

Composition and Fatty Acid Distribution of Bovine Milk Phospholipids from Processed Milk Products

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The aim of this work was to assess the accuracy of different extraction methods of phospholipids and to measure the effect that processing has on phospholipid composition. Four methods of extracting phospholipids from buttermilk powder were compared to optimize recovery of sphingomyelin. Using the optimal method, the phospholipid profile of four dairy products (raw milk, raw cream, homogenized and pasteurized milk, and buttermilk powder) was determined. A total lipid extraction by the Folch method followed by a solid-phase extraction using the Bitman method was the most efficient technique to recover milk sphingomyelin. Milk processing (churning, centrifuging, homogenization, spray-drying) affected the profile of milk phospholipids, leading to a loss of sphingomyelin and phosphatidylcholine after centrifugation for cream separation. A corresponding decrease in the saturation content of the raw cream phospholipids and a loss of phosphatidylethanolamine after spray-drying to produce buttermilk powder were also observed.

KEYWORDS: Phospholipids; lipid extraction; solid-phase extraction; milk processing; milk fat globule membrane; sphingomyelin

INTRODUCTION

Phospholipids account for about 1% of the total bovine milk lipids (1), and about 60% of these are found as part of the milk fat globule membrane (MFGM). Residual MFGM material is found in the skim milk phase, probably released during or after secretion of the lipid droplet (2). Upon processing, the MFGM is altered and loses some phospholipids, and the serum proteins tend to associate with the MFGM around the lipid core (3).

Milk can be processed by centrifugation to produce cream, by homogenization, heat treatment, or spray-drying to produce milk powder, and by churning to produce butter. Buttermilk is a by-product of the butter-making process and is generally considered to be a low-value product; however, it is rich in MFGM material (4). The surfactant properties of the buttermilk phospholipids could be of considerable importance for the nutritional and technological use of buttermilk and MFGM extracts, for example, in emulsions or for liposome formation.

The proteomics of the MFGM has been extensively studied, whereas lipidomics still lacks some important information. Morin et al. (5) studied the effects of cream pasteurization and spray-drying of buttermilk on the phospholipid content of MFGM and buttermilk. Many studies have reported the phospholipid content of raw milk and buttermilk powder but, in general, there is a lack of data on the phospholipid composition of processed dairy products. In light of increasing knowledge about the health and nutritional benefits of the MFGM phospholipids, it is important

to compare the efficiency of different methods of phospholipid extraction and determine the phospholipid profile of dairy products.

One important distinction of milk-derived phospholipids from all other plant sources is that they contain sphingomyelin. Sphingomyelin has been associated with many health benefits. Its biological activity is due to its metabolites ceramide, sphingosine, and sphingosine phosphate (6). MFGM phospholipids have been shown to have anticarcinogenic properties and cholesterolemia-lowering effects and to be efficient in the prevention of Alzheimer's disease, gastric disease, stress, and depression and in the suppression of multiple sclerosis (6). Sphingomyelin has been implicated in microdomain formation in cell membranes, playing a role in the regulation of several cellular processes (7).

Different methods of extraction of phospholipids from the same buttermilk powder were examined to determine the optimal recovery of sphingomyelin. The phospholipid profile, especially the sphingomyelin content and degree of saturation of the phospholipids present, from four processed dairy products (raw milk, raw cream, homogenized and pasteurized milk, and buttermilk powder) was also examined using the optimal extraction method as a function of four milk-processing conditions: churning, homogenization, pasteurization, and spray-drying.

MATERIALS AND METHODS

Two sets of extractions were carried out: the purpose of the first set was to compare the methods of extraction of phospholipids from the same buttermilk powder, and the second set was to compare the phospholipid profile of the four dairy products using the same extraction method.

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Samples and Reagents. Bovine raw milk was collected from a bulk tank of milk from the Holstein and Jersey dairy herd at the Dairy Products Technology Center (California Polytechnic State University, San Luis Obispo, CA). Processed whole milk from Producers Dairy Foods, Inc. (Fresno, CA; pasteurized at 76 °C for 15 s and homogenized at 14.2 MPa in the first stage and at 10.1 MPa in the second), was bought at a local campus market. The first set of extractions was carried out on buttermilk powder purchased from Dairy America, Inc. (Fresno, CA). Buttermilk powder for the second set of extractions was provided by Land O'Lakes, Inc. (Arden Hills, MN). Deionized water was used unless specified otherwise. All solvents (chloroform, methanol, hexane, diethyl ether, petroleum ether, ethyl alcohol, and ammonium hydroxide) were from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. Supelco Discovery DSC-Silica gel-based solid-phase extraction (SPE) cartridges (bed weight = 2 g, volume = 12 mL) for the first set of extractions on buttermilk powder were purchased from Sigma-Aldrich (St. Louis, MO) and Strata silica SI-1 normal phase SPE cartridges (bed weight = 2 g, volume = 12 mL) for the second set of extractions on four dairy products from Phenomenex (Torrance, CA). Silica gel plates coated with glass (Fisher Scientific) were used for thin-layer chromatography (TLC).

Total Lipid Extraction: Folch Method versus Mojonier Method. Total lipids from Dairy America buttermilk powder were extracted according to the modified Mojonier ether extraction method (8). To 5 g of powder (diluted in 5 mL of deionized water) was added 1.5 mL of NH_4OH to neutralize acids and dissolve caseins. The first extraction was carried out with 10 mL of ethyl alcohol, 25 mL of ethyl ether, and 25 mL of petroleum ether. The second extraction was with 5 mL of ethyl alcohol, 15 mL of ethyl ether, and 15 mL of petroleum ether. The third extraction used 15 mL of ethyl ether and 15 mL of petroleum ether. The total lipids were recovered in hexane (100 mg mL^{-1}) after evaporation of the solvents at 65 °C in a vacuum oven.

A total lipid extraction according to the Folch (9) method was carried out on freeze-dried raw milk, freeze-dried processed milk, raw cream (from raw milk centrifuged at 3000g for 5 min at 20 °C in a benchtop Eppendorf centrifuge), and buttermilk powder from Dairy America (in the first set of experiments) and from Land O'Lakes (in the second set of experiments). The raw milk and raw cream phospholipids came from the same pool of milk, whereas the processed milk and buttermilk powder were purchased from a different source.

The raw milk and processed milk samples contained 25% total fat on a dry matter basis, the raw cream 50%, and the buttermilk powder 10%. After extraction with chloroform/methanol (2:1, v/v) and then addition of a NaCl solution (8.76%) to purify the extract, the total lipids were dried with a rotoevaporator and recovered in hexane to a concentration of 100 mg mL^{-1} . Methanol was first added to improve the dissociation of lipid-protein interactions, and then chloroform was added to solubilize the lipids.

Comparison of Solid-Phase Extraction Methods: Bitman Method versus Avalli Method versus Vaghela Method. It is necessary to remove the triglycerides and other neutral lipids prior to any quantitative analysis. Three methods of SPE, the Bitman method (10), the Avalli method (11), and the Vaghela method (12), were carried out to separate the neutral lipids from the phospholipids. Only the Vaghela method was modified, as follows: a silica cartridge was used instead of an aminopropyl stationary phase, as Kaluzny et al. (13) showed a loss of acidic lipids using an aminopropyl-bonded phase. Each cartridge was loaded with 200 mg of lipids.

Briefly, the Bitman method involved conditioning the cartridge with chloroform and, first, elution of the neutral lipids with 40 mL of hexane/ethyl ether (1:1, v/v) and, then, elution of the phospholipids with a 20 mL wash of methanol followed by a 20 mL wash of chloroform/methanol/water (3:5:2, v/v/v). The Avalli method utilized hexane to condition the cartridge, 6 mL of hexane/diethyl ether (8:2, v/v) and 6 mL of hexane/diethyl ether (1:1, v/v) to elute the neutral lipids, and, then, 8 mL of methanol and 4 mL of methanol plus 4 mL of chloroform/methanol/water (3:5:2, v/v/v). In the Vaghela method, the cartridge was conditioned with hexane. The neutral lipids were eluted first with 18 mL of chloroform/isopropyl alcohol (2:1, v/v), then the fatty acids with 18 mL of 2% (v/v) acetic acid in diethyl ether, and finally the phospholipids with 18 mL of methanol. After each SPE, the solvents were evaporated and the phospholipid extracts were dissolved in chloroform to a concentration of 10 mg mL^{-1} , transferred into

amber glass vials capped with Teflon-lined caps, and stored at $-20 \text{ }^\circ\text{C}$ until further analysis.

Phospholipid Analysis. A TLC (chloroform/methanol/water, 65:25:4, v/v/v) was carried out to qualitatively identify the phospholipids present in each extract and check the presence of any residual traces of neutral lipids. Samples ($20 \mu\text{L}$) were spotted on the plates with a Hamilton gastight syringe (Fisher Scientific). Iodine vapor was applied overnight for the revelation of spots.

Electrospray Ionization—Tandem Mass Spectrometry (ESI-MS/MS) Lipid Profiling. The phospholipid extracts were taken to dryness under a gentle stream of nitrogen. A complete quantitative analysis of the phospholipid composition was performed by the Kansas Lipidomics Research Center (Manhattan, KS). An automated ESI-MS/MS approach was used, and data acquisition, analysis, and acyl group identification were carried out as described previously (14) with modifications. The dried samples were dissolved in 1 mL of chloroform, and an aliquot of $15 \mu\text{L}$ of extract in chloroform was used. Precise amounts of internal standards, obtained and quantified as previously described (15), were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.60 nmol of di12:0-phosphatidylcholine (PC), 0.60 nmol of di24:1-PC, 0.60 nmol of 13:0-lysoPC, 0.60 nmol of 19:0-lysoPC, 0.30 nmol of di12:0-phosphatidylethanolamine (PE), 0.30 nmol of di23:0-PE, 0.30 nmol of 14:0-lysoPE, 0.30 nmol of 18:0-lysoPE, 0.30 nmol of 14:0-lysophosphatidylglycerol (lysoPG), 0.30 nmol of 18:0-lysoPG, 0.30 nmol of di14:0-phosphatidic acid (PA), 0.30 nmol of di20:0(phytanoyl)-PA, 0.20 nmol of di14:0-phosphatidylserine (PS), 0.20 nmol of di20:0(phytanoyl)-PS, 0.23 nmol of 16:0–18:0-phosphatidylinositol (PI), and 0.16 nmol of di18:0-PI. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300:665:35, and the final volume was 1.2 mL.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple-quadrupole MS/MS (4000QTrap, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at $30 \mu\text{L min}^{-1}$.

Lipid species were detected with the following scans: PC, sphingomyelin (SM), and lysoPC, $[\text{M} + \text{H}]^+$ ions in positive ion mode with a precursor of 184.1; PE and lysoPE, $[\text{M} + \text{H}]^+$ ions in positive ion mode with a neutral loss (NL) 141.0; PI, $[\text{M} + \text{NH}_4]^+$ in positive ion mode with NL 277.0; PS, $[\text{M} + \text{H}]^+$ in positive ion mode with NL 185.0; and PA, $[\text{M} + \text{NH}_4]^+$ in positive ion mode with NL 115.0. SM was determined from the same mass spectrum as PC (precursors of m/z 184 in positive mode) (16, 17) and by comparison with PC internal standards using a molar response factor for SM (in comparison with PC) determined experimentally to be 0.39. The scan speed was 50 or 100 units s^{-1} . The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC (and SM), and +25 V for PI, PS, and PA. Declustering potentials were +100 V. Entrance potentials were +15 V for PE and +14 V for PC (and SM), PI, PA, and PS. Exit potentials were +11 V for PE and +14 V for PC (and SM), PI, PA, and PS. The mass analyzers were adjusted to a resolution of 0.7 atomic mass unit full width at half-height. For each spectrum, 9–150 continuum scans were averaged in multiple-channel analyzer mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 or -4.5 kV was applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas were integrated using a custom script with Analyst software (Applied Biosystems).

Statistical Analysis. The phospholipids' compositions and their fatty acid profiles were evaluated for five replicates. Analysis of variance (ANOVA) was calculated using SPSS version 14.0 software (SPSS Inc., Chicago, IL), and results were considered to be significantly different at $P < 0.05$.

RESULTS

Comparison of Methods To Extract Phospholipids from the Same Buttermilk Powder. *Phospholipid Profile.* A purity check

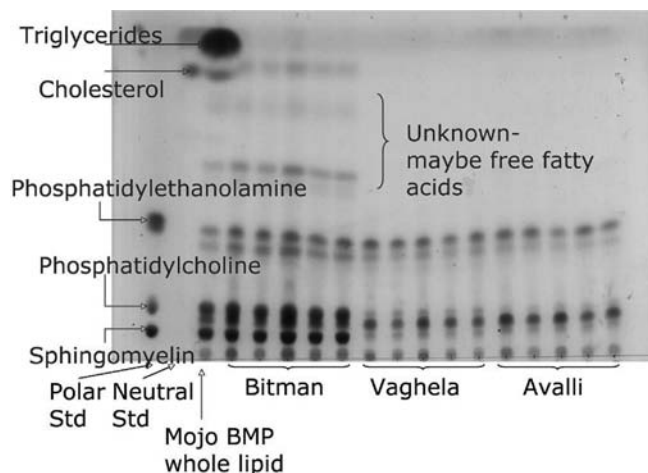


Figure 1. Thin-layer chromatography plate showing the migration of the phospholipids of buttermilk powder extracted by a total lipid extraction following the Mojonnier method, followed by an SPE using either the Bitman, Vaghela, or Avalli method (Std, standard; BMP, buttermilk powder; Mojo, Mojonnier).

Table 1. Phospholipid Composition of Buttermilk Powder Obtained Following Four Methods of Extraction^a

sample phospholipid	Mojo/Vaghela mol %	Mojo/Avalli mol %	Mojo/Bitman mol %	Folch/Bitman mol %
lyso-PC	0.1 ± 0.1	0.14 ± 0.03	0.6 ± 0.1	1.5 ± 0.1
PC	14.2 ± 1.5	9.7 ± 0.8	47.0 ± 0.7	35.3 ± 1.7
SM	2.0 ± 0.3	0.6 ± 0.2	22.6 ± 3.2	20.8 ± 0.4
ePC	1.8 ± 0.2	1.2 ± 0.1	7.0 ± 0.2	5.7 ± 0.2
lyso-PE	0.5 ± 0.4	3.1 ± 0.6	0.2 ± 0.1	0.7 ± 0.1
PE	36.3 ± 4.7	34.5 ± 2.6	9.6 ± 1.6	8.8 ± 0.2
PE-cer	0.018 ± 0.018	0.013 ± 0.019	0.001 ± 0.002	0.000 ± 0.001
ePE	1.1 ± 0.4	1.2 ± 0.12	0.3 ± 0.1	0.24 ± 0.02
PI	35.9 ± 6.0	38.3 ± 2.2	10.0 ± 2.1	16.4 ± 2.8
PS	6.8 ± 2.0	9.3 ± 0.5	2.5 ± 0.4	10.0 ± 0.9
PA	1.3 ± 0.2	2.0 ± 0.1	0.3 ± 0.1	0.5 ± 0.2

^a Abbreviations: lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; ePC, ether phosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PE-cer, phosphoethanolamine-ceramide; ePE, ether phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; Mojo, Mojonnier.

for each extracted phospholipid sample was carried out by TLC; the presence of triglycerides was not detectable in any of the three extracts (**Figure 1**). The phospholipid profile and fatty acid composition of the individual phospholipid species in buttermilk powder extracted following four methods of extraction were determined by ESI-MS/MS. The Mojonnier extraction followed by the Bitman SPE gave 33.6 nmol mg⁻¹ of lipid weight, the Mojonnier extraction followed by the Vaghela extraction gave 20.7 nmol mg⁻¹ of lipid weight, the Mojonnier extraction followed by the Avalli extraction gave 29.6 nmol mg⁻¹ of lipid weight, and the Folch extraction followed by the Bitman SPE gave 46.5 nmol mg⁻¹ of lipid weight.

The Avalli and Vaghela methods yielded lower amounts of PC, ePC, and SM (**Table 1**) compared to the Bitman method, but higher amounts of PE, ePE, PS, and PI. The Bitman method gave low PE values (**Table 1**), likely due to a loss in PE recovery. Lyso-PE, lyso-PC, and PA were detected in all of the extracted samples. At this stage it is not possible to say if they were originally present in the buttermilk powder resulting from the degradation of PE, PC, and other phospholipids over processing and storage or if the methods of extraction were responsible for this degradation.

Christie et al. (18), using the Bligh and Dyer (19) total lipid extraction and Bitman SPE methods, reported the following concentrations of phospholipids in buttermilk: 29.5% w/v PC, 25.1% w/v SM, and 38.1% w/v PE. Morin et al. (20), using the Mojonnier method, reported different concentrations of phospholipids in buttermilk: 33.2% w/w PC, 15.7% w/w SM, 33.9% w/w PE, 11.1% w/w PI, and 6.2% w/w PS.

The low amount of lipids in the buttermilk powders (about 10% on dry basis), the high protein content, and the presence of lipid-protein complexes make it hard to extract the lipids fully without altering them (21). The Mojonnier method involves a strong base, NH₄OH, and heating of the samples to evaporate the solvents, which both lead to hydrolysis or oxidation of phospholipids and their unsaturated fatty acids (22). Therefore, cold extractions, such as the Folch method, are preferred. In addition to the processing conditions, such as spray-drying, the storage of the buttermilk powder may have influenced the degradation of some phospholipids into lysophospholipids. The Folch method, involving the use of an alcohol, allows the complete solubilization of the MFGM polar lipids (6). The alcohol denatures the proteins and degrades the hydrogen bonds of the protein-lipid complexes.

Morin et al. (5) studied the effect of buttermilk processing on the phospholipid content; they used the Mojonnier method to extract the total lipids and analyzed the lipid content using a high-performance liquid chromatograph (HPLC)-evaporative light scattering detector. They observed a similar decrease in the PE content of buttermilk and MFGM isolates due to spray-drying, suggesting that the formation of protein-phospholipid complexes during spray-drying renders some phospholipids non-extractable by the Mojonnier technique. The Folch method was also not successful in extracting all PE (**Table 1**).

Fatty Acid Distribution. ESI-MS/MS was employed to determine the structural information of a specific species. The method of extraction significantly affected the ratio of saturation/unsaturation of the fatty acids (**Table 2**). The polyunsaturation of a phospholipid means that the phospholipid contains either two unsaturated fatty acids or one saturated and one polyunsaturated. Slightly fewer saturated phospholipids were found using the Folch technique compared to the Mojonnier method, meaning that degradation may have occurred during the Mojonnier method when the solvents were evaporated by heat. The Vaghela and Avalli methods were not efficient in the extraction of saturated fatty acids (**Table 2**), mainly due to poor extraction of SM (**Table 1**).

The Folch total lipid extraction method followed by the Bitman phospholipid SPE was chosen as it recovered more phospholipids (46.5 nmol mg⁻¹ of lipid weight), more SM, and a higher amount of saturated fatty acids and was more reproducible.

Comparison of the Phospholipid Composition of Raw Milk, Raw Cream, Processed Milk, and Buttermilk Powder Using the Same Extraction Method.

Phospholipid Profile. For a rapid and qualitative control of the efficiency of the SPE in separating the phospholipids and the neutral lipids, a TLC was performed on mixtures of phospholipids from four products (**Figure 2**). The TLC shows that the SPE did not totally eliminate all neutral lipids (**Figure 2**). Some cholesterol was still detected, and other spots might correspond to free fatty acids. Although cholesterol is hydrophobic, it contains a polar 3 β -hydroxyl group (23), and this could be why cholesterol was found in the samples after SPE. However, no further analysis was carried out as the main goal was to remove most of the triglycerides.

The phospholipid profile and fatty acid composition of the individual phospholipid species in raw milk, raw cream, processed milk, and buttermilk powder that were extracted following the Folch extraction method and the Bitman SPE method are

Table 2. Degree of Unsaturation of the Phospholipids Present in Buttermilk Powder Obtained following Four Methods of Extraction^a

		Mojo/Vaghela mol %	Mojo/Avalli mol %	Mojo/Bitman mol %	Folch/Bitman mol %
lyso-PC	saturated	0.03 ± 0.01	0.03 ± 0.01	0.23 ± 0.02	0.56 ± 0.05
	monounsaturated	0.03 ± 0.01	0.06 ± 0.01	0.24 ± 0.02	0.65 ± 0.06
	polyunsaturated	0.03 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.34 ± 0.03
PC	saturated	1.5 ± 0.1	0.8 ± 0.1	7.3 ± 0.5	5.7 ± 0.3
	monounsaturated	4.5 ± 0.5	2.3 ± 0.3	16.4 ± 1.0	12.4 ± 0.8
	polyunsaturated	8.1 ± 0.8	6.6 ± 0.9	23.3 ± 1.2	17.3 ± 0.9
ePC	saturated	0.33 ± 0.05	0.20 ± 0.04	1.6 ± 0.1	1.3 ± 0.1
	monounsaturated	0.9 ± 0.1	0.5 ± 0.1	3.7 ± 0.2	3.1 ± 0.2
	polyunsaturated	0.6 ± 0.1	0.5 ± 0.1	1.8 ± 0.1	1.3 ± 0.1
SM	saturated	1.7 ± 0.3	0.5 ± 0.2	21.2 ± 1.6	19.6 ± 1.4
	monounsaturated	0.29 ± 0.05	0.03 ± 0.01	1.4 ± 0.1	1.2 ± 0.1
	polyunsaturated	nd	nd	nd	nd
lyso-PE	saturated	0.010 ± 0.002	0.07 ± 0.02	0.003 ± 0.001	0.03 ± 0.01
	monounsaturated	0.27 ± 0.04	1.2 ± 0.2	0.08 ± 0.01	0.41 ± 0.04
	polyunsaturated	0.2 ± 0.1	1.8 ± 0.4	0.07 ± 0.01	0.22 ± 0.03
PE	saturated	0.23 ± 0.04	0.31 ± 0.05	0.04 ± 0.01	0.04 ± 0.01
	monounsaturated	6.1 ± 0.5	4.9 ± 0.5	1.4 ± 0.1	1.5 ± 0.1
	polyunsaturated	30.0 ± 2.4	29.3 ± 2.0	8.2 ± 0.6	7.2 ± 0.2
PE-cer	saturated	0.018 ± 0.007	0.007 ± 0.004	0.0006 ± 0.0004	0.0002 ± 0.0002
	monounsaturated	0.0003 ± 0.0002	0.007 ± 0.003	0.0001 ± 0.0001	nd
	polyunsaturated	nd	nd	nd	nd
ePE	saturated	0.02 ± 0.01	0.07 ± 0.03	0.004 ± 0.002	0.004 ± 0.002
	monounsaturated	0.33 ± 0.05	0.35 ± 0.05	0.10 ± 0.01	0.07 ± 0.01
	polyunsaturated	0.7 ± 0.1	0.8 ± 0.1	0.18 ± 0.03	0.17 ± 0.04
PI	saturated	3.7 ± 0.4	4.2 ± 0.2	1.1 ± 0.1	1.7 ± 0.2
	monounsaturated	12.2 ± 1.2	11.5 ± 0.6	3.3 ± 0.2	5.1 ± 0.5
	polyunsaturated	20.0 ± 2.6	22.6 ± 1.7	5.6 ± 0.4	9.6 ± 1.5
PS	saturated	0.003 ± 0.002	0.07 ± 0.02	0.002 ± 0.001	0.01 ± 0.01
	monounsaturated	3.1 ± 0.2	3.3 ± 0.4	0.9 ± 0.1	4.0 ± 0.3
	polyunsaturated	3.7 ± 0.3	5.92 ± 0.05	1.5 ± 0.1	6.0 ± 0.2
PA	saturated	0.002 ± 0.001	0.018 ± 0.003	0.003 ± 0.001	0.004 ± 0.002
	monounsaturated	0.08 ± 0.02	0.05 ± 0.01	0.025 ± 0.003	0.029 ± 0.007
	polyunsaturated	1.2 ± 0.1	2.0 ± 0.1	0.31 ± 0.03	0.51 ± 0.05
PL	saturated	7.6 ± 0.9	6.3 ± 0.7	31.4 ± 2.3	28.9 ± 2.2
	monounsaturated	27.8 ± 2.6	24.2 ± 2.2	27.6 ± 1.7	28.4 ± 2.1
	polyunsaturated	64.6 ± 6.4	69.5 ± 5.3	41.0 ± 2.6	42.7 ± 3.1
	saturated/unsaturated	0.1	0.1	0.5	0.4

^a Abbreviations: PL, phospholipids; nd, not detected.

presented in **Table 3**. A statistical analysis of differences could not be carried out on lyso-PC and PG due to unequal variance in the results. Results of buttermilk powder phospholipid profile extracted by the Folch/Bitman methods in the first set of experiments (**Table 1**) and in the second set of experiments (**Table 3**) differ, likely due to the buttermilk powders used in the two sets having derived from different sources of milk and processing conditions, especially spray-drying, as different protein aggregates were observed by confocal laser scanning microscopy (results not shown). In addition to the processing, the storage of the two powders may have affected the degradation of some phospholipids into lysophospholipids.

Before extraction, the raw milk was pumped and kept in a refrigerated holding tank at 4 °C for a few hours. This step may lead to hydrolysis of the neutral and polar lipids of the

membrane (24), explaining the presence of lyso-PC, lyso-PE, and PA in the raw milk phospholipid extracts. During processing of the buttermilk powder, the sheet-like MFGM material (25) and other components are subjected to long holding time and drastic conditions such as spray-drying and high pasteurization temperatures. The buttermilk powder components are therefore likely to oxidize or denature. Buttermilk powder also contains more lyso-PC than the other three dairy products (**Table 3**), meaning that PC is degraded during the churning process or the spray-drying or during the storage of the buttermilk powder, rather than during the Folch and Bitman extractions.

Deeth (26) used phospholipases to determine the asymmetrical distribution of the MFGM phospholipids. Centrifugation leads to a loss of phospholipids from the membrane surface (27). As SM and PC are polar lipids and mainly located in the outer leaflet

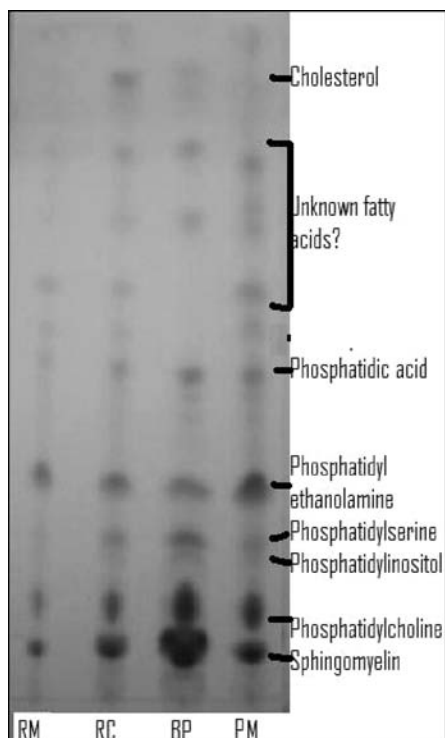


Figure 2. Thin-layer chromatography plate showing the migration of the phospholipids of raw milk (RM), raw cream (RC), buttermilk powder (BP), and processed milk (PM) extracted by a total lipid Folch extraction followed by an SPE using the Bitman method.

Table 3. Phospholipid Composition of Raw Milk, Raw Cream, Processed Milk, and Buttermilk Powder^a

sample phospholipid	raw milk mol %	raw cream mol %	processed milk mol %	buttermilk powder mol %
total lyso-PC	1.0 ± 0.1	0.7 ± 0.1	1.08 ± 0.02	3.6 ± 0.2
total PC	36.6 ^b	31.1 ^a	40.3 ^c	44.3 ^d
total SM	21.8 ^b	17.7 ^a	24.1 ^b	23.9 ^b
total ePC	5.4 ^b	4.4 ^a	5.2 ^b	6.8 ^c
total lyso-PE	0.5 ^b	0.4 ^a	0.5 ^b	0.4 ^a
total PE	22.6 ^c	24.8 ^d	20.8 ^b	7.3 ^a
total PE-cer	0.0087 ^b	0.0074 ^{ab}	0.0055 ^{ab}	0.0019 ^a
total ePE	1.0 ^c	0.9 ^{bc}	0.9 ^b	0.3 ^a
total PI	2.9 ^b	8.8 ^d	2.3 ^a	7.1 ^c
total PS	1.7 ^a	6.7 ^c	1.3 ^a	4.0 ^b
total PG	4.6 ± 0.8	3.6 ± 0.5	2.3 ± 0.1	0.51 ± 0.03
total PA	1.8 ^c	0.8 ^a	1.2 ^b	1.8 ^c

^aThe phospholipids were extracted following the Folch method for total lipid extraction and the Bitman method for the solid-phase extraction. Means within a row with different letters differ ($P < 0.05$). Abbreviation: PG, phosphatidylglycerol.

of the bilayer (26), they are likely to be more easily released to the milk serum upon centrifugation. The lipidomics data confirmed this hypothesis because SM and PC are less abundant in raw cream than in raw milk. This has also been reported by Christie et al. (18), who observed a higher proportion of SM in skimmed milk than in whole milk. The significantly lower amounts of SM and PC and higher amounts of PI and PS observed between the raw cream and raw milk phospholipids (Table 3) can also be explained by the difference in composition according to the size of the globules. Michalski et al. (28) found that small milk fat globules, separated by microfiltration, had different chemical and functional properties than large fat globules. This size separation could be advantageous for the quality of cheeses and the production of new dairy products.

The raw cream, obtained after centrifugation of the raw milk, mainly consisted of the larger milk fat globules. After homogenization and pasteurization, a part of the MFGM rearranges at the surface of the globule with the absorbed whey proteins and caseins (29). The amount of the major phospholipids PC, PE, and PI was significantly affected by these two processing steps (Table 3). Keenan (30) reported no alteration of the phospholipid ratios in MFGM and skim milk after homogenization. This leads to the conclusion that pasteurization may be responsible for the different phospholipid profiles in raw and processed milk.

The amount of PE in raw milk, raw cream, and processed milk phospholipids is within the range of values reported in the literature (31), indicating that the Bitman method is not likely responsible for the low amount of PE found in buttermilk powders (Tables 1 and 3) but, rather, that the powders have a low PE content due to spray-drying as observed by Morin et al. (5). The relative composition of the buttermilk powder phospholipids has 3 times less PE than the other samples (Table 3). Another explanation is that this phospholipid is more hydrophobic than PC and SM and might be located on the layer surrounding the triglyceride core of the globule and on the inner leaflet of the bilayer and, thus, partitions into the butter phase during butter manufacture. Keenan and Patton (24) reported that not all of the membrane material goes into the buttermilk serum during churning; some MFGM material remains on the surface of the fat globules in the butter phase. This could indicate that PE is found in high quantity in the monolayer surrounding the lipid core.

The SM content was constant for raw milk, processed milk, and buttermilk powder (Table 3). Only centrifugation significantly affected the relative amount of SM in the total phospholipid extract (Table 3). The amounts of PC and PE were significantly affected by processing (Table 3). PA and lyso derivatives of PE and PC were found in all four samples. Keenan and Patton (24) reported the presence of lyso derivatives of phospholipids in milk, but these lysophospholipids, as well as PA, are also considered by others as artifacts due to degradation during extraction or phospholipase activity during handling and storage (18). Sánchez-Juanes et al. (31) did not detect the presence of lyso-PE in raw whole milk but did in MFGM extracts. The values of raw milk and buttermilk powder phospholipids found in the literature are usually expressed as weight percentages and present a very wide variation. Pasteurized cream phospholipid data have been reported in the literature (32); however, only the main phospholipids, PC, PE, SM, PI, and PS, were reported (18, 31, 32). Sometimes, PS and PI amounts are combined due to poor chromatographic separation, as seen in the study by Sánchez-Juanes et al. (31). These authors reported higher amounts of PE, PI, and PS and lower amounts of PC and SM in MFGM than in whole milk. The raw cream phospholipids (Table 3) had the same profile as the MFGM phospholipids, as the raw cream phospholipids mainly contain MFGM phospholipids.

In the literature, the content of major phospholipids in raw milk varies greatly: 19.2–37.3% w/w PC, 19.8–42.0% w/w PE, 18.0–34.1% w/w SM, 0.6–11.8% w/w PI, and 1.9–10.5% w/w PS (1, 6, 11, 32). Jensen and Clark (33) reported the phospholipid and sphingolipid composition of bovine milk as 34.5 mol % PC, 31.8 mol % PE, 25.2 mol % SM, 3.1 mol % PS, 4.7 mol % PI, 3 mol % plasmalogens, and traces of lyso-PC, lyso-PE, ceramides, and diphosphatidylglycerol. Hay and Morrison (34) reported the presence of ePE and ePC in buttermilk powder phospholipids, 4% of alkenyl ethers in PE, and 1.3% in PC. These variations can be attributed to the differences in the method of extraction and analysis of the phospholipids, the handling of milk, and animal

Table 4. Degree of Unsaturation of the Phospholipids Present in Raw Milk, Raw Cream, Processed Milk, and Buttermilk Powder Obtained following the Folch Method for Total Lipid Extraction and the Bitman Method for Solid-Phase Extraction^a

		raw milk mol %	raw cream mol %	processed milk mol %	buttermilk powder mol %
total lyso-PC	saturated	0.6	0.4	0.6	1.4
	monounsaturated	0.3	0.2	0.3	1.5
	polyunsaturated	0.1	0.1	0.2	0.7
total PC	saturated	11.7	7.8	9.8	10.2
	monounsaturated	11.5	9.6	13.1	11.6
	polyunsaturated	13.3	13.5	17.2	22.3
total ePC	saturated	1.3	1.0	1.2	1.5
	monounsaturated	3.4	2.8	3.2	4.0
	polyunsaturated	0.7	0.7	0.7	1.3
total SM	saturated	20.9	16.8	22.6	22.5
	monounsaturated	0.9	0.8	1.5	1.4
	polyunsaturated				
total lyso-PE	saturated	0.1	0.0	0.1	0.0
	monounsaturated	0.3	0.2	0.3	0.3
	polyunsaturated	0.2	0.1	0.1	0.1
total PE	saturated	0.1	0.1	0.1	0.0
	monounsaturated	4.5	4.6	4.0	1.1
	polyunsaturated	18.1	20.1	16.7	6.2
total PE-cer	saturated	0.006	0.005	0.004	0.001
	monounsaturated	0.003	0.002	0.001	0.000
	polyunsaturated				
total ePE	saturated	0.0	0.1	0.0	0.0
	monounsaturated	0.3	0.3	0.3	0.1
	polyunsaturated	0.6	0.6	0.6	0.2
total PI	saturated	0.0	0.0	0.0	0.0
	monounsaturated	1.1	2.9	0.8	2.1
	polyunsaturated	1.8	5.9	1.5	5.0
total PS	saturated	0.0	0.0	0.0	0.0
	monounsaturated	0.7	2.4	0.5	1.3
	polyunsaturated	1.0	4.3	0.9	2.6
total PG	saturated	1.9	1.6	0.8	0.0
	monounsaturated	1.7	1.3	0.8	0.2
	polyunsaturated	1.0	0.7	0.7	0.3
total PA	saturated	0.0	0.0	0.0	0.0
	monounsaturated	0.1	0.1	0.1	0.1
	polyunsaturated	1.6	0.7	1.0	1.7
total PL	saturated	36.6 ^b	27.9 ^a	35.2 ^b	35.7 ^b
	monounsaturated	24.9 ± 3.3	25.3 ± 3.0	24.9 ± 2.1	23.6 ± 3.1
	polyunsaturated	38.5 ^a	46.7 ^c	39.7 ^{ab}	40.5 ^b
	saturated/unsaturated	0.6 ^b	0.4 ^a	0.5 ^b	0.6 ^b

^a Means within a row with different letters differ ($P < 0.05$).

factors such as breed, stage of lactation, diet, and season of milking.

Fatty Acid Distribution. SM is a highly saturated phospholipid (**Table 4**). The main fatty acids in SM are C16:0, C22:0, and C24:0 for all four samples (**Table 5**). These fatty acids have also been reported in the literature as major dairy SM fatty acids (1, 35). PE-cer is mainly composed of the fatty acids C16:0, C18:1, and C24:0 (results not shown). PC is the most saturated glycerophospholipid (**Table 4**). PE is the most polyunsaturated phospholipid and has very low degree of saturation

(**Table 4**). Raw cream phospholipids were significantly less saturated than the phospholipids from raw milk, processed milk, and buttermilk powder (**Table 4**), mainly due to a lower amount of SM in raw cream phospholipids (**Table 3**). The degree of monounsaturation was not significantly different across the range of processed dairy products. The values observed here differ from the values reported by Sánchez-Juanes et al. (31), possibly due to different techniques of extraction and analysis of phospholipids; the cold extraction method they used consisted of a total lipid extraction with a longer time that

Table 5. Fatty Acid Distribution of Sphingomyelin Present in the Four Dairy Extracts Obtained following the Folch Method for Total Lipid Extraction and the Bitman Method for Solid-Phase Extraction^a

sample description	raw milk mol %	raw cream mol %	processed milk mol %	buttermilk powder mol %
SM 16:1	0.29 ± 0.01	0.24 ± 0.02	0.38 ± 0.02	0.35 ± 0.02
SM 16:0 (or DSM 16:1)	8.20 ± 0.15	6.09 ± 0.22	8.58 ± 0.89	7.76 ± 0.41
DSM 16:0	nd	nd	0.33 ± 0.73	nd
SM 18:1	0.11 ± 0.08	0.08 ± 0.07	0.26 ± 0.04	0.04 ± 0.10
SM 18:0 (or DSM 18:1)	0.69 ± 0.09	0.69 ± 0.30	1.44 ± 0.47	0.72 ± 0.67
DSM 18:0	nd	nd	nd	nd
SM 22:1	nd	nd	nd	nd
SM 22:0 (or DSM 22:1)	6.02 ± 0.97	5.10 ± 0.61	6.03 ± 1.60	7.11 ± 1.75
DSM 22:0	1.10 ± 0.49	0.64 ± 0.49	1.08 ± 0.48	1.04 ± 0.38
SM 24:1	0.53 ± 0.05	0.52 ± 0.09	0.86 ± 0.06	1.02 ± 0.13
SM 24:0 (or DSM 24:1)	4.80 ± 0.47	4.32 ± 0.10	5.17 ± 0.55	5.76 ± 0.40
DSM 24:0	0.10 ± 0.07	nd	0.01 ± 0.02	0.09 ± 0.06
total SM	21.85 ± 1.08	17.68 ± 0.85	24.14 ± 2.06	23.89 ± 2.17

^a Abbreviation: DSM, dihydrosphingomyelin.

may have involved different degrees of recovery of certain classes of phospholipids (36). They did not use a SPE to separate the phospholipids from neutral lipids and determine the classes of phospholipids by two-dimensional TLC (31). The analysis of phospholipid fatty acids, in their case, was carried out by gas chromatography–mass spectrometry.

The ESI-MS/MS technique used in this study to determine the fatty acid profile of the phospholipids does not structurally differentiate between the two attached fatty acids. The results for SM, PE-cer, and lysophospholipids give the chain length of the single fatty acid attached to the polar headgroup, whereas the results for the other glycerophospholipids correspond to the summation of the individual chain lengths of the total acyl chains. The chain length of the fatty acids in SM (Table 5), PE-cer, lyso-PE, and lyso-PC (results not shown), varied between C16 and C24. The added chain length of the acyl chains of the other glycerophospholipids in the four phospholipid mixtures was 26–44 carbons in length (results not shown). The MFGM phospholipids have longer chain fatty acids than the triglycerides of the lipid core (37). The milk phospholipid acyl chains have been reported to be 10–24 carbons in length with the major ones being C14:0, C16:0, C18:0, C18:1, and C18:2 (1, 31, 38). Sánchez et al. (31) reported the presence of medium-chain fatty acids (C10–C17), long-chain fatty acids (C18–C20), and very long-chain fatty acids ($C > 20$) in phospholipids from MFGM extracts and whole milk; long-chain fatty acids were the most abundant in the glycerophospholipids, whereas SM contained more of the very long-chain fatty acids. The presence of very long-chain fatty acids (>20 carbons in length) in phospholipids and the high saturation content of SM fatty acids contribute to the structure of the milk fat globule membrane by maintaining its rigidity. The high degree of unsaturation of the glycerophospholipids (Table 4) may play an essential role in the fluidity of the MFGM (39). Again, the differences observed in the literature arise from animal factors such as the diet and breed of cows and the methods of extraction and analysis of the phospholipids.

DISCUSSION

In this work, we found that for a more accurate estimation of the amount of lipids present in a sample, hydration of the sample prior to extraction is necessary (18). This was carried out in the Mojonnier extraction, but it was not possible in the Folch extraction as it led to the formation of a stable suspension that was almost impossible to filter. The Folch method tends to give values on the low side (21).

Eder et al. (36) studied the effect of solvent and period of extraction on the recovery of extracted phospholipids from erythrocyte membranes. The recovery of PC and SM was not influenced by these two factors, whereas PS, diacyl PE, and plasmalogen PE and their fatty acid compositions were greatly influenced by the solvent and the time chosen for the extraction. This could be related to the presence of PC and SM in the outer leaflet of the MFGM (26), making them readily extracted, and to the presence of PE and PS in the inner leaflet, requiring more effective extraction conditions.

The extraction of phospholipids is a difficult task as they are usually associated with proteins or polysaccharides (18) and they are very sensitive to hydrolysis and oxidation. The cold extraction methods seem less oxidative of the polyunsaturated fatty acids (PUFA). The heating step in the Mojonnier technique may explain the partial degradation of the fatty acids of the phospholipids. The oxidative degradation of PUFA leads to undesirable oxidized flavors in dairy products (24). The analysis of phospholipids is often carried out by HPLC (40–42) or densitometric TLC (1), and the fatty acid composition is determined by gas–liquid chromatography of fatty acid methyl esters or butyl esters (1, 35) measuring fatty acids, respectively, from C:4 to C:22 with an improved detection of short-chain fatty acids by conversion into butyl esters (37). In this study, variation observed with the literature may arise from the use of a different technique of analysis, tandem mass spectrometry. ESI-MS/MS allows the identification of both phospholipid species and their fatty acid content, requiring a smaller quantity of phospholipid extracts than TLC followed by gas chromatography or gas chromatography–mass spectrometry, for example (43).

Milk processes such as spray-drying lead to considerable oxidation of the phospholipids (18). It has been suggested that spray-dried buttermilk powders may not contain any phospholipids because of autoxidation during spray-drying processing. During churning, MFGM material binds to air cells, leading to possible oxidation (24). The lower amount of PE in buttermilk powder due to spray-drying is also an important factor to consider when sourcing phospholipids to form vesicles or liposomes. Indeed, Waninge et al. (44) reported the impact of PE concentration on the phase behavior of phospholipid mixtures, shifting from a lamellar phase to a reversed hexagonal phase at high PE concentration.

The distribution of the fatty acids and the headgroup of the individual phospholipid species in the four dairy products under study determine the physical characteristics of the products and, in particular, the MFGM structure. The variability of the

phospholipids present and their fatty acids content are a response of the MFGM to the surrounding environment. Saturated phospholipids such as SM are involved in lipid raft formation by association with cholesterol in biomembranes. PUFA play key roles such as providing essential fatty acids, eicosanoid precursors, and membrane components. The degree of saturation and the length of the fatty acids of phospholipids play a key role in phase separation in membrane systems (45) and are thus involved in the fluidity/rigidity of the MFGM surrounding the liquid lipid core of the fat globule.

We can conclude that, after two major total lipid extractions and three common phospholipid SPEs were compared, the Folch cold extraction followed by the Bitman SPE was the most efficient and reproducible method in the recovery of sphingomyelin, phosphatidylinositol, and phosphatidylserine (compared to the Mojonnier/Bitman methods). Additionally, this method demonstrated more consistent results in the recovery of phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine (compared to the Mojonnier/Vaghela and Mojonnier/Avalli methods). The phospholipid profile of the four dairy products selected to exemplify different stages of milk processing was evaluated to study the effect of milk processing on their total phospholipid composition. We have focused on observed differences in sphingomyelin content as this component seems to be implicated in beneficial health aspects. The presence of long-chain and very long-chain fatty acids in all milk phospholipid extracts plays a role in the rigidity of the MFGM. Milk processing indeed affects the profile of milk phospholipids on the MFGM, causing a redistribution between the membrane and the milk serum or to other fractions. We observed most significantly a loss of sphingomyelin by centrifugation and a loss of phosphatidylethanolamine after spray-drying of buttermilk. In some applications, sphingomyelin is an important factor to consider when sourcing dairy phospholipids as it is likely involved in valuable biological and structural functions. Sphingomyelin is also implicated in the formation of lipid rafts in cells; thus, a loss of sphingomyelin (a saturated polar lipid) after centrifugation may induce changes in the fluidity of the MFGM and the structure of the lipid microdomains on the surface of the MFGM.

ABBREVIATIONS USED

MFGM, milk fat globule membrane; SPE, solid-phase extraction; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; DSM, dihydro sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; lyso-PG, lysophosphatidylglycerol; ePC, ether phosphatidylcholine; ePE, ether phosphatidylethanolamine; PE-cer, phosphoethanolamine-ceramide; PL, phospholipids; nd, nondetected; ESI-MS/MS, electrospray tandem mass spectrometry; NL, neutral loss; TLC, thin-layer chromatography; Std, standard; BMP, buttermilk powder; Mojo, Mojonnier; RM, raw milk; RC, raw cream; BP, buttermilk powder; PM, processed milk; PUFA, polyunsaturated fatty acid; HPLC, high-performance liquid chromatography.

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