Quantitative Analysis of Phospholipids from Whey Protein Concentrates by High-Performance Liquid Chromatography with a Narrow-Bore Column and an Evaporative Light-Scattering Detector

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ABSTRACT: A procedure for separation and quantitative determination of phospholipid classes by high-performance liquid chromatography with a narrow-bore column and a light-scattering detector was developed. Cerebrosides (CER), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH), and lysophosphatidylcholine (LPC) were base-line resolved, and column life was improved due to low back pressure and low alkalinity of the solvents. Solvent consumption was reduced by 80%, and the detection limit was improved more than tenfold, compared to an analytical column. A gradient elution with pH modifier was necessary for good resolution of acidic phospholipids. A binary solvent system, consisting of A: chloroform/methanol, 80:20 and B: chloroform/methanol/water/ammonium hydroxide (20%), 60:34:6:0.25, was used. Analysis was completed in 36 min, and repeated injections of the samples were possible. The method was applied to the analysis of phospholipids from whey protein concentrates (WPC). Phospholipids in WPC (75% protein) contained (%, w/w) 3.57 ± 0.13 CER; 18.13 ± 1.23 PI; 4.45 ± 0.21 PE; 7.45 ± 0.58 PS; 30.54 ± 1.84 PC; 35.82 ± 1.16 SPH; and no detectable LPC. *JAOCS 72*, 729–733 (1995).

KEY WORDS: Evaporative light-scattering detector, high-performance liquid chromatography, narrow-bore column, phospholipids, whey protein concentrates.

Whey protein concentrates (WPC) are whey powders that contain 35 to 75% proteins, and they are gaining acceptance as a functional ingredient in food formulations. However, lack of consistency in the gross composition, and thus the functionality of WPC, has limited their acceptance by food processors (1–4). Functional properties of a food protein are those physicochemical properties that influence rheological and organoleptic qualities of the food. Among the constituents of WPC, total lipids have a significant effect on functionality (5,6). In general, the higher the total lipid content, the poorer the functionality of WPC. Little is known about the lipids of whey and WPC (7). Most available data relate to the total lipid content of WPC. However, Theodet and Gandemer (7) and de Wit *et al.* (8,9) also determined total phospholipids, triacylglycerols, total free fatty acids, and diacylglycerols. More information about lipid classes and subclasses of phospholipids is vital to understand their role in functionality of WPC.

Since the last decade, high-performance liquid chromatography (HPLC) has become the method of choice for separation and quantitative determination of phospholipid classes (10–14). Some major limitations of the HPLC method are detection, column life, resolution of acidic phospholipids, and collection of large quantities of toxic solvents (15). Ultraviolet detectors require expensive solvents with high spectral transparency, and the quantitative results are not reliable, due to the varying degrees of unsaturation in phospholipids from different sources (13). Differential refractometry does not allow an elution gradient, which is necessary for resolution of common phospholipid classes in a single run. Recent development of the evaporative light-scattering detector (ELSD) has solved the problem of detection (15–18). Mobile-phase modifiers, such as phosphoric acid (19) and ammonium hydroxide (15), are necessary to prevent peak broadening and to allow base-line resolution of acidic phospholipids. The only limitation with ELSD is that the mobile phase, including modifiers, if any, should be volatile (20–22). This eliminates the use of phosphoric acid or phosphate butter as solvent modifiers. Ammonium hydroxide, though volatile, at high concentration dissolves the silica packing and thus reduces life of the column (23). Hence, it is important that a minimal concentration of ammonium hydroxide be used. Use of narrow-bore HPLC columns (2 mm i.d.) could reduce the toxic solvent waste by 80% and increase the detection sensitivity as compared to regular analytical columns (4.6 mm i.d.).

This study was planned to develop an HPLC procedure for separation and quantitative determination of phospholipid classes from WPC by means of ELSD and a narrow-bore column.

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EXPERIMENTAL PROCEDURES

Reagents. Optima-grade methanol and ammonium hydroxide (20–22%), and HPLC-grade chloroform (stabilized with ethanol and not pentene) were obtained from Fisher Scientific Co. (Pittsburgh, PA). A standard kit of phospholipids that contained cerebrosides (CER), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH), and lysophosphatidylcholine (LPC) was purchased from Matreya Inc. (Pleasant Gap, PA).

Samples for analysis. Dry whey powder (WP), acid whey WPC 34% protein, and cheese whey WPC 75% protein were obtained from Commonwealth Scientific and Industrial Research Organization (Hyatt, Australia), New Zealand Milk Products (Santa Rosa, CA), and New Zealand Dairy Research Institute (Palmerston North, New Zealand), respectively. Total lipids from above dry whey products were extracted and separted into phospholipids and other lipid classes by following the procedure of Vaghela and Kilara (unpublished data). WPC 75%, WPC 34%, and WP were hydrated to a water-to-sample ratio (w/w) of 2:1, 2:1.5, and 2:2.5, respectively, and gently mixed for 15 min with a magnetic stirrer. For extraction of total lipids, 3, 4, and 5.4 g of hydrated solutions of WPC 75%, WPC 34%, and WP, respectively, were mixed with 35 mL of 1:1 (vol/vol) chloroform/methanol mixture, and homogenized at 6000 rpm for 1 min in an Omni-mixer 17150 (Sorvall Inc., Newtown, CT). The contents were transferred to a 50-mL centrifuge tube and centrifuged at 2800 × *g* for 5 min in a Mistral 3000i centrifuge (Curton Matheson Sci. Inc., Houston, TX). Supernatant was collected, and the pellet was re-extracted with 35 mL of 2:1 (vol/vol) chloroform/methanol as above. Supernatant of the second extraction was collected and pooled with the previous supernatant. Solvents from the supernatants were evaporated in a Buchi Rotavapor - R (Brinkmann Instruments, Westbury, NY) rotary vacuum evaporator at 35°C. The crude extract of lipids was vacuum-desiccated for a minimum of 6 h. Nonlipid contaminants were removed by a gel filtration procedure (24). Dry crude lipids extract was dissolved in less than 5 mL of 19:1 (vol/vol) chloroform/methanol mixture saturated with water. The contents were filtered through a sintered-glass funnel (40–60 μm pore size), and the filtrate was loaded onto a gel filtration column. Total lipids, except gangliosides, were eluted with about 50 mL of 19:1 (vol/vol) chloroform/methanol mixture saturated with water and collected in a preweighed vacuum evaporator flask. Solvents from the gel filtration eluent were evaporated in a rotary vacuum evaporator at 35°C, and the total lipids were dried completely in a vacuum desiccator for a minimum of 4 h. Total lipids were separated into phospholipids, neutral lipids, and free fatty acids with a solid-phase extraction technique. Disposable Mega Bond Elut (2 g) aminopropyl SPE columns from Varian Sample Preparation Products (Harbor City, CA) were used. A vacuum manifold with twelve individual flow control valves and stainless-steel guide needles (Supelco, Bellefonte, PA) was used to elute the solvents through the SPE columns under 10–12 kPa vacuum. The Mega Bond Elut column was placed on the vacuum manifold and washed twice with 8 mL hexane. Total lipids were dissolved in less than 2 mL chloroform and applied to the column under vacuum. Chloroform was eluted, leaving total lipids on the column. Then, the column was sequentially eluted with 18 mL of each of 2:1 chloroform/isopropyl alcohol, 2% (vol/vol) acetic acid in diethyl ether, and methanol, to separate neutral lipids, free fatty acids and phospholipids, respectively. After separation, solvents from each SPE eluent were evaporated in a rotary vacuum evaporator, followed by vacuum desiccation until constant weight (usually 4 h). Phospholipids so separated were used as samples for this study.

HPLC procedure and detection system. To use a less viscous, good resolving, and lowest possible solvent modifier concentration, the solvent system of Becart *et al.* (15) was modified. The modified method used the following binary gradient: A: chloroform/methanol, (80:20, vol/vol); B: chloroform/methanol/water/20% ammonium hydroxide, (60:34:6:0.25, by vol).

A Perkin-Elmer HPLC system with Series 410 pump and LCI-100 integrator (Perkin-Elmer, Norwalk, CT) and a Varex ELSD II A ELSD (Varex Corp., Rockville, MD) were used. A few changes in the HPLC system were made for use of the narrow-bore column. The mixing loop was removed, and all tubing after the injector was replaced with 0.125-mm i.d. tubing to reduce the delay volume. Rheodyne 1-μL internal loop injector was used (Alltech Associates, Inc., Deerfield, IL). After optimization of the parameters for the ELSD, the drift tube temperature was set at 115°C. The flow of the carrier gas (nitrogen) was set at 22 mm on the flowmeter of the detector. Separations were carried out on a 250 × 2.1 mm, 5-μm Inertsil silica column (Alltech Associates Inc.) at room temperature (23 ± 3°C). The mobile phase cycle is illustrated in Figure 1. The flow rate of the mobile phase was 0.22 mL/min with back pressure of 85 to 105 kg/cm^2. It was important to

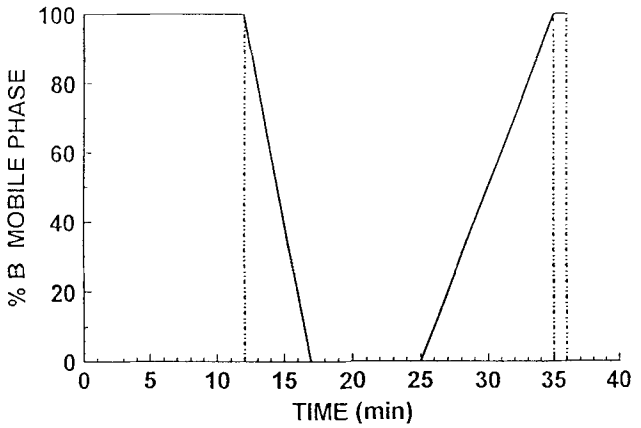

FIG. 1. Percentage of solvent B during the high-performance liquid chromatography analysis of phospholipids in a narrow-bore column with a binary gradient cycle.

observe the timings of the gradient run and column equilibration. Otherwise, significant variation in retention time can appear, which might even lead to poor resolution between some classes of phospholipids.

A response calibration curve for each class of phospholipids was prepared by injecting 1 μL chloroform solution containing 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg of the standard. Three injections for each concentration level were performed. The detection limits, which is a response of twice the noise level, for PC, PE, SPH, and CER, were determined by injecting 2 to 20 ng of each phospholipid standards. Samples of phospholipids from three different whey products were tempered at room temperature and dissolved in chloroform to give about 5 μg of total phospholipids per μL of the sample solution. One microliter of the prepared sample solution was injected with a Rheodyne 7125-675 sample injector (Alltech Associates Inc.).

RESULTS AND DISCUSSION

Initially, several reported procedures for separation of classes of phospholipids were tried to adapt them to a narrow-bore column coupled with ELSD (12,13,16). The two major problems encountered were: (i) poor resolution and peak broadening of PS and PI; and (ii) a rapid and irreversible increase in back pressure, leading to a short life of the column.

It was also observed that a solvent modifier, such as ammonium hydroxide, was essential for good resolution and peak symmetry of PS and PI. The solvent system of Becart *et al.* (15) was modified and optimized for the ammonium hydroxide concentration. The modified solvent system contained no ammonium hydroxide in solvent A, and solvent B contained less than half the concentration of the Becart *et al.* (15) solvent system. This low concentration of ammonium hydroxide improved the column life (23) and gave repeatable retention times for each class of phospholipids.

As the normal analytical HPLC system (capable of 0.1 to 10 mL/min flow rates) was modified for use with a narrow-bore column, it was necessary to calculate the delay volume, which is the amount of solvent between the mixing chamber of the pump and the injector. The delay volume becomes insignificant with normal analytical columns (4.6 mm i.d.) because the flow rate of the mobile phase is usually 1 mL/min or higher. However, with a narrow-bore column (2 mm i.d.), for an acceptable linear velocity of the mobile phase, it would require 0.2 mL/min flow rate. Hence, the delay volume becomes significant for narrow-bore columns. Modification of the HPLC system by removing the mixing loop and replacing the tubing with the shortest possible length of 0.125-mm i.d. tubing reduced the delay volume significantly. The reduction in delay volume would never be total and required adjustment in gradient timings. Figure 1 shows that the gradient starts with 100% of the more polar solvent B as against 100% of solvent A for a gradient cycle with conventional analytical columns. This is because the HPLC system that we modified for use of the narrow-bore column had a delay volume of 2.42 mL or 11 min. In other words, it took 11 min for initial solvent of the gradient to travel from the pump to the inlet end of the column. Theoretically, we injected the sample when 100% of B was being pumped; however, the actual solvent composition at the inlet of the column was that of the gradient cycle at 25 min. Thus, it is essential that after modifying the present HPLC system for a narrow-bore column, the delay volume be determined and then the solvent cycle be run accordingly. After determining the solvent cycle, it is important that each sample is strictly injected on time, or variation in retention times and/or poor resolution of the classes of phospholipid will be encountered. This also requires that the first gradient cycle be run without injection of the sample.

As mentioned in the Experimental Procedures section, chloroform used in the mobile phase must not be stabilized with pentene. Pentene is extremely volatile (b.p. 30.1°C). In the presence of ammonium hydroxide the silica from the column started dissolving rapidly, as a white mass of silica could be seen accumulated on the orifice of a nebulizer. This resulted in an unstable base line with hundreds of ghost peaks and a short life of the column. Use of chloroform stabilized with ethanol solved the problem.

Figure 2 shows the chromatogram of the test mixture that contained 1 μg of each class of phospholipid standard. All the classes of phospholipids were base-line resolved. CER, being the least polar, eluted first and gave two peaks. The first and second peaks represented CER with and without hydroxy-

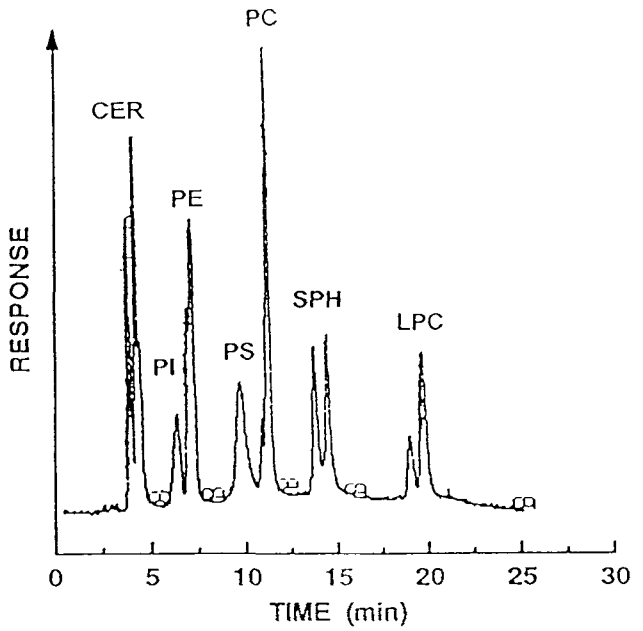

FIG. 2. Separation of a standard test mixture of seven classes of phospholipids. High-performance liquid chromatography conditions: flow rate 0.22 mL/min; Inertsil silica column (Alltech Associates, Inc., Deerfield, IL) 250 × 2.1 mm; evaporative light-scattering detector conditions: drift tube temperature: 115°C; nebulizer gas flow: 22 mm. Abbreviations: CER: cerebrosides; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PS: phosphatidylserine; PC: phosphatidylcholine; SPH: sphingomyelin; LPC: lysophosphatidylcholine.

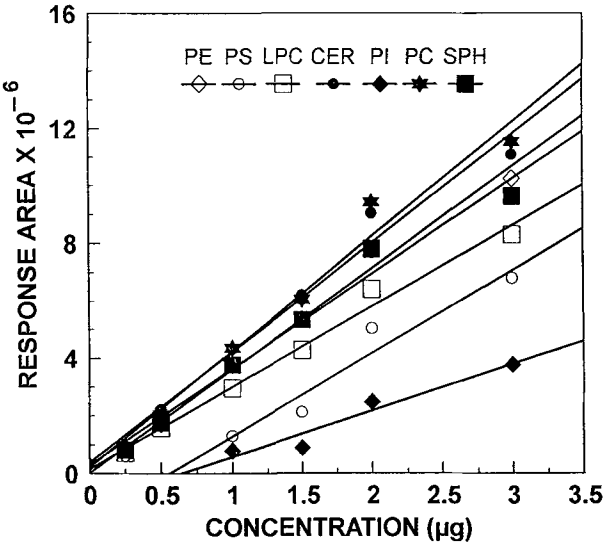

FIG. 3. Calibration curves for classes of phospholipids. See Figure 2 for abbreviations.

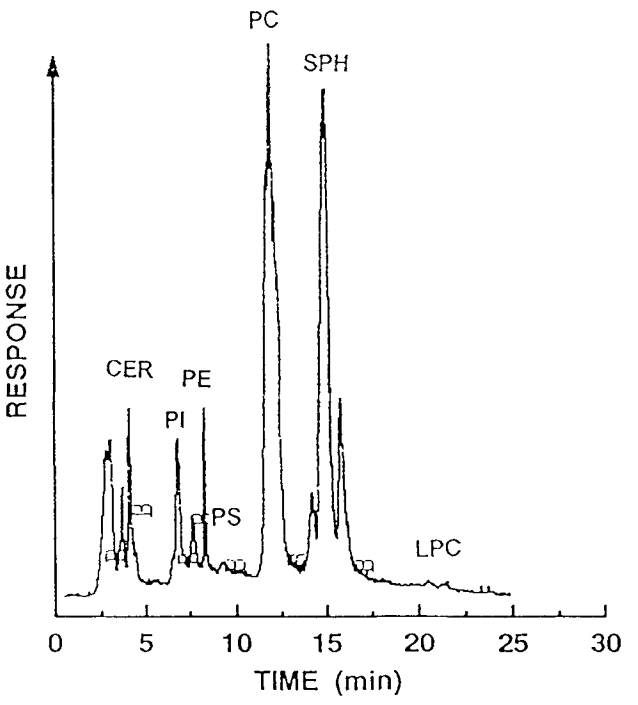

FIG. 4. High-performance liquid chromatography (HPLC) chromatogram of phospholipids from whey protein concentrates. HPLC conditions: flow rate 0.22 mL/min; Inertsil silica column 250 × 2.1 mm; evaporative light-scattering detector conditions: drift tube temperature: 115°C; nebulizer gas flow: 22 mm. See Figure 2 for abbreviations and Inertsil company source.

lated fatty acids, respectively (15). SPH and LPC also gave two peaks each. Figure 3 shows the calibration curves for different phospholipid classes. The responses were linear for PC, PE, SPH, LPC, and CER (with $R^2 = 0.99$) in the concentration range of 0.25 to 3.0 µg, and for PI and PS in the range of 1 to 3 µg. The detection limit for PC, SPH, PE, and CER in the narrow-bore column was 10 ng, as against 100 ng reported for PC with an analytical column (15). Detection sensitivity was improved, due to both lower dilution of the sample with mobile phase in the narrow-bore column and improved sensitivity of the ELSD with lower flow rate of the mobile phase as compared to the conventional analytical column.

The HPLC chromatogram of the phospholipids from WPC is shown in Figure 4. Again, all the classes of phospholipid were base-line resolved, and nonpolar lipids eluted as the first peak preceding the CER peaks. PE and SPH gave two and three peaks as against one and two peaks for the standards. This could be due to the fact that standards were not derived from milk but from egg and plant sources. Data presented in Table 1 shows each phospholipid class as percent of total phospholipids from WP, WPC-75, and WPC-34. Although all three dry whey products were manufactured from different whey sources, they contained statistically similar proportions of all phospholipid classes, except for CER and PE. WPC-34 and WP contained similar proportions of PE, while WPC-75 contained almost double the proportion of PE. There are no data for classes of phospholipids from WPC in the literature for comparison. However, when these results were compared with the proportion of phospholipid classes in milk (25), proportions of PC and SPH were comparable, PI and PS were higher, and PE was lower for all three whey products analyzed.

TABLE 1
Composition of Phospholipids from Dry Whey Products

Classes of phospholipid	[% (w/w) of the total phospholipids]		
	WPC-75	WPC-34	Whey powder
Cerebrosides	3.57 ± 0.13[a]	2.87 ± 0.35[a]	3.97 ± 0.41[a]
Phosphatidylinositol	18.13 ± 1.23[a]	21.95 ± 2.51[b]	18.41 ± 1.85[a]
Phosphatidylethanolamine	4.45 ± 0.21[a]	2.21 ± 0.18[b]	1.94 ± 0.17[b]
Phosphatidylserine	7.48 ± 0.58[a]	9.56 ± 1.66[a]	8.35 ± 0.64[a]
Phosphatidylcholine	30.54 ± 1.84[a]	31.59 ± 1.15[a]	33.34 ± 1.11[a]
Sphingomyelin	35.82 ± 1.16[a]	31.83 ± 1.98[a]	33.99 ± 2.01[a]
Lysophosphatidylcholine	ND[c]	ND	ND

[a,b]Reported values are means ± standard deviations. Values within a row followed by a different letter are significantly different ($P < 0.05$); WPC, whey protein concentrates.
[c]Not detected.

In conclusion, a narrow-bore column, in combination with an ELSD and ammonium hydroxide as a solvent modifier, allows rapid, repeatable base-line separation of all classes of milk phospholipids. Detection sensitivity was improved by tenfold or more, and solvent consumption was reduced by 80%. Use of ammonium hydroxide as a solvent modifier was essential for sharp peaks and base-line resolution of the acidic phospholipids. However, at high concentration, it also slowly dissolved silica from the column and thus shortened column life. Concentration of ammonium hydroxide required in the mobile phase as a solvent modifier in this procedure was less than half of the reported levels and thus improved the column life significantly.

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