Separation and Analysis of Phospholipids in Different Foods with a Light-Scattering Detector

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ABSTRACT: A method for the separation of phospholipids (PL) from total lipids by solid-phase extraction (SPE) with reverse-phase C_8 cartridges is described. The method was validated with a standard mixture of PL and applied to natural food matrixes, such as egg, chicken meat, salami, and ripened cheese. The recovery of PL ranged between 93 and 99.7% and was evaluated by an organic phosphorus spectrophotometric determination. The egg powder PL fraction obtained by SPE contained about 20% (w/w) nonpolar PL material when 100–150 mg of lipids were loaded onto the cartridge. Higher percentages of nonphospholipid components (30–43%) were obtained when the amount of lipids loaded was below or above the 100–150 mg range. The purified PL fractions were analyzed by high-performance liquid chromatography (HPLC) with an evaporative light-scattering detector. Good HPLC performance was observed even with low-purity SPE fractions (43% nonphospholipid material).

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KEY WORDS: Evaporative light-scattering detector, HPLC analysis, phospholipids, purification, solid-phase extraction.

Phospholipid (PL) isolation from total lipid mixtures has been done traditionally by thin-layer chromatography (TLC) (1,2) or column chromatography (3–7). With the advent of disposable cartridges for solid-phase extraction (SPE), several purification methods have been developed for different types of lipids (8–14) and, more specifically, for PL (15–17). In general, SPE allows for fast analysis and has good reproducibility; however, for more difficult separations, the results and recoveries vary, depending on the type of phase used and the nature of the sample matrix and composition. Some methods that were originally used for PL separation from meat (9,16) gave low recoveries when applied to other matrixes, such as cheese. This paper compares the performance of several solid phases with respect to the isolation of PL from various food products. PL recovery was compared to an organic phosphorus spectrophotometric determination of the lipids and the PL fractions obtained; these fractions were then analyzed by high-performance liquid chromatography (HPLC) with an evaporative light-scattering detector (ELSD).

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EXPERIMENTAL PROCEDURES

Samples and reagents. Samples of egg powder, chicken meat, ripened cheese, and salami were obtained from an Italian market. All chemicals were purchased from Carlo Erba (Milano, Italy). Methanol and chloroform were HPLC-grade; ammonium hydroxide (30%), *n*-hexane, diethyl ether, acetic acid, anhydrous sodium sulfate were analytical-grade reagents. Freshly deionized and bidistilled water was used. The following PL standards were from Sigma Chemical Co. (St. Louis, MO): L-α phosphatidylcholine (PC) from bovine liver; L-α phosphatidylethanolamine (PE), L-α phosphatidyl-L serine (PS), and sphingomyelin (Sph) from bovine brain; L-α phosphatidylinositol (PI) from soybean; L-α phosphatidyl-D,L-glycerol (PG) from egg yolk lecithin.

SPE. Total lipids extraction from food samples was carried out by the Folch method (18). The lipid extract was purified by using normal-phase (silica, aminopropyl) and reversed-phase (C_{18} and C_8) SPE cartridges. The cartridge capacity was 500 mg, and they were supplied by Bond Elut Varian (Harbor City, CA). Before conditioning, a small amount of anhydrous sodium sulfate was deposited onto the cartridge. Column elution was carried out at a temperature below 20°C with a Vac Elut holder (Analytichem International; Harbor City, CA) under a constant gentle vacuum.

Silica SPE. The cartridge was conditioned with 3 mL *n*-hexane. Lipid sample (200 mg), dissolved in 0.5 mL chloroform, was loaded onto the cartridge and eluted with the following solvent mixtures: 4 mL of *n*-hexane/diethyl ether (8:2, vol/vol), 4 mL of *n*-hexane/diethyl ether (1:1, vol/vol), and 4 mL methanol. Alternatively, 4 mL of 1–5% solution of acetic acid in methanol was used as the third elution solvent.

Aminopropyl SPE. The SPE cartridge was conditioned with 3 mL *n*-hexane. Lipid sample (200 mg) was dissolved in 0.5 mL chloroform and was loaded onto the cartridge. Elution was carried out by adding 2.5 mL of chloroform/isopropanol (2:1, vol/vol) (two times), 2.5 mL of a 2% (vol/vol) solution of acetic acid in diethyl ether (two times), and 1 mL methanol (four times) (9).

C_{18} SPE. The SPE cartridge was conditioned with 3 mL methanol. Lipid sample (200 mg) was dissolved in 0.5 mL of chloroform/methanol (2:1, vol/vol) and was loaded onto the cartridge. Elution was carried out by adding 4 mL of

methanol/water (4:1, vol/vol), 4 mL methanol, and 4 mL of methanol/chloroform (4:1, vol/vol).

C_8 SPE. The SPE cartridge was conditioned with 3 mL methanol. Lipid sample (200 mg) was dissolved in 0.5 mL of chloroform/methanol (2:1, vol/vol) and applied onto the cartridge. Elution was carried out by adding 4 mL methanol, 5 mL of chloroform/methanol (3:2, vol/vol), and 5 mL chloroform.

Different amounts of lipids, extracted from egg powder, were loaded on C_8 cartridges (10, 50, 100, 150, 200, and 250 mg); all analyses were performed in duplicate. The phosphorus content of each PL-containing fraction was determinated by the spectrophotometric method described by Morrison (19); the analysis was carried out twice for each fraction.

A standard solution of PE, PG, PI, PC, and Sph (100 μg/mL of each PL) was purified by C_8 SPE cartridges.

After elution, the cartridges can be regenerated by washing with chloroform (3 mL), chloroform/methanol (2:1, vol/vol) (3 mL), and methanol (5 mL).

HPLC. The HPLC system consisted of the following components: Degasser, ERC, Erma Inc. (Tokyo, Japan); injector, Rehodyne model 7125 (Cotati, CA); pump, model 64 Knauer (Berlin, Germany); gradient controller, M-300, Autochrom Inc. (Milford, MO); and ELSD, Sedex, model 45 Sedere (Vitry sur Seine, France). The HPLC system was equipped with an on-line filter (Rehodyne). The HPLC column was a silica Lichrosorb Si 60, 10 μm (25 cm × 4.6 mm i.d.) from Wellington House (Macclesfield, United Kingdom). The chromatograms were recorded with a Spectra Physics 4290 integrator (San José, CA). The ELSD was set at 60°C for evaporation temperature and at 2 atm pressure for nebulization gas (compressed air).

A methanolic ammonium hydroxide gradient in chloroform was chosen as eluent because of its low viscosity and good solvent properties. After optimization, the following binary gradient was used: solvent A, chloroform/methanol/ammonium hydroxide 30% (80:19.5:0.5, by vol), and solvent B, chloroform/methanol/water/ammonium hydroxide 30% (60:34:5.5:0.5, by vol). Flow rate was 1.5 mL/min. The solvent program is shown in Table 1.

Time required for returning to initial conditions was 15 min, thereby avoiding significant variations in the retention volumes.

TABLE 1
High-Performance Liquid Chromatography Solvent Program for a Binary Gradient[a]

Time (min)	A%	B%
0	100	0
8	45	55
15	40	60
20	40	60
35	100	0

[a]Solvent A, chloroform/methanol/ammonium hydroxide 30% (80:19.5:0.5, by vol); Solvent B, chloroform/methanol/water/ammonium hydroxide 30% (60:34:5.5:0.5, by vol).

Standard curves for HPLC analysis were run with the commercial standards of PC, PE, PS, PI, PG, and Sph. Solutions contained 0.25–4 μg of PL and were injected in an increasing concentration order in each run. Three replicates were run for each concentration. Regression analyses were done with the quadratic function: $y = (a + bx)^2$; r^2 varied between 0.9333 for PS and 0.9811 for PE (20).

RESULTS AND DISCUSSION

Extraction of total lipids was performed by the Folch method, which allows a complete extraction of PL. These types of components are the most difficult to be extracted due to their chemical structure, which has both hydrophilic and lipophilic character, and to the fact that they are membrane constituents and are, therefore, more structured and strongly bonded than other lipid fractions.

PL in the lipid extract were quantitated by using the phosphorus spectrophotometric method suggested by Morrison (19), in which blue color is developed because of the formation of ammonium phosphomolibdate. This determination gave a linear response at a phosphorus amount range between 0.25 and 13 μg. This range corresponds to a PL quantity between 6 and 300 μg, considering a conversion factor equal to 24.5 (mg P × 2.5 = mg PL).

Phosphorus determination of the polar lipid fraction was also carried out. During the setting up of the method, the fractions that eluted before and after the polar lipids were also subjected to phosphorus determination to ensure that band broadening had not occurred during elution, which could have resulted in a PL distribution among the fractions. It was also confirmed that, in the silica and the NH_2 cartridges, the PL eluted in the third fraction, whereas in the C_8 and C_{18} phases they eluted in the first fraction. Three replicates were run for each type of SPE cartridge.

Table 2 compares the average concentration (mg/g of fat) of PL in egg powder obtained with different SPE purification methods; the results regarding the other food matrixes are not included in this table. Three replicates were run for each type of SPE cartridge. Good repeatability was observed for all purification methods; however, the PL recoveries were significantly different.

Regarding the silica cartridges, the results varied greatly, depending on the PL content of the sample. For egg powder, which has a high PL content (over 20% of lipids), the recov-

TABLE 2
PL Percentage Recoveries Obtained with the Different SPE Methods[a]

Phases	PL in crude lipids (mg)	PL in SPE fraction (mg) (x ±σ[b])	Recovery (%)
C_8	224.8	217.9 ± 10.1	97.3
C_{18}	224.8	123.9 ± 4.0	55.2
Silica	224.8	157.9 ± 4.7	70.0
NH_2	224.8	119.1 ± 3.2	53.2

[a]PL, phospholipid; SPE, solid-phase extraction. [b]σ, standard deviation.

ery was 70%, while in the ripened cheese (data not shown), which contains a much lower amount of PL (1% of lipids), the recovery was about 60%. These data correspond to the test in which a 5% solution of acetic acid in methanol was used as third elution solvent; lower recoveries were obtained with a lower percentage of acetic acid in methanol. An elution mixture with a higher concentration of acetic acid could not be used though because it could have caused several problems in the HPLC analysis.

Although NH_2 cartridges have been suggested for lipid fractionation and PL purification from meat, the PL recoveries in most samples tested were extremely low, especially in cheese and egg powder (30 and 53%, respectively).

On the other hand, the C_{18} reversed-phase cartridges gave a 55% PL recovery; losses may have been due to an interaction between the diacylglycerol side of the PL and the octadecyl chain of the solid phase. Because this interaction could be diminished by reducing the length of the alkyl chain, the C_8 cartridge performance was evaluated, and a significant increase in the PL recovery was observed (97%).

To ensure that purification by SPE did not show certain selectivity for the different PL, a standard mixture that contained similar amounts of PL and lipids extracted from egg powder were purified by using four different solid phases (C_8, C_{18}, silica, and NH_2) and then analyzed by HPLC. Table 3 reports the concentration of the single PL present in egg powder, obtained by purification with C_8, C_{18}, silica, and NH_2 SPE. The quantitation of each PL was performed by using a calibration curve with PL standards (20). The amount of PE, PC, and Sph recovered with C_{18} cartridges was low, whereas the silica-phase fraction showed a particularly low content of PC. On the other hand, PG and PI were not detected in the PL fraction obtained from the NH_2 cartridge. Total recoveries of the C_{18}, silica, and NH_2 SPE were lower than 50%, though. For the C_8 phase, the amounts of PC and Sph present in this fraction were much higher than those found in the other ones; moreover, no PL selectivity was observed, resulting in a 100% total recovery. This fact was confirmed by the HPLC analysis of the PL standard mixture before (a) and after SPE (B) (Fig. 1).

As observed from the previous results, the C_8 phase gave the best qualitative and quantitative PL recovery and was, therefore, selected as the most adequate phase for SPE purification of PL. To evaluate the effect of the load of total lipids on the PL recovery, different amounts of total lipids from egg powder, ranging between 10 and 250 mg, were loaded onto the 500 mg C_8 cartridges (Table 4); all tests were performed in duplicate. The recoveries were determined by comparison of the phosphorus content before and after SPE purification. Table 4 shows the concentration of PL detected (mg PL/g lipids), the percent recovery, and coefficient of variation (CV) (%) for each load of total lipids. The average recovery, without including test A (10 mg), was quite high (95.4%). The best CV were those for the higher loads of lipids; in fact, this

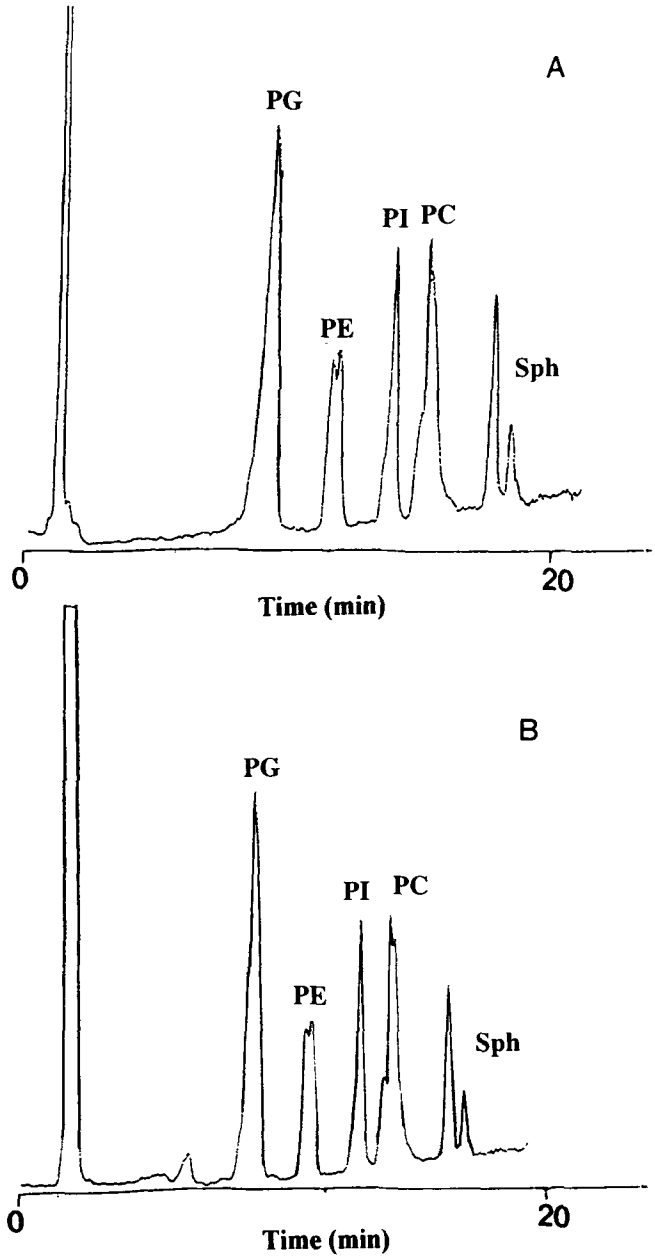

FIG. 1. High-performance liquid chromatography traces of phospholipid standards. A, before SPE purification; B, after SPE purification.

TABLE 3
Comparison of the Average Concentrations (mg/g of fat) of Single PL in Egg Powder, Obtained with Different SPE Purification Methods[a]

Phases	PG	PE	PI	PC	Sph	LisoPC	Total	Total recovery (%)
C_8	0.6	17.0	1.3	79.0	9.4	2.9	110.2	98.0
C_{18}	0.6	8.3	1.4	34.0	1.8	1.8	47.9	42.6
Silica	0.2	12.0	1.3	9.6	1.1	1.3	25.5	22.7
NH_2	—	15.6	—	20.1	1.4	1.3	38.4	34.1

[a]PG, L-α phosphatidyl-DL-glycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; Sph, sphingomyelin; see Table 2 for other abbreviations.

TABLE 4
PL Recoveries After Purification by C_8 SPE for Different Loads of Total Lipids from Egg Powder

Test	Lipid load (mg)	PL load[a] (mg)	Weight of SPE fraction (mg)	PL in SPE fraction (mg)	Non-PL in SPE fraction (%)	PL recovery (%)	CV[b] of PL recovery (%)
A	10	2.3	4.9	5.5	—	157.1	20.9
B	50	11.2	17.0	10.0	41.2	93.2	20.7
C	100	22.5	27.6	21.4	22.5	97.8	15.5
D	150	33.7	40.3	30.8	23.6	95.6	14.6
E	200	45.0	58.7	42.6	27.4	97.4	6.7
F	250	56.2	83.2	47.4	43.0	91.8	5.6

[a]Determinated by quantitation of phosphorus.
[b]CV (%) corresponds to the percentage coefficient of variation, i.e., the % ratio between the standard deviation and the average value of three replicates. See Table 2 for other abbreviations.

parameter seems to have an important effect on the performance of the chromatographic analysis. Considering both the recovery and the CV, the 200-mg load of lipids (test E) seems to provide the best working conditions for SPE purification of PL.

Test A was not considered for the calculation of the general average because it gives a 157% recovery, which seems to be an anomalous result. This may be due to the fact that, at this lipid concentration, solvent impurities in the form of phosphates become significant and increase the phosphorus content, therefore resulting in a false high recovery. If little sample is available, as in biological samples, 50-mg cartridges could be used instead of the 500-mg ones.

Table 4 also shows the total weight of the SPE fractions, as well as the amount of PL found therein. The weight difference between these two values corresponds to the amount of nonphospholipid material present in the PL purified fraction; as observed from the data, the percentage content of nonphospholipid material in the fraction varies according to the initial lipid load. In fact, the 100- and 150-mg loads gave the lowest percentages (~20%) of nonphospholipid compounds in the purified fractions. Higher values (30–43%) were found when the amount of lipids loaded was below or above the 100–150 mg range. These values are, nevertheless, much lower than the percentage of nonpolar lipids in the crude lipid extract (85–90%) (21). However, no significant effects were observed in the phosphorus determination, or in HPLC performance, when the low-purity SPE fractions (43% of nonphospholipid material) were analyzed.

On the other hand, temperature and vacuum intensity seemed to play an important role on SPE fractionation. The highest purity level was achieved at 20–22°C with a gentle vacuum. Under these conditions, it was possible to get the highest adsorption of the sample into the solid phase before the addition of the eluent, thereby increasing the retention of the nonpolar components.

After evaluation of the C_8 selectivity and purification capacity, the C_8 method was tested on various food matrixes. Table 5 reports the percentage recoveries obtained with the C_8 cartridges for four different food products (egg powder, chicken meat, salami, and ripened cheese); the recoveries, which are expressed as the percentage ratio between the phosphorus content in the lipid fraction before SPE purification and that present in the polar lipids after SPE, ranged from 92 to 99.3%. The SPE fractionation was run in triplicate for each type of food sample; good reproducibility was obtained. As observed from this table, the recoveries of samples with low triglyceride contents were high. In fact, the salami gave the lowest recovery, which might be due to its highly saturated triglyceride content; an improvement may be achieved by using a solid phase with a shorter alkyl chain.

Figure 2 shows an HPLC trace of PL from chicken meat purified by C_8 SPE. Table 6 reports the amounts of each PL, as well as the total amount of PL found in each food product. The results obtained from HPLC quantitation were much lower than those from the spectrophotometric determination. This difference is due to the fact that HPLC analysis only quantifies the main PL, while the spectrophotometric method determines even the PL degradation products (such as Liso-PL and PL oxidation products). These degradation products elute together with PL when separated with C_8 cartridges.

One of the main advantages of this SPE reversed-phase method, with respect to normal-phase SPE, is that it gives good recovery of polar lipids, which are of great importance in food preservation and ripening control. However, to obtain a reliable quantitation, it is important to carry out the HPLC

TABLE 5
PL Content of Different Foods Before and After C_8 SPE and Corresponding PL Recovery[a]

Foods	Phospholipids (mg/g of fat)				Recovery (%)
	Crude lipids		After SPE purification		
	Average	σ	Average	σ	
Egg powder	224.8	5.27	219.6	14.87	97.7
Chicken meat	77.3	5.00	77.0	6.16	99.3
Ripened cheese	7.5	0.28	7.3	0.70	97.3
Salami	23.8	0.85	22.0	1.59	92.4

[a]See Table 2 for abbreviations.

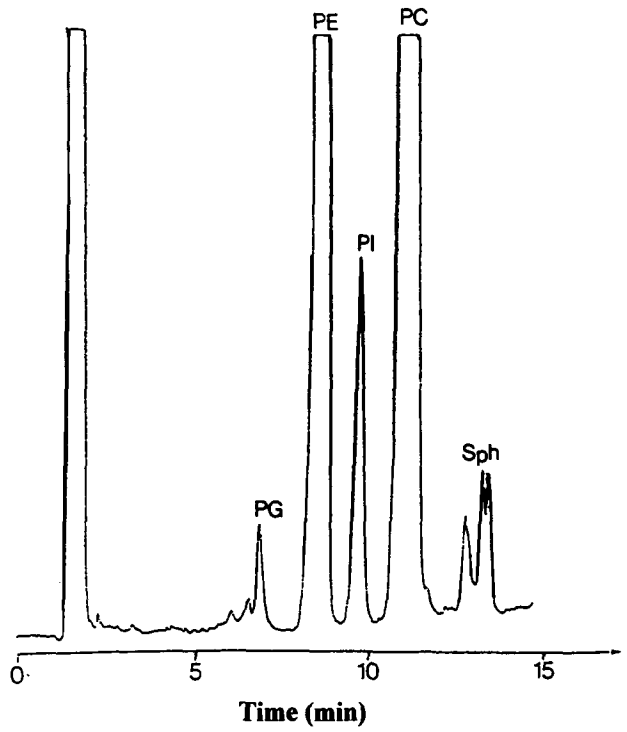

FIG. 2. High-performance liquid chromatography trace of phospholipids from chicken meat.

analysis of the purified PL fractions without frozen storage that would lead to selective insolubilization of some components, especially PE. This factor could have strongly influenced some previous results obtained with standard PL mixtures and fresh products, such as meat (9,16).

Fast analysis and good reproducibility are achieved with the described method. This method can be applied to all types of food products, resulting in good recovery of PL and their degradation products. However, diglycerides, monoglycerides, and free fatty acids are not completely eliminated from the HPLC fraction (~20% of the purified fraction); in fact, partial glycerides elute at the beginning of the chromatogram, while free fatty acids elute just before PG, which leads to overlapping if present in high concentrations. Another advantage of this method is that C_8 cartridges can be reused. After PL elution, they must be washed with chloroform, to elute neutral lipids and then with chloroform/methanol (2:1, vol/vol) and methanol; no changes in cartridge performance have been observed after being used at least three times.

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TABLE 6
Average PL Composition of Different Foods (mg/g of fat) and Corresponding Standard Deviation

Foods	PG	PE	PI	PC	Sph	Total[a]
Egg powder	0.9 ± 0.10	24.4 ± 1.49	1.1 ± 0.05	157.6 ± 11.29	2.9 ± 0.35	186.8 ± 19.09
Chicken meat	1.2 ± 0.14	15.6 ± 0.37	1.6 ± 0.03	32.7 ± 5.63	2.2 ± 0.01	53.3 ± 5.38
Ripened cheese	0.1 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.01	1.8 ± 0.001	2.7 ± 0.02
Salami	4.9 ± 0.21	1.3 ± 0.21	0.3 ± 0.02	7.8 ± 0.37	0.8 ± 0.17	15.1 ± 0.56

[a]Mean values of three replicates. See Tables 2 and 3 for abbreviations.

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