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Analysis of Phospholipids in Infant Formulas Using High Performance Liquid Chromatography−Tandem Mass Spectrometry

Bertram Fong,* Lin Ma, and Carmen Norris

Fonterra Research Centre, Dairy Farm Road, Private Bag 11029, Palmerston North 4412, New Zealand

ABSTRACT: Phospholipids have been used widely in the food industry as emulsifiers, but it is their biological and nutritional function that has been the focus over recent years. The recognition of the importance of phospholipids and sphingomyelin for infant development has led to an increase in the number of infant formulas claiming to contain these complex lipid components. Therefore, the ability to measure these lipids in infant formulas and dairy-derived complex lipid ingredients for fortification purposes is important. A high-performance liquid chromatography−tandem mass spectrometry method that quantifies phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin found in infant formulas and dairy-derived complex lipid ingredients is described. The method uses external standards of similar fatty acid profile for calibration. The recovery of phospholipids ranged from 92% to 102% with a method repeatability coefficient of variation of 6−10%. In addition to the specificity and selectivity of the method, details of the molecular species in the individual phospholipid classes are available using this method.

KEYWORDS: *phospholipids, infant formulas, HPLC−MS/MS*

INTRODUCTION

Phospholipids (PLs) and sphingolipids are classes of polar lipids that are recognized to play important roles in cell membrane integrity and other biological functions such as cell signaling, cell proliferation, brain development, and important components of the inflammatory response.[1−8] In milk, PLs form part of the outer membrane that surrounds and stabilizes the milkfat globule in solution and also provides an important nutrition source for the growing infant. The recognition of the importance of PLs for infant growth[4,9] has led to the development of infant formulas claiming to contain these complex lipid components. It is therefore important to be able to measure these PL components not only in the complex lipid ingredient but also in the final formula for formulation work and label claim verification purposes.

Numerous publications identify methodologies that have been used to characterize and quantify PLs in both biological and food matrixes. These extend from the more traditional thin layer chromatography (TLC) method to more sophisticated techniques such as mass spectrometry (MS).

TLC, and subsequently high-performance TLC, was one of the earliest techniques that was used for PL characterization and quantification. Both these techniques are still used today for the quantification of different PL classes, either alone[10−13] or in conjunction with more sophisticated techniques such as MS.[14−16] However, TLC−MS methods are time-consuming because the PL bands must be extracted from the TLC plates and re-extracted into an appropriate solvent before MS analysis.

^{31}P nuclear magnetic resonance (NMR) determines the mole amount of phosphorus from a PL species, and the PL concentration is calculated using an average molecular weight of the PL species from that source. This method has been used to measure PLs in various food matrixes[17−19] and recently used to measure PLs in dairy-derived complex lipid ingredients[20] with minimal sample preparation. The reconstituted samples were analyzed without any need for prior solvent extraction, which is typical for TLC and high-performance liquid chromatography (HPLC) methods. However, the lower sensitivity of the ^{31}P NMR method means that, for the analysis of PLs in infant formulas, a solvent extraction step of the lipids is still required to enrich the PLs before ^{31}P NMR analysis.

HPLC methods coupled to ultraviolet (UV) detection for the separation and quantification of PLs have been used,[21,22] but by far the most commonly reported analytical method for PL quantification is HPLC coupled to either MS or an evaporative light-scattering detector (ELSD) for detection. LC separation coupled to an ELSD is probably the most extensively used method for PL analysis in food including dairy products and infant formulas.[23−35] The PLs are typically separated using normal phase chromatography, or more recently by hydrophilic interaction liquid chromatography and cyanopropyl chromatography, followed by ELSD detection. The nonlinearity of the ELSD creates complicated calibrations with linearity over only small concentration ranges.[26,30,36−39]

A Corona charged aerosol detector (CAD) has also been reported for use with PL analysis.[40] The detector gives a response that is independent of the chemical structure of the analyte, which is an advantage for PLs because multiple species within each class of PLs are measured. The detector has also been reported to have three times the sensitivity compared with an ELSD, but the response is generally nonlinear with a second-order polynomial fit for PLs.[40]

HPLC−MS has also been used extensively for both the quantification and the characterization of PLs in biological matrixes.[41−43] This method has been well reviewed by Peterson

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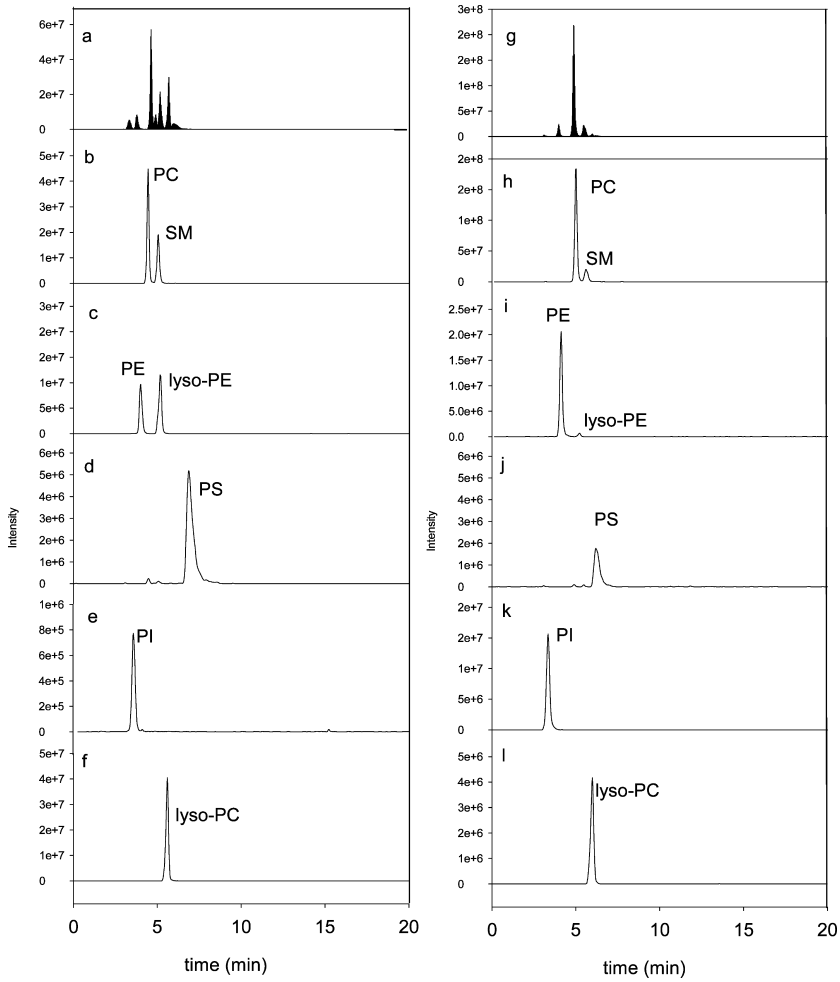

Figure 1. A typical total ion current (TIC) trace of a phosholipid standard mixture (a) and infant formula (g), followed by the extracted ion chromatograms for each phospholipid (b–f) and (h–l), respectively.

and Cummings,[44] and the PL species in food matrices including dairy products have been characterized.[30,45−52]

The advantage of the MS detection of PLs is that it offers greater specificity, selectivity, and, in many cases, sensitivity than UV, ELSD, ^{31}P NMR, and CAD techniques; in addition, it also provides molecular species information, unlike ^{31}P NMR. In this study, we validated our previously reported HPLC−MS method[53] for the quantification of five PLs (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS)) and sphingomyelin (SM) in infant formulas and PL-enriched dairy ingredients using external calibration standards that were sourced naturally. To our knowledge, this is the first time that LC−MS/MS has been used to quantify PLs in infant formulas or specialty dairy ingredients.

MATERIALS AND METHODS

Standards and Chemicals. Naturally sourced external calibration standards: PC and PI (both from bovine liver), PE (from bovine heart), and SM (from bovine milk) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) while PS standard (from bovine brain) was purchased from Sigma-Aldrich (St. Louis, MO). Synthetic PC standards for the MS response study: 1-palmitoyl-2-myristoyl-*sn*-glycerol-3-phosphocholine, 1-palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine, and 1-palmitoyl-2-stearoyl-*sn*-glycerol-3-phosphocholine were purchased from Avanti Polar Lipids Inc. (Alabaster, AL), while the dipalmitoyl-glycerol-3-phosphocholine was purchased from Sigma-Aldrich (St. Louis, MO). All solvents used were of LC grade (Merck, Darmstadt, Germany), except for chloroform which was analytical grade (ethanol stabilized).

Samples. All infant formulas were commercial whey-protein-dominant powder (for infants aged 0−6 months) samples except for infant formula 1 (IF1), which was for infants aged 0−3 months and contained bovine colostrum powder. The homogenized milk sample (3.5% fat) was also a commercial sample. The complex lipid ingredient samples (buttermilk powder concentrate (BPC50), G600, and PC700) were proprietary polar lipid ingredients that are manufactured from milk by Fonterra Co-operative Group Ltd. (New Zealand).

Sample Preparation. All powder samples were rehydrated in water prior to lipid extraction to give a solids content of 30% w/v for infant formulas and 0.25% w/v for PL-enriched ingredients (G600, BPC50, and PC700). Liquid samples were extracted directly. Lipid extraction was carried out using the Svennerholm and Fredman[54] extraction protocol. Briefly, 0.5 mL of the sample was extracted using a chloroform/methanol mixture twice, and the lower organic phase layer was pooled and made up to 10 mL with chloroform/methanol (1:2). Prior to HPLC−MS/MS analysis, the samples were diluted a further 10 times for IF and 40 times for the PL-enriched ingredients.

External Calibration Standard Preparation. PL standard stock and intermediate solutions were prepared at 1 mg/mL and 20 μg/mL, respectively, in chloroform/methanol (1:2) and stored at −80 °C under nitrogen. Serial dilutions of the intermediate solution in chloroform/methanol (1:2) were made to give a six-point calibration curve for each PL class.

HPLC–MS/MS. HPLC–MS/MS was carried out on an ACQUITY UPLC system (Waters, Milford, MA) interfaced to a tandem quadrupole mass spectrometer (TSQ Quantum Ultra EMR, Thermo Scientific, Waltham, MA) via a heated electrospray ionization source. The PLs were separated on an APS-2 Hypersil hydrophilic column (150 mm × 2.1 mm, 3 μm, ThermoFisher Scientific, Waltham, MA) with MS detection. The HPLC–MS/MS detection of the PLs was based on precursor ion or neutral losses that occurred during fragmentation, hence allowing the summation of molecular species within each PL class for quantification. The HPLC–MS/MS run conditions are described in detail by Norris et al.[50]

Method Validation. The accuracy of the method was evaluated by performing recovery studies. The between-day precision and the within-day precision were assessed by repeated analysis of an infant formula (IF1) sample. Recovery experiments, in which the reconstituted IF1 sample was spiked with PL at 100% of endogenous PL levels, were also carried out. The accuracy of the HPLC–MS/MS method was also assessed against a ^{31}P NMR method[17] using a selected number of infant formula samples, BPC50, and the PC700 complex lipid ingredient sample.

RESULTS AND DISCUSSION

HPLC–MS/MS. The separation of the bovine PLs according to headgroup was achieved using the conditions described by Norris et al.[53] Typical HPLC–MS traces of a calibration mixture and an infant formula sample are shown in Figure 1. Although relatively sharp peaks were obtained for PI, PE, and PC, the PS and SM peaks in the IF were significantly broader because of the partial separation of the individual PS and SM species, with longer chain fatty-acid-containing species eluting later than shorter chain fatty-acid-containing species, as has been previously discussed.[53] Although we were able to separate and identify the lyso-PL products using this method (Figure 1), we did not quantify them in this study.

Ionization Efficiencies. The MS response, including fragmentation and neutral loss efficiencies of different molecular species within a class of PL, has been shown to decrease with increasing acyl chain length[55–57] and to increase with unsaturation, the latter being more pronounced at higher lipid concentrations.[57,58] Conversely, Buyukpamukcu et al.[46] found that different PC molecular species at equimolar concentration gave the same MS response irrespective of the degree of unsaturation and the acyl chain length. The nature of the phospho-headgroup has been reported to have a greater influence than either the acyl chain length or the degree of unsaturation on the ionization efficiency of PLs.[59,60]

In our study, we found that PC had the highest MS response, with PI having the lowest response on an equimolar basis (Figure 2). Using a limited number of synthetic PC standards, we were able to confirm that the choline precursor loss response (m/z 184) was also influenced by the fatty acid acyl chain length and the degree of unsaturation (Figure 3). However, the influences of acyl chain length and unsaturation were only significant at high PC concentrations, in the nonlinear region of the response curve. Below a concentration of 10 nmol/mL, the molar response did not appear to be significantly influenced by either the acyl chain length or the degree of unsaturation (Figure 3). Although our data is limited to PC, these findings are consistent with those of Koivusalo et al.[58] and Delong et al.,[60] who reported that the effects of acyl chain length and unsaturation on the MS response are significantly reduced with dilution, with a recommended linear range up to 5 nmol/mL. Although we investigated only PC in our study, as reported by Koivusalo et al.[58] and Delong et al.,[60] we expected the other PLs to behave in a similar fashion.

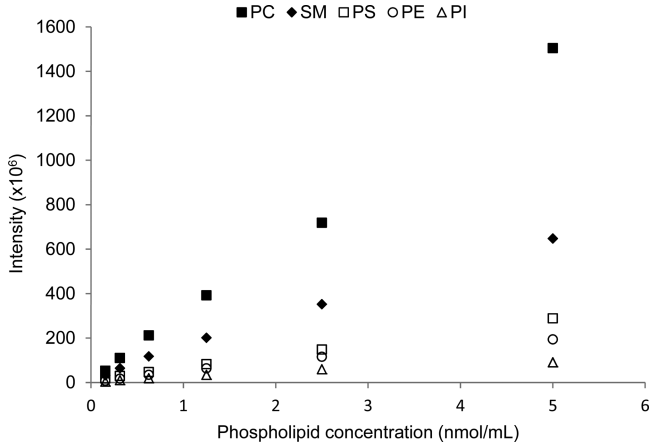

Figure 2. Typical PL calibration curves generated using naturally sourced standards containing a mixture of PL molecular species (Figure 4). The linear regressions (r^2) were typically above 0.9. The different PL MS response is due to different neutral loss and precursor scan events used for detection.[53]

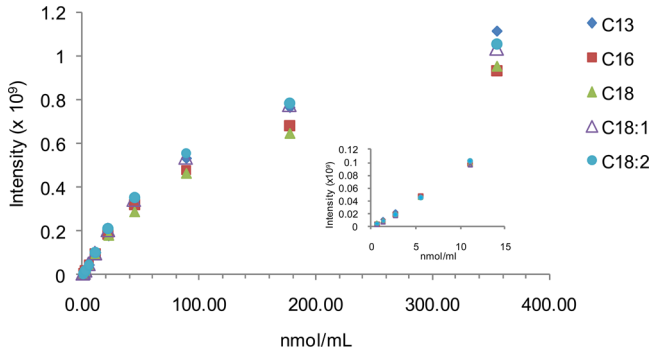

Figure 3. The effect of the acyl fatty acid chain length and the degree of unsaturation of PC on its MS reponse. Five different synthetic PC molecular species, containing C16:0/C13:0, C16:0/C16:0, C16:0/C18:0, C16:0/C18:1, and C16:0/C18:2 fatty acids in the *sn*-1 and *sn*-2 positions repectively were elevated. The inserted graph is the expanded concentration region from 0 to 10 nmol/mL.

To ensure minimal response variation, as caused by acyl chain length or degree of unsaturation, our six-point calibration curve ranged from 5 to 0.16 μg/mL (approximately from 5.5–6.5 to 0.2 nmol/mL) for each of the five PLs. In addition, we endeavored to use naturally sourced PL calibration standards that had molecular ions and relative abundances that most closely matched those present in our samples. Typical mass spectra showing the distribution of the PL molecular ions in a standard and in samples is provided in Figure 4.

Method Validation. *Matrix Effects.* Matrix effects are one of the major concerns with MS; components in the sample matrix can suppress the ionization efficiency of the analyte. In this study, we evaluated the presence of matrix effects by comparing the infant formula results obtained using the described method with those obtained using standard addition, in which standard solutions are spiked into the sample matrix at increasing concentration within the calibration range. For infant formula, the standard addition results (Table 1) were not significantly different from the results obtained using an external standard curve, which gave us confidence that we were not getting any significant matrix suppression effects that affected the total amount of PLs measured during the

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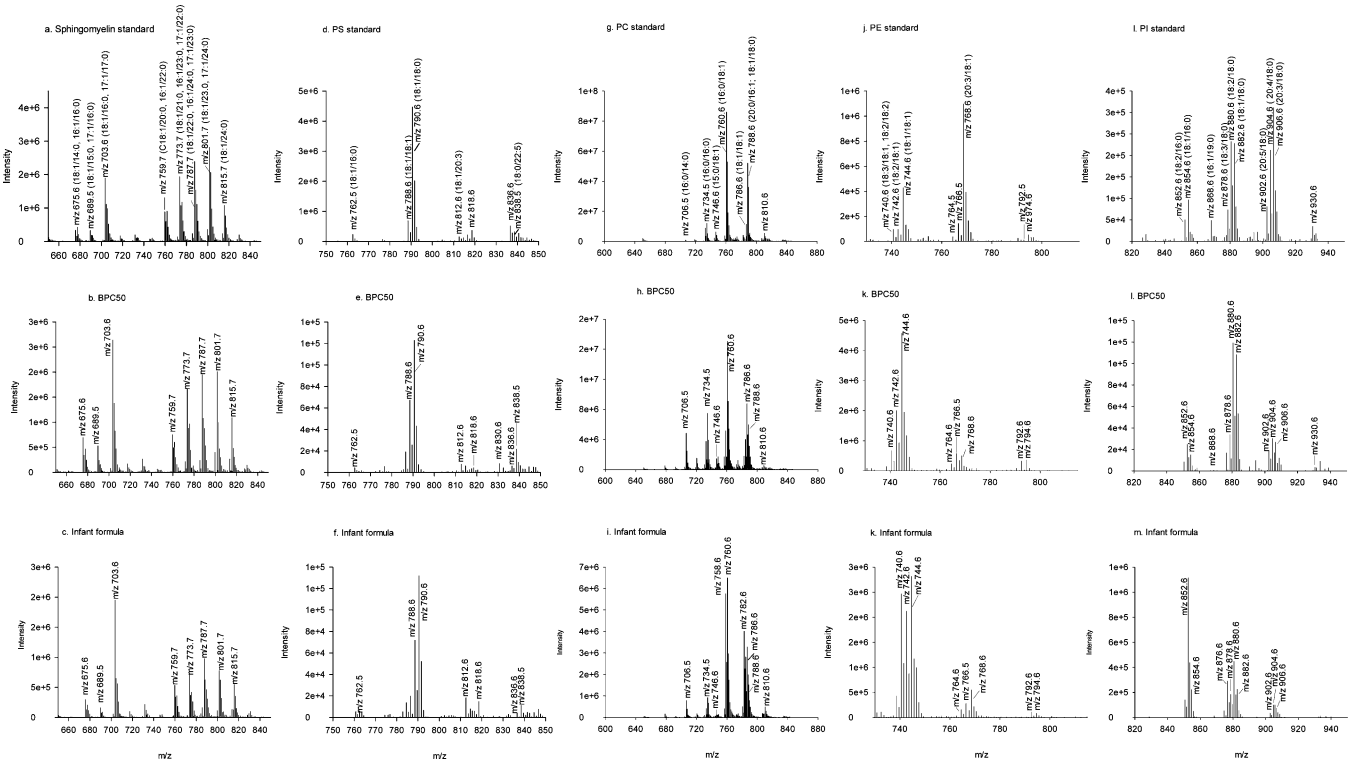

Figure 4. A typical PL mass spectrum showing the molecular ion distribution for the calibration standard (a), BPC50 (b), and infant formula 1 samples. Some of the dominant molecular ions are also tentatively identified with the possible fatty acid composition based on data reported by Fong et al.[45] Note that the PI molecular ions were present as M + NH_4^+/H^+ adducts.

Table 1. Comparison of PL Concentration Determined by the Standard Addition Technique with That Determined by the Current External Standard Method, and Recoveries of PL Standards Spiked into the IF1 Sample

	PC	PE	PI	PS	SM
IF1	2.53 ± 0.25	1.43 ± 0.11	0.6 ± 0.1	0.26 ± 0.03	1.25 ± 0.08
standard addition (n = 2)	2.41 ± 0.06	1.46 ± 0.04	0.71 ± 0.01	0.28 ± 0.1	1.36 ± 0.07
recovery (%), (n = 4)	98 ± 7 (7, 4)	97 ± 4 (4, 4)	93 ± 11 (11, 4)	92 ± 7 (8, 4)	102 ± 2 (2, 8)

Table 2. PL Concentration in Various Commercial Infant Formulas[a,b]

sample	PC	PE	PI	PS	SM
infant formula 1	2.53 ± 0.25 (9.7, 50)	1.43 ± 0.11 (7.6, 50)	0.6 ± 0.1 (10, 25)	0.26 ± 0.03 (10, 50)	1.25 ± 0.08 (6, 50)
infant formula 2	0.79 ± 0.02 (2, 2)	0.71 ± 0.01 (1, 2)	0.44 ± 0.03 (8, 2)	0.28 ± 0.02 (2, 6)	0.82 ± 0.004 (0.5, 2)
infant formula 3	0.74 ± 0.02 (3, 2)	0.64 ± 0.01 (2, 2)	0.40 ± 0.03 (6, 2)	0.23 ± 0.01 (3, 2)	0.74 ± 0.01 (2, 2)
infant formula 4	0.71 ± 0.05 (6, 2)	0.75 ± 0.01 (2, 2)	0.26 ± 0.01 (2, 2)	0.19 ± 0.01 (4, 2)	0.63 ± 0.01 (1, 2)
infant formula 5	0.63 ± 0.0 (0, 2)	0.60 ± 0.04 (6, 2)	0.26 ± 0.02 (9, 2)	0.18 ± 0.01 (6, 2)	0.61 ± 0.03 (5, 2)
infant formula 6	0.81 ± 0.07 (8, 7)	0.67 ± 0.05(8, 7)	0.34 ± 0.03 (8, 7)	0.27 ± 0.02 (7, 7)	0.59 ± 0.03 (5, 7)
^{31}P NMR (n = 2)	0.74 ± 0.02	0.63 ± 0.01	0.28 ± 0.01	0.22 ± 0.00	0.49 ± 0.01
infant formula 7	0.84 ± 0.03 (9, 8)	0.62 ± 0.03 (5, 8)	0.46 ± 0.03 (7, 8)	0.13 ± 0.01 (10, 8)	0.31 ± 0.02 (6, 8)
^{31}P NMR (n = 2)	0.82 ± 0.00	0.61 ± 0.02	0.41 ± 0.01	0.14 ± 0.00	0.27 ± 0.02
infant formula 8	0.73 ± 0.0 (0, 2)	0.61 ± 0.01 (2, 2)	0.36 ± 0.04 (11, 2)	0.23 ± 0.01 (3, 2)	0.50 ± 0.03 (7, 2)
^{31}P NMR (n = 1)	0.75	0.63	0.31	0.20	0.47

[a]Selected samples were also analyzed using the ^{31}P NMR method. [b]The coefficient of variation followed by n is provided in parentheses: units, mg/g ± standard deviation.

electrospray ionization process of the PLs present in the samples.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD and LOQ were estimated by loading decreasing amounts of the standard onto the HPLC−MS system until the peak signal was approximately 3−4 times and 10 times the signal of the noise level, respectively. The estimated LOD was 100 pg for PI and PS, 50 pg for PE, 24 pg for SM, and 12 pg for PC. The LOQ was estimated at 230 pg for PI and PS, 100 pg for PE, 50 pg for SM, and 25 pg for PC.

Recovery. Spiked recovery experiments using infant formula samples were also conducted. The recovery of 92−102% (Table 1) gave us confidence that the Svennerholm and Fredman extraction protocol[54] (variation of the traditional Folch extraction method[61]) that we adopted was effective in recovering the PLs from the sample matrix.

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Table 3. PL Concentration in Various PL-Enriched Dairy-Derived Complex Lipid Ingredients and Homogenized Milk[a,b]

sample	PC	PE	PI	PS	SM
PC700	191 ± 17 (9, 4)	169 ± 7 (4, 4)	16 ± 0.2 (1, 4)	24 ± 1 (5, 4)	161 ± 10 (7, 4)
^{31}P NMR (n = 2)	182 ± 1	161 ± 2	21 ± 3	24 ± 1	153 ± 3
BPC50	44 ± 4 (8, 4)	44 ± 3 (7, 4)	9 ± 0.3 (3, 4)	21 ± 1 (2, 4)	36 ± 3 (8, 4)
^{31}P NMR (n = 2)	42 ± 0.2	43 ± 0.4	12.9 ± 0.1	18 ± 0.1	35 ± 0.9
G600	21.4 ± 1 (3, 2)	40.8 ± 0.1 (0.2, 2)	28.3 ± 0.4 (1, 2)	49.6 ± 0.8 (2, 2)	25.8 ± 0.7 (3, 2)

[a]Selected samples were also analyzed using the ^{31}P NMR method. [b]The coefficient of variation followed by n is provided in parentheses: units, mg/g ± standard deviation.

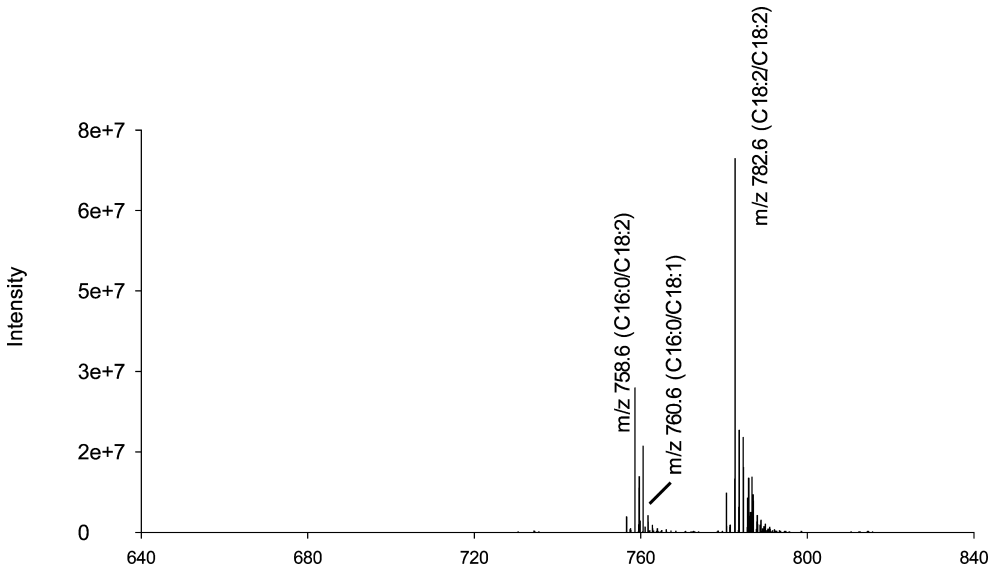

Figure 5. A typical PC mass spectrum showing the molecular ion distribution for the soya lecithin. The various molecular species are also tentatively identified with the possible fatty acid composition based on data reported by Le Grandois et al.[62].

Method Repeatability. The overall method repeatability was assessed by the repeated analysis of one infant formula sample over a 3-month period. The coefficient of variation ranged from 6% to 10% (Table 2) across the different PL classes. The typically lower PI and PS concentrations in the infant formula samples meant that we generally found high coefficients of variation associated with these values (Table 2).

Comparison with the ^{31}P NMR Method. To further validate the method, we compared the PL results obtained using our HPLC–MS/MS method with those obtained using the ^{31}P NMR method[17] for three infant formulas (Table 2) and two complex lipid ingredients (PC700 and BPC50, Table 3). The ^{31}P NMR method was carried out by an independent external laboratory. The PC700 and BPC50 ingredients were analyzed without any further sample extraction, but the infant formula samples had to be extracted using the Svennerholm and Fredman extraction protocol,[54] and the PLs were further enriched by the removal of neutral lipids using solid-phase extraction prior to ^{31}P NMR. Overall, although the HPLC–MS/MS method tended to measure slightly higher results than the ^{31}PNMR method, these differences were not statistically significant ($p < 0.05$), except for PI, where the HPLC–MS/MS values were significantly lower for the two complex lipid ingredient samples (PC700 and BPC50). It is possible that the lower PI recovery (93%, Table 1) may contribute to this difference,

PL Concentration in Complex Lipid Ingredients. PC700, BPC50, and G600 are proprietary ingredients (Fonterra Co-operative Group Ltd., New Zealand) that are enriched in PLs. The PL levels in PC700 and BPC50 were also determined by ^{31}P NMR, as discussed above (Table 3). The PC700 sample was the most PL-enriched sample that we analyzed, containing up to 60% w/w PL, followed by G600 (16.5% w/w) and BPC50 (15% w/w). Our standardized milk sample contained approximately 0.037% w/v PLs whereas the PL level reported in the literature for raw milk is in the range 0.021–0.035% w/v.[11,23,25,28,30,31,35] Although these enriched complex lipid ingredients were sourced from milk, their PL distributions did not reflect that found in milk. This is due to selective extraction of the PLs during the enrichment process. For example, the PC700 ingredient contained less PS and PI relative to the other PL classes when compared with milk.

Phospholipid Concentration in Infant Formulas. Overall, our results indicate that the PL concentration in infant formulas varied significantly across the different brands of infant formula powders analyzed (Table 2). With the exception of IF1, PC ranged from 0.63 to 0.84 mg/g, PE ranged from 0.61 to 0.75 mg/g, PI ranged from 0.26 to 0.46 mg/g, PS ranged from 0.13 to 0.28 mg/g, and SM ranged from 0.31 to 0.82 mg/g. These results are consistent with the available PL data reported by Braun et al.[27] Much lower PL results were reported in earlier infant formulas by Kynast and Schmitz[21] (PC, 0.037–0.12 mg/g; PE, 0.034–0.07 mg/g; PI, 0.015–0.05 mg/g; SM, 0.067–0.074 mg/g). IF1 in our study had significantly higher PC, PE, PI, and SM concentrations than all other infant formulas analyzed in this study (Table 2), probably because of the added colostrum powder in this sample (as indicated).

Examination of the mass spectra for each of the PL classes showed that the PC in the infant formulas contained relatively higher levels of m/z 758.6 and m/z 782.6 molecular species (Figure 4) compared with those found in the standards and the dairy-derived complex lipid ingredient samples analyzed. These PC molecular ions have been tentatively identified as fatty acid combinations C16:0/C18:2 (or much less likely C16:1/C18:1) and C18:2/C18:2, respectively, and are PC molecular species that are commonly found in soya lecithin (Figure 5).[62] Infant formulas are generally instantized using soya lecithin.[62]

PLs in Milk. Finally, we were also able to apply the HPLC−MS/MS method to the analysis of PLs in homogenized milk. The PL composition of a single commercial homogenized milk (3.5% fat) was measured with a PC, PE, PI, PS, and SM concentration of 0.09 ± 0.004, 0.09 ± 0.01, 0.03 ± 0.01, 0.05 ± 0.003, and 0.11 ± 0.005 mg/mL, respectively. In raw milk, the PL concentration ranges reported in the literature[11,23,25,28,30,31,35] were 0.06−0.08, 0.03−0.12, 0.013−0.014, 0.01−0.02, and 0.05−0.07 mg/mL, respectively (where %PLs were converted to mg/mL based on PL data provided by respective authors). This shows in principle that this method can be extended to analysis of PLs in liquid milk samples.

In summary, we have described an HPLC−MS/MS method for the separation and quantification of PLs in infant formulas and dairy-derived lipid ingredient samples. In addition to its sensitivity and specificity, this method also allows the analyst to examine the PL molecular ions under the PL peaks and perhaps to deduce the fatty acid composition of these molecular ions and allow identification of sources of PLs. Other PL species such as lyso-PC and lyso-PE can be added to the quantification list provided appropriate standards are used. The method has proven to be sufficiently robust on equipment that is becoming routinely available.

AUTHOR INFORMATION

Corresponding Author

*Phone: +64 6 3504649. Fax: +64 6 356 1476. E-mail: bertram.fong@fonterra.com.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

^{31}P NMR, ^{31}phosphorus nuclear magnetic resonance; CAD, charged aerosol detector; ELSD, evaporative light-scattering detector; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin layer chromatography; UV, ultraviolet

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