Analysis of Soybean Lecithins and Beef Phospholipids by HPLC with an Evaporative Light Scattering Detector

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Linear ($r > 0.99$) calibration curves were obtained for 10–150 μg of phosphatidylethanolamine (PE), 10–75 μg of phosphaditylinositol (PI), phosphaditylserine (PS) and lysophosphatidylethanolamine, 10–100 μg of phosphatidic acid (PA) and 10–250 μg of phosphatidylcholine (PC) by high-performance liquid chromatography analyses with an evaporative light scattering detector, a Zorbax 7-μm silica column and gradient elution with two solvents. One solvent (A) contained 415 mL isooctane (IOCT), 5 mL tetrahydrofuran (THF), 446 mL isopropanol (IPA), 104 mL $CHCl_3$ and 30 mL H_2O; and the other solvent (B) contained 216 mL IOCT, 4 mL THF, 546 mL IPA, 154 mL $CHCl_3$ and 80 mL H_2O. The gradient in which 100% A linearly changed to 100% B in 20 min followed by 12 min of 100% B and then a linear change to 100% A during 5 min separated PE, PS and PC in soybean lecithins and beef lipids, but failed to resolve PI and PA. In these same samples, less polar lipids were separated from phospholipids (PL) by elution from Bond-Elut silica columns with diethyl ether/hexane (20:80, vol/vol), and PL were recovered by elution with methanol. This procedure is useful for concentration of minor lipid components. Levels of PE, PI-PA, PS and PC were higher in granular than in liquid lecithin, and PC was the most abundant PL in soybean lecithins and beef lipids.

KEY WORDS: HPLC analysis-phospholipids, phospholipid concentration-solid phase extraction, soybean lecithins.

Reports of high-performance liquid chromatographic (HPLC) analysis of phospholipid classes (PL) in vegetable oils (1–5) are fairly representative of HPLC methods for PL in general. However, problems still exist in the quantitative analysis of PL by HPLC, even though several methods have been used to quantitate PL after HPLC separation. These include collection of each PL class and determination of lipid phosphorus (6–8), measurement of radioactive isotopes (8–10), measurement in the 203–210 nm range by an ultraviolet (UV) HPLC detector (1,2,5) and measurement by a moving wire flame ionization HPLC detector (4) or by an HPLC evaporative light scattering detector (3).

The quickest and easiest methods for measuring PL are those that incorporate the HPLC detectors listed previously. However, problems exist with these detectors. The flame ionization detector is no longer available commercially (3), and it is difficult to obtain accurate results by using the UV detector because at 203–210 nm, the degree of unsaturation and not the number of molecules is measured (2,5,11). Also, the double bonds in the unsaturated fatty acids in the PL contribute more to the absorbance in this UV range than carbonyl ester or amine bonds (9,11–13). The evaporative light scattering detector has given promising results for PL quantitation, even though nonlinear response of this detector from 0 to 50 μg of phospholipids has been reported by Van Der Meeren *et al.* (3). Christie (14) reported that linear response of the evaporative light scattering detector dropped off drastically below 10 μg PL, but was almost linear from 10 to 200 μg of PL. Reasons for nonlinear response may include unsuitable HPLC conditions for the analyses. Oppenheimer and Mourey (15) reported that the flow rate of the nebulizer gas and composition and flow rate of the mobile phase influenced the response of the evaporative light scattering detector to increasing amounts of the sample. A second problem with the evaporative light scattering detector is the lack of sensitivity, particularly when compared to the UV detector (2).

The evaporative light scattering detector possibly would be more convenient for PL quantitation if HPLC conditions could be found that would result in a linear detector response to increasing amounts of PL. Also, methods are needed to concentrate minor amounts of phospholipids in oils/fats for measurement by the evaporative light scattering detector. Therefore, the objectives of this study were: i) To develop a two-solvent HPLC system that would give linear relationships between peak areas detected by the evaporative light scattering detector and increasing amounts of selected PL, as well as to separate the major PL classes; ii) to investigate a concentration procedure for minor constituents in oils/fats, and iii) to quantitate PL in vegetable oil lecithins and animal lipids.

EXPERIMENTAL PROCEDURES

Phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SPH) were purchased from Sigma Chemical Co. (St. Louis, MO). PE, PI, PC and LPC were from soybean sources, PA was synthetic, PS was from bovine brain, LPE was from bovine liver and SPH was from egg yolk. All solvents were HPLC-grade and were purchased from Scientific Products (Stone Mountain, GA). Bond-Elut silica columns (100-mg size) were purchased from Analytichem International (Harbor City, CA). The 7-μm Zorbax column (25 cm × 4.6 mm i.d.) was obtained from Alltech Associates (Deerfield, IL). A powdered and a liquid soybean lecithin sample were obtained from a supplier of lecithin products, and total lipids were extracted from a bovine longissimus by the method of Melton *et al.* (16).

Four 100-mg samples from each lecithin and two samples of beef total lipids were separately dissolved in isooctane/chloroform, (1:1, vol/vol) to a total volume of 1.00 mL. Each solution (5 or 10 μL) was analyzed by HPLC upon gradient elution with solvents A and B (Table 1), and the solvent program given in Table 2. The HPLC instrument was equipped with a UK injector, M6000A pumps, and an automated gradient controller (Waters Associates, Milford, MA). It also was attached to the column previously described, an ACS Model 750/14 evaporative light scattering (mass) detector (Peres Industries, State College, PA) and a Model C-R2AX Chromatopac data processor (Shimadzu, Columbia, MD). The parameter settings for the

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TABLE 1

Composition of HPLC Solvents for Phospholipid Analysis

Component	Solvent A (mL)	Solvent B (mL)
Isooctane	415	216
Tetrahydrofuran	5	4
Isopropanol	446	546
Chloroform	104	154
Water	30	80

TABLE 2

Gradient Elution Program for Phospholipid Analysis

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Type of change
Initial	1.60	100	0	
20.0	1.60	0	100	Linear
32.0	1.60	0	100	
37.0	1.60	100	0	Linear
45.0	1.60	100	0	

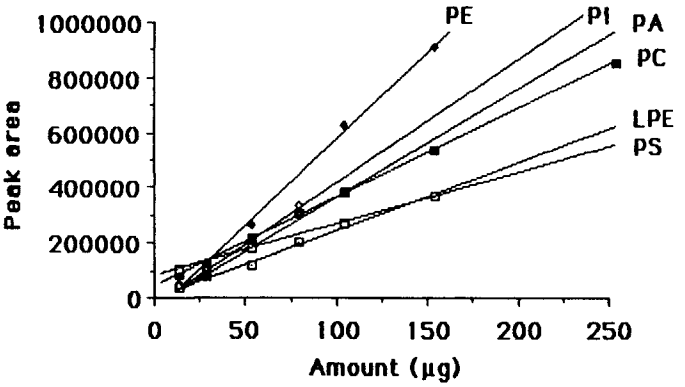

FIG. 1. Calibration curves for phospholipids analyzed by HPLC with an evaporative light scattering detector.

evaporative light scattering detector were as follows: External gas pressure, 40 psi; internal gas pressure, 26 psi; evaporator, 40; attenuation, 16; time constant, 5; and photomultiplier, 4. The mean levels (mg/100 mg sample) and the standard deviation for PE, PI-PA, PS and PC in the lecithins and beef lipids were determined. The sum of these PI concentrations was subtracted from 100 to determine the level of other lipids by HPLC.

The percentages (mg/100 mg total lipids) of phospholipids and other lipids in each lecithin and beef lipids (100 mg samples) also were determined by using solid phase extraction (SPE). Other lipids were eluted from a Bond-Elut silica column with 30 mL of hexane/diethyl ether (20:80, vol/vol), and the phospholipids (fraction 2) by 30 mL of methanol. The weight in each fraction was determined after the solvents had been evaporated on a steam bath under a flow of nitrogen, followed by drying in a 100°C oven for 30 min. The percentage of lipids in each fraction was then calculated. The dried lipids in each fraction were then dissolved in isooctane/chloroform (1/1, vol/vol) to a total volume of 1.00 mL, and were analyzed by HPLC.

Solutions of known concentrations of PE, PI, PA, PS, LPE and PC were analyzed by the HPLC procedure. Calibration curves were prepared for 10–150 µg of PE, 10–75 µg of PI, PS and LPE, 10–100 µg of PA and 10–250 µg of PC *vs.* the evaporative light scattering detector response for peak area in integrator counts. Correlation coefficients (r) were determined for all curves by linear regression. Solutions of LPC and SPH also were analyzed by the HPLC method, but were not quantitated in the samples.

RESULTS AND DISCUSSION

The solvents A and B (Table 1) used for gradient elution of phospholipids are composed of components used by Christie (17) for HPLC analysis of phospholipids. However, that study used a gradient elution program with three solvents and pumps instead of the two solvents and pumps used in the present study.

The calibration curves for PE, PI, PS, PA, PC and LPE are shown in Figure 1. Linear relationships ($r > 0.99$) between peak areas in integrator counts and amounts of phospholipid in µg were found for 10–75 µg of PI and LPE, for 10–100 µg PA, for 10–150 µg of PE and PS, and for 10–250 µg of PC. The linear slope of the PL calibration curves increased in the following order: LPE < PC < PA < PS < PI < PE. The results found for PC and PE are similar to those reported by Christie (17), who found that the peak area measured by the evaporative light scattering detector was approximately linear from 20 to 175 µg of both PE and PC, and that the slope of the PE calibration curve was geater than that of PC. This latter investigator did not report the calibration curves of the other phospholipids analyzed in the present study. However, the linear calibration curves for PC, PI, and PA and PE are contrary to results reported by Van Der Meeren *et al.* (3), who found nonlinear results of the evaporative light scattering detector for these respective PL from 0 to 50 µg.

The chromatograms of the lipids in Fractions 1 and 2 of liquid lecithin from the Bond-Elut silica column are shown in Figure 2. Phospholipids were absent from Fraction 1. The chromatograms in Figure 1 were obtained when the Zorbax silica column was new, and the retention times for the different phospholipids were as follows: 10.1 min for PE, 15.8 min for PI and PA (which coeluted), 17.1 min for PS and 27.3 min for PC. No LPE (21.8 min) or LPC (32.7 min) was detected.

The chromatogram of the phospholipids in a liquid lecithin sample is shown in Figure 3. The triacylglycerols eluted in 2.1 min. Retention times for PE (11.2 min), PI-PA (17.2 min), PS (22.0 min), LPE (25.9 min), PC (30.1 min) and LPC (36.1 min) were longer than for the respective phospholipids shown in Figure 2. This chromatogram was obtained after the column had been used for approximately one month. The retention times of lipids on a silica gel column have been found to increase when the column has been subjected to solvents containing alcohols or water. The polar alcohol and water molecules are continually incorporated into the stationary phase, making the column more polar (2). In the current study, washing the column with 50 mL of tetrahydrofuran removed alcohol and water

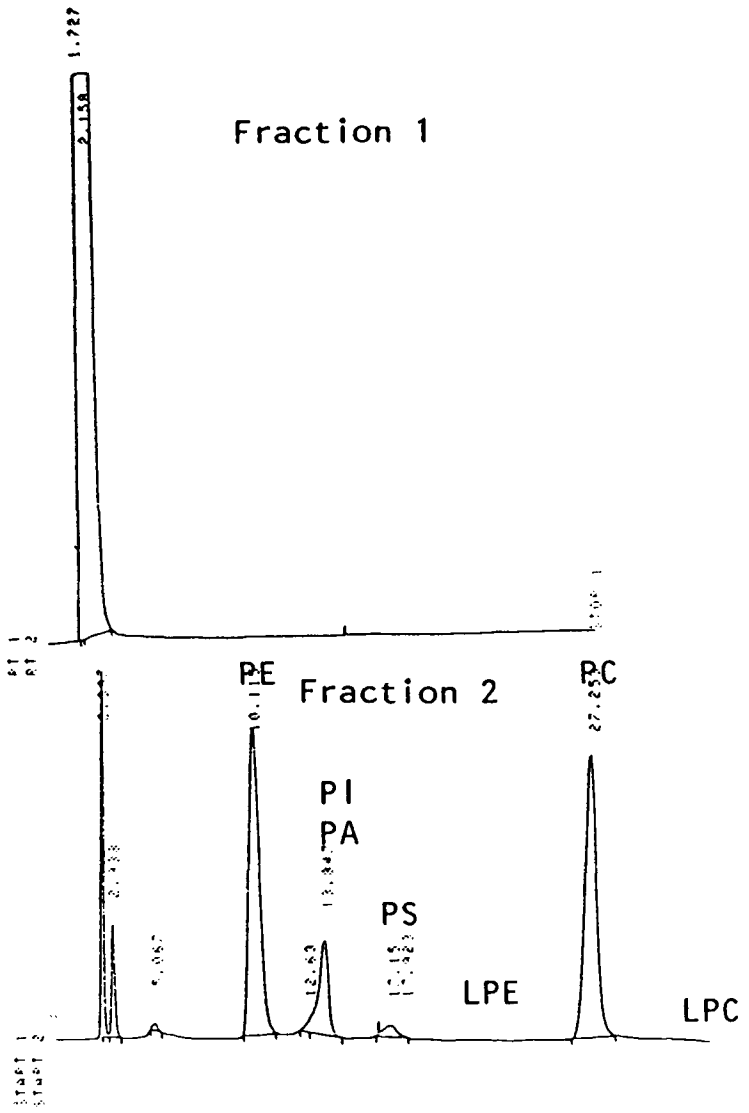

FIG. 2. HPLC chromatograms of lipid fractions from liquid soy lecithin (PE eluted at 10.1 min; PA and PI at 15.8 min; PS at 17.1 min; LPE, not detected, at 21.8 min; PC at 27.3 min and LPC, not detected, at 32.7 min).

molecules and reduced the retention times of the PL to approximately the same times obtained on the new column.

The chromatogram of beef phospholipids is presented in Figure 4. PE, PI-PA, PS, PC and SPH were found in beef lipids. LPE and LPC were not detected. The retention times given in Figure 4 for a specific phospholipid are between the retention times for the same phospholipid shown in Figures 2 and 3. Again, with increasing column use, the retention time of a phospholipid increased gradually and was restored as previously stated.

The mean levels of PE, PI-PA, PS and PC in lecithins and beef lipids are given in Table 3. Granular lecithin had the highest level of phospholipids, and the beef lipids contained the lowest levels of each PL. PS was detected in beef lipids but not quantitated. The most abundant phospholipid in the lecithins was PC. This is in agreement with results reported for some soy lecithins (1–4), but not for others (1,2,4). However, the levels of the various phospholipids in soybean lecithins are dependent upon many variables (5), making it difficult to compare one study to another. That PC is the most abundant phospholipid in bovine lipids is in agreement with previous reports (18).

The coefficients of variation (CV) among replicates analyzed by the HPLC method ranged from 1.4% for PC in the granular lecithin to 4.7% for PI-PA in the same sample (Table 3). These CV agree well with the 2.4–4.3% CV for HPLC quantitation by UV detection of PL in lecithins reported by Hurst and Martin (1).

The levels of phospholipids and other lipids determined by HPLC and by elution from the Bond-Elut column are given in Table 4. The percentage of other lipids obtained by difference for each sample agreed well with that determined by solid-phase extraction. In previous work, it was determined that the other lipids in crude vegetable oils eluted from silica gel columns by hexane/diethyl ether (20:80, vol/vol) consist of saturated and

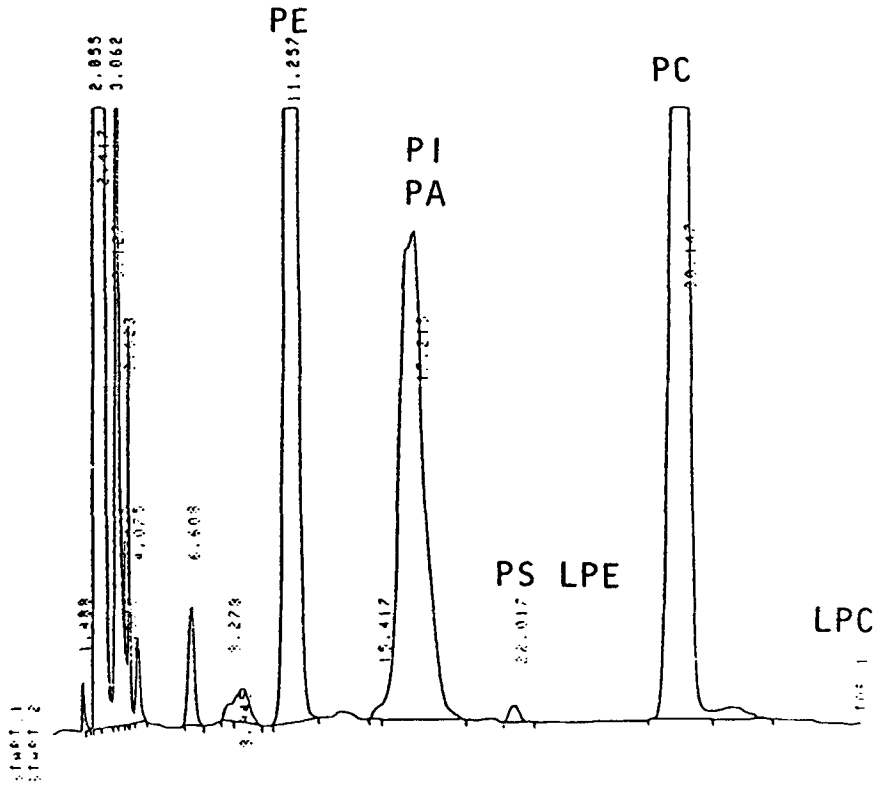

FIG. 3. HPLC chromatogram of phospholipids in liquid soy lecithin (triacylglycerols eluted at 2.1 min; PE at 11.2 min; PI and PA at 17.2 min; PS at 22.0 min; LPE, not detected, at 25.9 min; PC at 30.1 min and LPC, not detected, at 36.0 min).

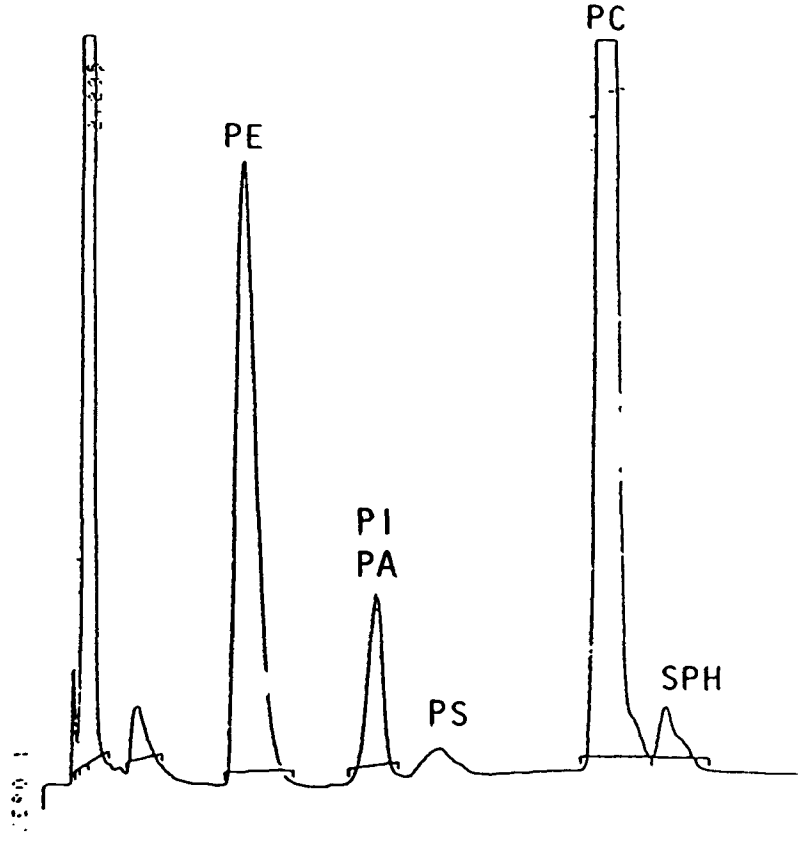

FIG. 4. HPLC chromatogram of beef phospholipids (PE eluted at 10.6 min; PI and PA eluted at 16.8 min; PS eluted at 19.8 min; PC eluted at 28.6 min and SPH eluted at 31.5 min).

TABLE 3

Mean Concentrations[a] (mg/100 mg) of Phospholipids in Soy Lecithins and Beef Lipids

Lipid[b]	Lecithins		Beef lipids
	Granular (n = 4)	Liquid (n = 4)	(n = 2)
PE	26.3 ± 0.5	17.9 ± 0.8	6.4 ± 0.3
PI-PA	19.3 ± 0.9	15.1 ± 0.2	15.1 ± 0.2
PS	0.9 ± 0.04	0.5 ± 0.03	—[c]
PC	42.6 ± 0.6	31.9 ± 0.6	27.9 ± 0.5

[a]Mean ± SD.
[b]PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; and PC, phosphatidylcholine.
[c]Not quantitated.

TABLE 4

Concentrations (mg/100 mg) of Lipids in Different Fractions of Soy Lecithins and Bovine Lipids

Lipids	Lecithins		Beef lipids
	Granular	Liquid	
PL[a]	89.1	65.4	37.0
Other[a] from HPLC	10.9	34.6	63.0
PL from SPE[b]	88.2	68.9	34.2
Other from SPE[b]	11.7	33.4	66.1

[a]PL equals the sum of concentrations for PE, PI-PA, PS and PC; other obtained by subtracting the sum from 100.
[b]Solid-phase extraction.

unsaturated hydrocarbons, mono-, di- and triacylglycerols, sterols, tocopherols and free fatty acids (19). It is also possible to concentrate low levels of phospholipids in oils/fats by this fractionation procedure. Although larger-sized silica columns are required for the quantitative fractionation of greater amounts of oils/fats, the 100-mg size can be used quantitatively to isolate minor constituents in up to one gram of oil/fat.

The analytical procedures given in this report allow rapid, accurate analysis of phospholipids in vegetable oil lecithins and in total lipids extracted from animal tissues. The solid-phase extraction method allows separation of PL from other lipids and quantitative concentration of minor amounts of PL in oils/fats for the HPLC analysis. However, further research is needed with HPLC silica columns of smaller particle size and/or modified solvents to separate phosphatidylinositol and phosphatidic acid.

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