connected in tandem, has been used to fractionate 200-μl samples of soybean PCs (50% in methanol) at an acetonitrile–methanol (10:90) flow-rate of 3.0 ml/min (isocratic).

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