Phospholipid analysis in whey protein products using hydrophilic interaction high-performance liquid chromatography-evaporative light-scattering detection in an industry setting

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ABSTRACT

The main objective of this work was to develop an analytical method that can be used in a dairy manufacturing facility for the quantitation of phospholipids in dairy products. Total lipids from a dairy matrix were obtained first by Folch extraction. The total lipid extract was then applied to a silica gel-based solid-phase extraction column, and triglycerides and other nonpolar lipids were separated from the phospholipids and sphingolipids. Quantitation was performed by hydrophilic interaction HPLC coupled to evaporative light-scattering detection using a quaternary separation method. The method was validated using a commercial whey protein phospholipid concentrate and was used to analyze phospholipid and sphingolipid composition in buttermilk, whey protein concentrate, whey protein phospholipid concentrate, and several other dairy ingredients. This method was sensitive and reproducible and can be used in the dairy industry as a research tool to develop new value-added dairy phospholipid products, then later as a standard protocol for quality assurance analysis of current and future products.

Key words: whey protein concentrate, whey protein phospholipid concentrate, whey protein phospholipid concentrate, sphingolipid, hydrophilic interaction high-performance liquid chromatography-evaporative light-scattering detection

INTRODUCTION

Milk fat globule membrane (MFGM) is used to compartmentalize triglycerides in the secretory cells of mammary glands. This membrane consists of 3 layers

of polar lipids (PL) and membrane-associated proteins. Fat droplets originate in the endoplasmic reticulum of the cell where they gain the first layer of PL. Then, once the lipid vesicles reach the apical end of the cell to be secreted as part of milk, a true phospholipid bilayer with membrane-associated proteins is added to the surface as the droplet “buds” through the cell membrane into the alveolar lumen (Rombaut and Dewettinck, 2006; Holzmüller and Kulozik, 2016).

The MFGM PL include 2 major groups: phospholipids and sphingolipids (Dewettinck et al., 2008). The major PL of importance in the dairy industry are glucosylceramide, lactosylceramide, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM; Rombaut et al., 2005). The nature of these PL is amphiphilic, which allows them to act as emulsifiers between the fat droplets and aqueous serum phases of milk (Contarini and Povolito, 2013). It is because of this functional property that PL have been of interest to the dairy industry as a dairy-based emulsifier. In addition to PL functional properties, the food industry also has great interest in associated nutritional properties and health benefits of polar lipids (Dewettinck et al., 2008; Spence et al., 2009b).

During the processing of milk, PL are concentrated into the cream and fat dominant fractions; thus, a large portion of research on dairy PL has been focused on cream, or buttermilk and buttermilk products (Astaire et al., 2003; Fong et al., 2007; Spence et al., 2009a; Gallier et al., 2010; Le et al., 2011; Barry et al., 2017a; Bourlieu et al., 2018). During the cheesemaking process, lipid droplets in milk are disrupted, which results in MFGM fragmentation and the transfer of PL into whey. The PL species in whey products were investigated by Vaghela and Kilara (1995), Boyd et al. (1999), and Levin et al. (2016).

The dairy industry has made great efforts to isolate and concentrate PL from various dairy processing streams, including cream washing and centrifugation

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(Holzmüller and Kulozik, 2016), membrane filtration, supercritical fluid extraction (Astaire et al., 2003; Spence et al., 2009a), enzymatic hydrolysis of proteins, and combinations of the former (Barry et al., 2017a). Research and development efforts are continuing to develop commercial processes for PL-enriched ingredients and products. In all of these endeavors, sensitive and reliable analytical methods are needed for PL determination.

Many analytical methods for the quantitation of PL have been developed over the years including thin-layer chromatography-densitometry (Bitman and Wood, 1990), HPLC (Rombaut et al., 2005; Russo et al., 2013), MS (Donato et al., 2011; Fong et al., 2013), and ^{31}P nuclear magnetic resonance (Donato et al., 2011; Fong et al., 2013). Thin-layer chromatography-densitometry is a semiquantitative method at most, and it cannot be used for quality control purposes. Use of ^{31}P nuclear magnetic resonance for detection of PL is an excellent research tool; however, it is a specialized technique not viable in a dairy industry setting due to high maintenance cost and required expertise. Likewise, MS is expensive, high maintenance, and requires expertise to operate.

The dairy industry needs an analytical procedure for PL detection using common techniques already present in the day-to-day routine testing laboratory. The technique of HPLC is already implemented in the dairy industry, specifically among whey processors, for the analysis of proteins and milk sugars. Many published HPLC methods in literature are difficult to recreate and apply directly as plug-and-play solutions for beginning analysis of PL in an industry setting. These methods normally require some degree of modification for acceptable results on the specific system being used. Additionally, methods such as those used by Spence et al. (2009a) involve the heavy use of chloroform, which is uncommon in dairy industry laboratories, poses new hazards in the laboratory environment, and requires additional chemical waste disposal.

In this study, an analytical method was developed and validated for the analysis of PL in multiple dairy products applicable to an industry setting, while minimizing the requirement for halogenated solvents. Chloroform extraction of lipids is a valuable tool providing quantitative recovery that we chose not to exclude from the study; however, modification to reduce solvent demand was necessary. The methods presented here include solvent extraction of PL from several dairy matrices, sample purification, and analysis by HPLC coupled with evaporative light-scattering detection (ELSD) including intermediate precision conducted among several industry scientists. This method can be

easily implemented in the dairy industry for product development and quality control purposes.

MATERIALS AND METHODS

Chemicals and Reagents

Chloroform, 2-propanol, methanol, n-hexane, and diethyl-ether were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and were of HPLC grade. Phospholipids phosphatidyl glycerol (PG), PI, PE, PS, PC, and SM were purchased from Sigma-Aldrich Chemical Company. Ammonium formate and acetonitrile were of chromatographic grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Whey protein concentrate 34 (WPC34) and whey protein phospholipid concentrate (WPPC) samples were supplied by Agropur Dairy Cooperative (Appleton, WI) and met American Dairy Product Institute standards. Extra-grade buttermilk powder was purchased from Dairy America (Fresno, CA), liquid refined soy lecithin and de-oiled soy lecithin powder were purchased from The Solae Company (St. Louis, MO), and de-oiled sunflower lecithin powder was purchased from Sternchemie Lipid Technology Company (Hamburg, Germany).

Lipid Extraction

Lipids were extracted from samples using the Folch method (Folch et al., 1957), modified for a smaller volume. Samples were weighed to load 0.1 g of fat in the extraction based on total fat content determined by the Mojonnier method (AOAC 989.05, AOAC International, 2016) or material composition data provided by the manufacturer for commercial samples. A 60- μL aliquot of 25 mg/mL PG in chloroform:methanol (2:1, vol/vol) was added to the sample as a surrogate standard. An aliquot (1.8 mL) of 125 mM sodium chloride solution was added and mixed on a laboratory vortex until uniform. A 12-mL volume of chloroform:methanol (2:1, vol/vol) was added to the mixture and vigorously mixed. The suspension was centrifuged at $2,000 \times g$ for 10 min at room temperature. The bottom layer (chloroform layer) was removed and reserved. Two subsequent extractions were performed by adding 8 mL of chloroform to the top aqueous layer, removing the bottom organic layer after each extraction and pooling with other organic phases. Pooled extracts were evaporated using a vacuum centrifuge and the residues were re-dissolved in 4.5 g of chloroform:methanol (2:1, vol/vol), noting the use of solvent mass to compensate for the high density and potential pipetting errors between scientists in an industry setting.

Table 1. HPLC-evaporative light-scattering detection gradient program for analysis of polar lipids¹

Time (min)	Step detail	% Solvent A	% Solvent B	% Solvent C	% Solvent D
0.00	Initial	5.0	19.0	3.0	73.0
1.00	Hold	5.0	19.0	3.0	73.0
1.50	Gradient	5.0	30.0	4.0	61.0
3.00	Gradient	5.0	34.0	4.5	56.5
5.00	Gradient	8.0	37.0	4.5	50.5
8.50	Gradient	15.0	39.0	4.5	41.5
10.00	Gradient	15.0	50.0	4.5	30.5
12.50	Hold	15.0	50.0	4.5	30.5
12.60	Equilibrate	5.0	19.0	3.0	73.0
25.00	End	5.0	19.0	3.0	73.0

¹Liquid chromatography solvent gradient program used for the analysis of polar lipids on a silica column (150 mm × 2.1 mm, 2.7 µm). Column flow of 0.3 mL/min, column temperature of 55°C, and autosampler temperature of 20°C. Solvent A = 180 mM ammonium formate; solvent B = isopropanol; solvent C = methanol; solvent D = acetonitrile.

Separation of Lipids with Solid-Phase Extraction

Solid-phase extraction (SPE) clean-up of PL was performed using the method of Avalli and Contarini (2005) to remove nonpolar lipids. A silica SPE cartridge with 1 g bed weight and 6 mL capacity was used in this study (Supelclean LC-SI, Sigma-Aldrich). The SPE cartridge was conditioned with 4 mL of hexane. Raw-lipid extract (0.5 mL) was loaded on the cartridge at a concentration of 30 mg/mL. Nonpolar lipids were eluted with 3 mL of hexane:diethyl-ether (8:2, vol/vol) followed by 3 mL of hexane:diethyl-ether (1:1, vol/vol). Phospholipids were eluted with 2 mL of methanol followed by 2 mL of chloroform:methanol:water (3:5:2, vol/vol/vol). Collected phospholipid solution was evaporated under a stream of nitrogen. Phospholipids were re-dissolved in 0.5 mL of chloroform:methanol (2:1, vol/vol) before injection on HPLC.

Hydrophilic Interaction HPLC-ELSD Analysis of Phospholipids

Extracted phospholipids were analyzed using an Acquity UPLC H-Class Plus system equipped with a quaternary pump, CH-A column heater, FTN Sample Manager, and an Acquity Evaporative Light Scatter Detector (Waters, Milford, MA). The analytical column was an Ascentis Express Silica HILIC LC column with dimensions of 150 mm × 2.1 mm, 2.7 µm bead, and 90 Å pore size purchased from Sigma-Aldrich. The method was run at 0.3 mL/min column flow with a column temperature of 55°C and injection volume of 6 µL. Samples were maintained at 20°C in the autosampler. Separation of phospholipids was performed using a quaternary, multistep gradient in Table 1. Solvent A consisted of 180 mM ammonium formate, solvent B was isopropanol, solvent C was methanol, and solvent D was acetonitrile. Evaporative light-scattering detector conditions were set at 70°C drift tube temperature, 40

psi nitrogen nebulizer pressure, signal gain setting of 50, and 12°C nebulizer temperature.

Mixed calibration standards were prepared in chloroform:methanol (2:1 vol/vol) by diluting from individual phospholipid standard stock solutions (25 mg/mL PG, PE, PS, PC, and SM, and 20 mg/mL PI). The PG was selected as a reference for the method and was prepared the same as the other chemical standards. Quantitation was performed by a quadratic regression curve of ELSD response versus PL concentration. Calibration standards were prepared daily from stocks stored at −20°C.

Method Validation Procedures

Method validation including analyte spiking and recovery, injection stability, and intermediate precision were performed. Phospholipid standards (PI, PE, PS, PC, and SM) in chloroform:methanol (2:1, vol/vol) were spiked into WPPC before lipid extraction. In addition, recovery of the SPE process was analyzed by spiking pure phospholipid standards (2.83 mg/g PI, 3.52 mg/g PE, 3.60 mg/g PS, 3.63 mg/g PC, and 2.92 mg/g SM at sample matrix concentration) into the lipid extract of WPPC in chloroform:methanol (2:1, vol/vol). Due to the volatility of solvents used in preparation of phospholipids, injection stability was analyzed using statistical control charts (individual value chart, moving range chart) according to guidelines set by ASTM International (2016). A mid-level mixed phospholipid standard with 0.39 mg/g PI, 1.04 mg/g PE, 1.25 mg/g PS, 0.51 mg/g PC, and 0.51 mg/g SM in chloroform:methanol (2:1, vol/vol) was injected on the HPLC consecutively for 6 h and 40 min, equaling a total of 18 repeated injections. Intermediate precision analysis was performed to include multiple calibration curves (n = 2) and sample preparations (n = 3) of WPPC by 3 analysts each day, over 2 d.

Table 2. HPLC-evaporative light-scattering detection method validation data

Phospholipid ¹	Mean ²	RSD ³ (%)	LLOD ⁴	LLOQ ⁴	ULOQ ⁴	Recovery 1 ⁵ (%)	Recovery 2 ⁶ (%)
PI	3.5	8.4	0.11	0.35	1.48	104.1	100.5
PE	30.2	4.7	0.26	0.87	3.90	109.8	96.7
PS	9.3	3.6	0.22	0.73	4.68	94.4	89.3
PC	24.0	5.4	0.11	0.37	1.89	95.5	108.5
SM	18.3	10.2	0.06	0.20	1.90	101.1	111.0

¹PC = phosphatidylcholine; PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, and SM = sphingomyelin.

²Mean in units of mg/g in raw whey protein phospholipid concentrate (WPPC; n = 18).

³RSD = relative standard deviation from method intermediate precision of WPPC (n = 18).

⁴LLOD = lower limit of detection; LLOQ = lower limit of quantitation; ULOQ = upper limit of quantitation; limits reported at test concentration in units of mg/g.

⁵Recovery 1: polar lipids recovery through the Folch lipid extraction sample preparation process (n = 3).

⁶Recovery 2: polar lipids recovery through the SPE only sample preparation process for lipids extracted from WPPC (n = 3).

RESULTS AND DISCUSSION

HPLC Calibration and Limits

A chromatogram of a typical phospholipid calibration standard injection is shown in Figure 1. Quantitation of phospholipids was performed using a quadratic regression curve ($R^2 > 0.999$) of detector response (peak area) versus concentration over the quantitation range used in this study. All analytes of interest are baseline resolved and elute within 13 min. The additional time in the method (gradient program t = 12.60 to t = 25.00) shown in the chromatogram as baseline signal after the elution of SM is required to equilibrate the silica column for reproducible immediate subsequent injections.

The upper limit of quantitation is arbitrarily set to 125% of the highest phospholipid calibration standard concentration for each standard. Lower limit of detec-

tion and lower limit of quantitation were determined by the signal-to-noise (S/N) ratio method (Snyder, 1997) using the S/N of phospholipid chromatographic peak regions in a blank injection. Thresholds of 3 times and 10 times the S/N were used to determine lower limit of detection and lower limit of quantitation, respectively; for each PL, these results are presented in Table 2.

Spiking and Recovery

The potential loss of PL during sample preparation was monitored at the 2 major extraction steps by quantifying the PL recovered from the Folch extraction and the SPE clean-up. Table 2 reports the recoveries of each PL species from the 2 extraction steps. The lowest recovery was seen in PS during the SPE clean-up (89.3%). All other values exceed 90% recovery and we conclude that the Folch and SPE procedures are suit-

Figure 1. Typical chromatogram of polar lipids (PL) separation using hydrophilic interaction HPLC quaternary solvent gradient. PG = phosphatidylglycerol; PI = phosphatidylinositol; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin; LSU = light-scattering units.

able for the purification of PL from dairy matrices. The SPE method presented here is useful for the removal of nonpolar lipids and thus a concentrating step for the PL; however, it was not a necessary step for successful PL quantitation. Combinations of Mojonner or Folch extractions with SPE methods including the Avalli method used in this study have been compared in literature. (Gallier et al., 2010). It was concluded that the cold operating conditions of Folch are preferred to Mojonner, which uses a strong base and direct heat, for the quantitation of PL. The Folch method used in this study was modified to minimize the required halogenated solvents, while still delivering quantitative lipid recovery from samples of varying protein and fat contents.

Intermediate Precision

Intermediate precision is the measure of precision within a laboratory using defined methods and procedures. For this study intermediate precision was conducted using the methods above among 3 industry scientists, and exact procedures were prepared in protocol format and distributed to the scientists in advance of conducting the experiment. Measurements were made in triplicate across 2 d using the same sample of WPPC. Acceptable limits were set at 10% relative standard deviation (**RSD**) for detection of each PL analyte among the scientists. Reproducibility within the target RSD was met for the detection of all analytes. The resulting RSD for the PL analytes were 8.4% for PI, 4.7% for PE, 3.6% for PS, 5.4% for PC, and 10.2% for SM.

Phospholipid Sample Stability

To assess the ability of the method to report results that are stable throughout an analysis by HPLC individual value and moving range control charts were used to monitor the stability of injections of a single phospholipid calibration standard mixture over 6 h and 40 min (data not shown). Control limits were set using the guidelines of ASTM E2587 (ASTM International, 2016). Across the total data collection period, signal response for each analyte was monitored to determine the point at which response moved outside of control limits. This would suggest a loss of stability due to factors other than random chance. The prepared sample was observed to remain stable for 5 h and 35 min including sample preparation time (estimated as 30 min). It is recommended that samples prepared using this method be prepared quickly and analyzed within the observed stability time of 5 h and 35 min. This allows for analysis of 5 to 6 samples after calibration standards before loss of PL stability. If analysis cannot be completed within

the recommended time, samples should be stored at -20°C or lower until analysis.

Phospholipid Quantitation of Production and Commercial Products

To demonstrate the capability of the method for determining the PL content of varying dairy products for new product development and quality assurance, the analytic method was applied to buttermilk powder, WPC34, and WPPC. Buttermilk powders were used to challenge the method with a dairy-based high-fat matrix, and WPC34 was selected for its high lactose content. The WPPC product was selected to challenge the method with a high protein content matrix, while also being enriched in PL.

Plant-based lecithin products were also tested to challenge the method with another high lipid matrix, specifically high in PL content. The ability of measuring PL content of plant-based lecithin and dairy PL enriched streams using the same procedures is of great interest to the dairy industry for future applications in production of a dairy-based lecithin equivalent. Plant lecithin products are used widely in the dairy industry for a variety of products including instantized dairy powders and ice cream. Being able to source PL from under-utilized dairy process streams would allow the dairy industry to become more independent of plant-based lecithin and have a cleaner label by removing an allergen.

As expected, the sampled dairy products had higher levels of SM, widely understood to be more prevalent in animal lipids, compared with the plant lecithin products. In general, the PL profiles for the dairy products (Table 3) have similar percent composition among PL species. Buttermilk powder was found to have $1.71 \pm 0.03\%$ PL by mass, which agrees with the total PL value reported by Barry et al. (2017b) of 1.30% wt/wt. Spence et al. (2009a) reported a higher value of 2.2% PL for regular cream buttermilk.

The analysis of PL in WPC products is less common in literature; however, Vaghela and Kilara (1996) studied the effects of production variables on PL composition in several WPC. The control production (non-variable/untreated sample) of WPC35 and WPC75 were reported to contain $13.49 \pm 0.47\%$ and $13.35 \pm 0.40\%$ PL of total lipid mass, respectively (Vaghela and Kilara, 1996). These reported values differ greatly from the value reported in this study for WPC34 ($1.93 \pm 0.12\%$ of total lipid mass, Table 3). The exact reason for this disagreement was not investigated in this study but is hypothesized to be caused by major differences in production between the samples of WPC, specifically lower protein content concentrates. The WPC34 (or

Table 3. Phospholipid content ($\pm$ SD) of commercial dairy products and plant-based lecithin products¹

Product	Total PL %	% PI	% PE	% PS	% PC	% SM
BMP	1.71 $\pm$ 0.03	7.9 $\pm$ 0.6	25.3 $\pm$ 1.1	17.2 $\pm$ 0.8	34 $\pm$ 3	15.1 $\pm$ 1.3
WPC34	1.93 $\pm$ 0.12	5.53 $\pm$ 0.06	23.0 $\pm$ 0.7	15.8 $\pm$ 1.9	35.8 $\pm$ 1.6	20 $\pm$ 2
WPPC	8.5 $\pm$ 0.5	4.1 $\pm$ 0.2	35.4 $\pm$ 0.6	11.0 $\pm$ 0.7	28.2 $\pm$ 0.7	21.4 $\pm$ 1.2
LSL	36.2 $\pm$ 0.8	18 $\pm$ 2	35.5 $\pm$ 1.0	3.0 $\pm$ 0.3	41 $\pm$ 3	1.94 $\pm$ 0.17
PSL	48.4 $\pm$ 1.6	22 $\pm$ 2	16.1 $\pm$ 0.7	3.5 $\pm$ 0.5	55 $\pm$ 3	2.7 $\pm$ 0.4
SunL	56 $\pm$ 2	18.7 $\pm$ 1.9	25.4 $\pm$ 1.9	2.3 $\pm$ 0.5	51 $\pm$ 4	2.5 $\pm$ 0.3

¹Total polar lipids (PL) % reported as % PL of total sample mass; PL species reported as % of total PL content (n = 3). PI = phosphatidylinositol, PE = phosphatidylethanolamine, PS = phosphatidylserine, PC = phosphatidylcholine, SM = sphingomyelin. BMP = buttermilk powder; WPC34 = whey protein concentrate 34; WPPC = whey protein phospholipid concentrate. WPPC results are from the larger intermediate precision study (n = 18). LSL = liquid soy lecithin; PSL = powder soy lecithin; SunL = powder sunflower lecithin.

WPC35) can be made differently between processors, and in some cases low protein concentrate products are often back blended from effluent of other processes to meet the minimum required composition of the product identity.

The largest concentration of PL in a dairy matrix in this study was seen in WPPC (8.5 $\pm$ 0.5%, Table 3). This matrix is the most promising source of PL from dairy and has been investigated by Price et al. (2018) and Levin et al. (2016). Price et al. (2018) reported a PL content of 29.1 $\pm$ 0.7% PL of the total fat content of WPPC (wt/wt), and total fat reported as 5.5% by Folch extraction. Levin et al. (2016) reported a range of PL contents from 4 different WPPC from 4 suppliers of 0.57 to 0.74% PL of total fat content (wt/wt). This large variance of PL content in WPPC products again is most likely due to differences in production. The PL content is not a requirement of any whey product standard of identity established by the American Dairy Product Institute. However, the differences in PL composition seen in the literature support the need for an industry-applicable method for PL analysis to better understand the additional value products have from PL content.

CONCLUSIONS

The method presented in this study allows for sensitive, reproducible detection of PL in a variety of dairy matrices. The combination of hydrophilic interaction HPLC separation and small-scale Folch extraction was successful in reducing the halogenated solvent requirements for analysis. Acceptable reproducibility was seen in the intermediate precision experiment and is expected to improve as the method continues to be used. These procedures are suitable for the dairy industry and are currently being used to research PL composition throughout a cheese-whey process flow. This method will allow for new insights into dairy product composition and the production of new value-added ingredients.

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