Phospholipid enrichment in sweet and whey cream buttermilk powders using supercritical fluid extraction

A. J. Spence,* R. Jimenez-Flores,†¹ M. Qian,* and L. Goddik*

*Department of Food Science and Technology, Oregon State University, Corvallis, 97330

†Dairy Products and Technology Center, California Polytechnic State University, San Luis Obispo, 93407

ABSTRACT

Milk fat globule membrane contains many complex lipids implicated in an assortment of biological processes. Microfiltration coupled with supercritical fluid extraction (SFE) has been shown to provide a method of concentrating these nutritionally valuable lipids into a novel ingredient. In the dairy industry there are several by-products that are rich in phospholipids (PL) such as buttermilk, whey, and whey cream. However, PL are present at low concentrations. To enrich PL in buttermilk powders, regular buttermilk and whey buttermilk (by-product of whey cream after making butter) were microfiltered and then treated with SFE after drying. The total fat, namely nonpolar lipids, in the powders was reduced by 38 to 55%, and phospholipids were concentrated by a factor of 5-fold. Characterization of the PL demonstrated specific molecular fatty amide combinations on the sphingosine (18:1) backbone of sphingomyelin with the greatest proportion being saturated; the most common were 16:0, 20:0, 21:0, 22:0, 23:0, and 24:0. Two unsaturated fatty amide chains, 23:1 and 24:1, were shown to be elevated in a whey cream buttermilk sample compared with the others. However, most unsaturated species were not as abundant.

Key words: supercritical fluid extraction, phospholipid, buttermilk lipid, sphingomyelin

INTRODUCTION

Milk fat consists of small globules surrounded by a surface layer or membrane, termed the milk fat globule membrane (MFGM). This membrane has a complex composition and structure and functions to prevent coalescence of the fat globules (Deeth, 1997; Walstra et al., 1999). In addition to maintaining globule structure, the MFGM features several complex lipids, phospholipids (PL), sphingolipids (SL), and glycolipids shown

to be involved with a variety of biological processes (Molkentin, 2000; Dacaranhe and Terao, 2001; Berra et al., 2002). During the process of churning cream into butter, fat globules coalesce as a result of air beaten into the cream. The surrounding MFGM, as well as some globule fat, remains in the liquid buttermilk fraction. Milk fat globule membrane contains 70% of membrane protein; the approximate ratio of proteins, lipids, and carbohydrates within the membrane is 4:3:1. The phospholipid fraction of the membrane is 25% (Walstra et al., 1999). The major PL fractions consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS) (Renner et al., 1989; Barenholz and Thompson, 1999). Sphingolipid in particular is known to have essential roles in cell-to-cell interactions, differentiation, proliferation, immune recognition, and transmembrane signaling (Okazaki et al., 1989; Kim et al., 1991; Huwiler et al., 2000; Lightle et al., 2000). There are no nutritional requirements for PL or SL; however, recent studies implicate a relationship between dietary consumption and health, suggesting that they are functional components of food (Berra et al., 2002).

Typically found in membrane-rich tissues such as pancreas, liver, brain, and neural, SL and their breakdown products not only play a structural role in lipid bilayers, but have profound effects on cell regulation (Barenholz and Thompson, 1999; Vesper et al., 1999; Cinque et al., 2003). Sphingolipids consist of a long-chain sphingoid backbone may represent the most structurally diverse, as well as complex, group of lipids in nature (Vesper et al., 1999; Berra et al., 2002). Along with PC, SM form one of the major classes of bovine milk polar lipids and comprise more than 50% of membrane polar lipids (Barenholz and Thompson, 1999). Until recently, SM was thought to be metabolically inert and only functioned as a structural component (Hanahan, 1997). However, the roles that SM and its metabolites play in cellular apoptotic pathways give evidence that they may have potential uses as anticancer agents or for regulating disorders in which apoptosis plays a crucial role (Perry et al., 1996; Nava et al., 2000). Many of their roles in

Received July 7, 2008.

Accepted November 25, 2008.

¹Corresponding author: rjimenez@calpoly.edu

signaling pathways are due to their structures, varying in polar head group and fatty acid components as well as in their association with other molecules such as glycoproteins (Sullards, 2000). Because of the unique composition and concentration of these lipids, MFGM in buttermilk could be used as a source of SL.

Several attempts in fractionating buttermilk components have been made. An investigation by Rombaut et al. (2006) determined the effects of MFGM separation from butter serum via microfiltration (MF) upon addition of sodium citrate for casein micelle disassociation. Destabilization of the micelle is thought to enable transmission of the protein through the membrane to decrease fouling and separate MFGM material. Although disassociation of the micelles was found to improve their permeation flux, a high fouling rate of PL was observed. As such, microfiltration, even with the aid of destabilizing the casein micelles, is insufficient in MFGM purification from butter serum.

Morin et al. (2004) reported on the effects of temperature and pore size in MF of fresh and reconstituted buttermilk. Higher amounts of retained fat coupled with a large amount of protein transmission were observed when using a temperature of 25°C. Buttermilk fractionation was also affected by pore size: both protein and lipid contents were increased in the retentate when using a 0.1- μ m membrane. In addition, improved separation between lipids and proteins was shown using fresh rather than reconstituted buttermilk.

Whey cream (WC) is primarily used to standardize milk fat before cheese making and can be used to make whey butter and, in turn, whey buttermilk (Fox et al., 2000). Sodini et al. (2006) indicated that whey buttermilk is a potentially novel ingredient showing higher emulsification properties and lower foaming ability, along with stable levels of protein solubility, viscosity, and emulsifying capacity over a pH range of 4 to 6. Morin et al. (2006) compared the volumetric concentration (VCMF) and diafiltration (DFMF) of both regular and WC buttermilk in terms of separation efficacy and permeation flux. A 2-fold MF concentration by both processing methods increased the PL content in WC buttermilk by 50%.

Moreover, coupling the MF process with supercritical fluid extraction (SFE) can selectively remove fat from a complex mixture. A previous study by Astaire et al. (2003) found that by combining MF and SFE, the triglyceride could be effectively removed from buttermilk, whereas bioactive lipids of the MFGM could be concentrated in buttermilk. To our knowledge, using these techniques in conjunction to develop a novel buttermilk ingredient has not been explored further. Additionally, there are no current uses for buttermilk obtained from WC; therefore, a process to enrich MFGM

components could lead to a promising utilization of this by-product.

The objective of the present work was to enrich the PL content in buttermilk powder obtained from both regular cream and WC. The effectiveness of the coupled microfiltration and SFE processes was based on the comparison of compositional differences (lipids, proteins, phospholipids) of initial and final products. Sphingomyelin structure was described to show the unique composition of the final products.

MATERIALS AND METHODS

Chemicals and Reagent

Lipid standards, SM, PC, PS, PI, and PE were purchased from Sigma Chemical Co. (St. Louis, MO). Silica-gel thin layer chromatography (TLC) plates (60 Å) were from EMD Chemicals (Darmstadt, Germany). All solvents and other reagents were of analytical grade and purchased from Fisher Scientific (Tustin, CA).

Buttermilk Production

For each trial, 200 L of fresh cream or WC was processed. Fresh regular manufacturing cream (RC) was provided by Foster Farms (Modesto, CA) and composition was as follows: protein, $3.81 \pm 0.04\%$; lipid, $74.47 \pm 4.19\%$; ash, $0.85 \pm 0.10\%$. Whey cream was graciously donated by Hilmar Cheese Co. (Hilmar, CA) and was derived from the production of a variety of different types of cheeses; WC composition was as follows: protein, $1.34 \pm 0.23\%$; lipid, $50.69 \pm 24.52\%$; ash, $0.39 \pm 0.13\%$. Butter was made using a continuous pilot-scale butter churn (Egli, Switzerland). Butter fines were removed from the buttermilk by filtration into milk cans through cheese cloth. Regular buttermilk production was repeated 3 times and WC buttermilk was repeated twice with different lots of both creams. The composition of RC buttermilk was as follows: protein, $25.01 \pm 0.76\%$; lipid, $12.22 \pm 1.56\%$; phospholipid, $0.14 \pm 0.04\%$ ash, $5.60 \pm 0.16\%$. The composition of WC buttermilk was as follows: protein, $13.10 \pm 1.65\%$; lipid, $27.42 \pm 20.87\%$; phospholipid, $0.10 \pm 0.10\%$; ash, $4.89 \pm 2.22\%$.

Microfiltration and Diafiltration. Microfiltration and diafiltration were performed according to the procedures described previously (Astaire et al., 2003; Morin et al., 2006). Briefly, the microfiltration unit consists of 2 tubular membranes containing Tami Sunflower Design ceramic membranes (Tami Industries, Nyons, France) fitted in parallel on the module. Total surface area was 0.7 m². A membrane with a pore size of 0.45 μ m was used and fitted in stainless steel

housings (US Filters, Warrendale, PA). All runs were carried out at low temperature (8–10°C) at a transmembrane pressure of 80 to 95 kPa. Previous studies have shown that for reconstituted buttermilk, a cold MF process had a more efficient retention of MFGM lipids; additionally, fluxes were lower and more stable during cold processing (Astaire et al., 2003; Morin et al., 2004). The microfiltration process termed VCMF consisted of concentrating the buttermilk by microfiltration to a volumetric concentration factor (**VCF**) of 2×. In the diafiltration DFMF process, chilled tap water was continuously added to the retentate in order to replace the extracted permeate until a dilution factor (**DF**) of 2× was reached (i.e., twice the amount of the starting volume). Transmission (**Tr**) of proteins, lipids, phospholipids and ash through the membrane was calculated according to the equation:

$$\text{Tr (\%)} = (\text{Cp/Cr}) \times 100,$$

where Cp is the concentration of a component in the permeate and Cr the concentration of the same component in the retentate (Morin et al., 2006).

Spray-Drying. Final retentates from both processes were spray dried using a Niro Filterlab Spray Dryer (Hudson, WI) following the manufacturer's instructions. The water evaporation capacity of the equipment is 100 lb/h. The spray nozzles had a core of size 16, the orifice was size 70, and the pressure was 500 psi. The powdered product is termed buttermilk powder (**BMP**).

SFE. The SFE system and components were acquired from Thar Designs Inc. (Pittsburgh, PA) and operating conditions were described previously by Astaire et al. (2003). Briefly, the in-house unit included the following: 500-mL vessel, model P-50 high-pressure pump, automated back pressure regulator model BPR-A-200B, and PolyScience (Niles, IL) brand water bath and pump unit (model 9505). Circulated water at 5°C was used for cooling different zones in the SFE apparatus. Carbon dioxide was from Airgas West (San Luis Obispo, CA). The system conditions were controlled manually by Windows 2000-based software (Hewlett-Packard, Palo Alto, CA). Buttermilk powder (300 g) was mixed 1:1 with Teflon (polytetrafluoroethylene) beads and rings (24.2 g), 3 mm in diameter (Fisher Scientific), and placed in an inert bag made of Rapid-flow milk filter tubes (Filter Fabrics Inc., Goshen, IN). The running conditions were as follows: flow rate, 20 g/min; total vessel flushes 3.13 ± 0.23 ; total run time, 86.00 ± 0.06 min; and total CO₂ used, $1,560.0 \pm 116$ g. Following treatment, the fat in the SFE extraction vessel was drained and stored at 4°C until further

analysis; a portion of the fat was diluted to 10 mg/mL in chloroform-methanol (2:1, vol:vol). To ensure complete removal of fat, the vessel was rinsed with 50 to 100 mL of chloroform:methanol (2:1 vol/vol) and discarded. Concentration factor (**CF**) was calculated for phospholipids using:

$$\text{CF} = \text{Cf/Co},$$

where Cf is the final phospholipid concentration reached in the BMP following SFE treatment and Co is the original phospholipid concentration in the BMP before SFE treatment.

BMP Lipids

Lipid Profile Analysis. The total lipids (polar and nonpolar) were extracted from the SFE-treated BMP in duplicate by the Mojonnier method as described by Marshall (1992) for the analysis of BMP and buttermilk-derived powders. For each extraction, 1 g of powder was reconstituted in the Mojonnier tube with 8.5 mL of warm water.

Lipid profiles were analyzed by TLC. The polar lipids were separated using chloroform:methanol:water (65:25:4, vol/vol); nonpolar lipids were separated using petroleum ether:ethyl ether:acetic acid (85:15:2, vol/vol) (Astaire et al., 2003). Lipids were prepared in chloroform:methanol (2:1, vol/vol) to 10 mg/mL, and lipid standards were diluted to 1 mg/mL in preparation for TLC analysis. One hundred micrograms of sample and 5 µg of standard was applied using glass capillaries. Lipids were visualized by exposure to iodine vapor and then identified by comparison to standards.

Phospholipid Composition. Phospholipids from all powder samples were analyzed by HPLC using a Biologic DuoFlow (Bio-Rad Laboratories, Hercules, CA) upgraded with high-pressure pump (model F10). The column was a LiChrospher DIOL 100, 5 µm, 4.6 i.d. × 150 mm (Alltech, Deerfield, IL). Lipids were collected and analyzed with an evaporative light-scattering detector (Sedex 55, Sedere, Alfortville Cedex, France) and EZ-Logic software (Bio-Rad Laboratories). The injector valve (AVR 7-3) was fitted with a 25-µL injection loop. Lipid samples were diluted from 10 to 5 mg/mL before injection. The separation was performed at ambient temperature of 21°C by linear gradient elution described by Rombaut et al. (2005) with 87.5:12:0.5 (vol/vol/vol) of chloroform:methanol:triethylamine buffer (pH 3, 1 M formic acid) at time (*t*) = 0 to 28:60:12 (vol/vol/vol) at *t* = 16. At *t* = 17 min, the mobile phase was brought back to the initial conditions and the column was allowed to equilibrate until the next injection

at $t = 21$ min. The flow was maintained at 0.5 mL/min. For detection, the nebulizing gas of N_2 was used at a flow rate of 1.8 L/min, and the nebulizing temperature was 85°C. The gain was set at 4.5. Components were identified and quantified using calibration curves made with PE, PI, PC, PS, and SM standards. There was variability in PL content among the triplicate samples for the VCMF RC experimental point. The authors have excluded the PL data from the first buttermilk batch, as it was an outlier based on the high standard deviation.

Sphingomyelin Analysis. The Mojonnier extractions previously dissolved in chloroform:methanol (2:1), were dried under N_2 flow and resuspended in 3 mL of chloroform. Polar lipids were separated by solid-phase extraction using 500-mg silica columns (Strata SI-1 SPE silica, 55 μ m, Phenomenex, Torrance, CA). Columns were conditioned with 6 mL of chloroform at 3 to 4 mL/min flow, polar lipid samples concentrated by SPE were loaded at 1 to 2 mL/min, columns were washed with 6 mL of chloroform at 3 to 4 mL/min, and the polar lipids were eluted in 6 mL of methanol. Following SPE, samples were hydrolyzed in methanolic KOH (0.1 M, 40°C for 3 h) to saponify glycerolipids. Hydrolyzed samples were re-extracted by a procedure described by Merrill and Wang (1986). Specifically, a solution of 1:2 chloroform:methanol (1.5 mL) was added to the hydrolyzed lipids and mixed thoroughly; 1 mL each of chloroform and water were added and centrifuged (1,000 $\times g$ for 2 min); the upper phase, containing the hydrolyzed and saponified glycerolipids, was discarded and the chloroform phase, containing intact SM, was washed twice with water and dried under N_2 .

Sphingomyelin molecular species was analyzed using a liquid chromatography MS/MS system at the Linus Pauling Institute (Oregon State University). Liquid chromatography was performed with a Shimadzu system: 2 LC-10Advp pumps, a DGU-14A degasser, a SIL-HT autosampler, and a CTO-10Avp column oven (Shimadzu, Columbia, MD). The column used was a Discovery C18 column, 50 $\times$ 2 mm i.d. $\times$ 5 μ m (Supelco, St. Louis, MO). The flow rate was 0.3 mL/min, the column temperature was 30°C, and the injection volume was 5 μ L. The equilibration time of the column was 3 min. The binary solvent gradient was 1 min 100% A, 8 min 60% A, 13 min 30% A, and 20 min 30% A. Solvent A was methanol:water:formic acid (60:40:0.2 vol/vol/vol) + 10 mM ammonium acetate; solvent B was methanol:chloroform:formic acid (60:40:0.2 vol/vol/vol) + 10 mM ammonium acetate. The HPLC system was coupled through a turbo ion spray source to a triple-quadrupole mass spectrometer (model API 3000, Applied Biosystems/MDS SCIEX, Foster City, CA). High-purity nitrogen was used as the nebulizer

gas (Polar Cryogenics, Portland, OR) and supplied at 6 L/min. The declustering and collision potentials were 25 and 35 V, respectively. A precursor scan using the MS system was performed by scanning for m/z 184 at a flow rate of 10 μ L/min. Data handling was performed using the Analyst 1.4.1 software (Applied Biosystems/MDS SCIEX). Prior to liquid chromatography MS/MS analysis, samples were diluted to 100 μ g/mL in solvent A. Long-chain bases (**LCB**) and fatty amides are referred to in the format number of carbons: number of double bonds in the aliphatic chain.

Nitrogen and Protein Determination

The amount of total nitrogen, nitrogen soluble at pH 4.6, and NPN from the treated BMP retentates were measured by Kjeldahl method (AOAC, 1995). Samples were heated on a Digestion System 20, model 1015 digester (Tecator, Höganäs, Sweden). Samples were distilled with the Kjeldahl System, 1026 Distilling Unit, and FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific) were used as reagents. Titrations were done using 0.1 N HCl, and the percentage protein was calculated using the milk protein conversion factor, 6.38. Total protein measurements were taken in duplicate. The NPN, expressed in protein equivalents, was calculated as $NPN \times 6.38$. The soluble protein at pH 4.6 was calculated as $(\text{soluble N} - NPN) \times 6.38$. The insoluble protein at pH 4.6 was calculated as $(\text{total N} - \text{soluble N}) \times 6.38$ (21).

Total Solids, Moisture, and Ash

Total solids were determined using the direct oven-drying method for milk using a forced air oven at $100 \pm 1^\circ\text{C}$. Percentage moisture was calculated by subtraction. Percentage ash was determined by incineration at 550°C

Statistical Analysis

Statistical analyses were performed with Minitab 14.0 software (Minitab Inc., State College, PA). All comparisons were done by ANOVA with Tukey's pairwise comparison using Minitab 14.0 software. Results were considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

BMP Lipids

After 3 extractions, SFE processing reduced the total fat in the powders by 36 to 55%. The average total solids, total ash (% of DM), total lipid (% of DM),

Table 1. The average total solids, total ash, total lipid, total phospholipid (PL), ratio of PL to protein, final concentration factor (CF) of PL, and lipid reduced in supercritical fluid extraction treated regular and whey buttermilk powder

Sample ¹	Total solids	Total ash	Total lipid	Total PL	PL/protein (mg/mg)	CF of PL	Lipid reduced (%)
BMP before SFE			(% of DM)				
DFMF RC	96.1 ± 1.2	6.1 ± 0.2	21.8 ± 0.7 ^{ab}	2.2 ± 0.5 ^{ab}	0.04 ± 0.01 ^a	1.8 ± 0.0 ^{ab}	
VCMF RC	97.3 ± 0.8	6.2 ± 0.2	16.7 ± 1.2 ^a	2.2 ± 0.3 ^{ab}	0.06 ± 0.01 ^a	1.5 ± 0.1 ^a	
DFMF WC	95.3 ± 2.9	5.5 ± 1.2	35.3 ± 10.9 ^b	0.9 ± 0.4 ^a	0.04 ± 0.02 ^a	1.7 ± 0.2 ^{ab}	
VCMF WC	95.4 ± 0.8	6.0 ± 0.7	23.1 ± 8.3 ^{ab}	0.7 ± 0.1 ^a	0.04 ± 0.01 ^a	2.3 ± 0.7 ^a	
BMP after SFE							
DFMF RC	94.8 ± 0.3	7.3 ± 0.2	12.3 ± 0.9 ^a	7.8 ± 1.1 ^c	0.15 ± 0.02 ^b	3.5 ± 0.8 ^b	44.7 ± 2.5
VCMF RC	95.8 ± 1.4	7.1 ± 0.3	11.4 ± 1.7 ^a	9.2 ± 1.6 ^c	0.24 ± 0.01 ^c	4.2 ± 1.4 ^b	35.6 ± 5.7
DFMF WC	96.9 ± 2.4	7.2 ± 0.3	21.7 ± 8.8 ^{ab}	3.2 ± 0.2 ^b	0.12 ± 0.02 ^b	3.9 ± 1.5 ^b	38.5 ± 5.1
VCMF WC	95.6 ± 2.9	7.4 ± 0.2	9.8 ± 0.6 ^a	3.0 ± 0.2 ^b	0.15 ± 0.01 ^b	4.2 ± 0.8 ^b	55.3 ± 10.7

^{a-c}Values in same column for each product with different superscripts differ significantly ($P < 0.05$); $\pm$ indicates standard deviation.

¹BMP = buttermilk powder; SFE = supercritical fluid extraction; DFMF = diafiltration; RC = regular cream buttermilk; VCMF = volumetric concentration; WC = whey cream buttermilk.

total phospholipid (% of DM), ratio of phospholipid to protein (mg:g), and final CF of PL using SFE treatments is shown in Table 1. The total lipid (nonpolar and polar) observed in the initial DFMF and VCMF WC buttermilk have a high variability compared with the other buttermilks specifically because of commercial origin, because we did not select the kind of process parameters. Total lipids between the DFMF WC BMP before SFE treatment differs significantly from VCMF RC before treatment as well as DFMF RC, VCMF RC, and VCMF WC following SFE treatment ($P = 0.002$). Lipid results showed that there was a high variability between the samples for the VCMF filtered powders, both WC and RC (as seen by the percentage weight SD of 6 and 11, respectively). It is interesting to note that although the DFMF whey BMP had the highest value of total lipids, it also had a very low value for PL. The DFMF powders showed less variability in relation to percentage of lipid reduction for both WC and RC (SD of 5 and 3, respectively). However, there was no difference in percentage of lipid reduction between the powders ($P = 0.091$). Although the amount of total lipid varied among the different powders, we do not expect the ratio between nonpolar and polar lipids to differ significantly between the powders (Walstra et al., 1999). Theoretically, when the densities of the supercritical fluid and the target analyte (triglycerides in this case) are equal, then maximum solubility is achieved (Rozzi and Singh, 2002). Therefore, this result is expected because the SFE extractions were run at constant pressure, temperature, and extraction time.

The lipid variability seen in the BMP is due mainly to the initial variability in the starting material. Sources of variations in milk composition can be attributed to several factors, including stage of lactation, season, and herd (Walstra et al., 1999). High variations in whey cream before production of buttermilk have

been reported previously (Morin et al., 2006, 2007). This is caused by both the type of cheese that generates the whey and by differences in the operation conditions of the whey cream separator (Morin et al., 2006). The variations that were observed in the buttermilk powder are also related to the filtration process. Both the MF mode and buttermilk type had a significant effect on the amount of lipid transmission through the membrane (Morin et al., 2006). The average transmission of components through the membrane is shown in Table 2. Higher transmission was observed with DFMF, resulting from more stable fluxes and less fouling. In addition, whey buttermilk had lower lipid transmission, attributed to aggregation and presence of casein proteins in regular buttermilk (Morin et al., 2006). High variability of phospholipid transmission was due mainly to the difference in lipid distribution in the buttermilk. The portion of aggregates in WC is heterogeneous in size, and this globule mixture, upon churning, can rupture into a wide range of fragment sizes. Different phospholipid aggregates can also be attributed to the starter for the cheese process. The accumulation of these factors leaves the final product with high variability (Morin et al., 2006).

Concentration factor of the PL was calculated to illustrate the overall effectiveness of the SFE treatment. The CF was calculated on a dry basis by comparison of the PL concentration in the final BMP after SFE with the initial BMP. Results show that the calculated CF of the PL are very close, 3.49 to 4.24 ($P = 0.397$). These results are significantly different than the initial phospholipid CF ($P = 0.006$). Morin et al. (2006) demonstrated that MF of RC and WC buttermilk concentrated the PL by an average of 1.31 to 2.25. Our results indicate that an additional SFE treatment serves to further concentrate these bioactive lipids in the BMP, which is in agreement with others (Astaïre

Table 2. The average transmission (%; $\pm$ SD) of components through the membrane (adapted from Morin et al., 2006)

Component	Sample ¹			
	DFMF RC	VCMF RC	DFMF WC	VCMF WC
Lipids	8.50 $\pm$ 1.56 ^a	5.82 $\pm$ 1.70 ^{ab}	4.73 $\pm$ 2.03 ^a	3.02 $\pm$ 0.96 ^a
Phospholipids	39.13 $\pm$ 19.29 ^b	12.66 $\pm$ 2.70 ^a	9.88 $\pm$ 5.18 ^a	19.76 $\pm$ 14.66 ^{ab}
Protein	19.55 $\pm$ 4.29 ^{ab}	18.31 $\pm$ 2.79 ^a	25.04 $\pm$ 7.86 ^b	33.00 $\pm$ 4.79 ^c
Ash	65.53 $\pm$ 9.66 ^a	67.93 $\pm$ 2.84 ^a	88.02 $\pm$ 5.98 ^b	102.66 $\pm$ 7.64 ^c

^{a-c}Values in same row for each product with different superscripts differ significantly ($P < 0.05$).

¹DFMF = diafiltration; RC = regular cream; VCMF = volumetric concentration; WC = whey cream.

et al., 2003), and that there is no difference between CF for the type of starting material. The PL content increased significantly following the SFE treatment ($P = 0.000$) and the PL (mg) to protein (mg) ratio also increased significantly ($P = 0.000$). The percentage PL is important, but more relevant is the ratio of PL to protein. This directly relates the PL content to measured solids contained in the buttermilk powder. From Table 1, a $3\times$ increase in the ratio is observed following SFE treatment for all buttermilk samples.

The mean phospholipid distribution (as percentage of PL) in the SFE-treated BMP samples is shown in Table 3. The phospholipid distribution among the samples did not differ significantly ($P > 0.05$), although there were some interesting observations. Distribution for PS, PC, and SM are similar between samples and throughout the process; however, for these lipids, the amounts observed in the VCMF samples are elevated compared with DFMF, possibly attributed to the filtration method. The distribution of PE and PI differ among the powders. The PE content for the RC samples shows that the VCMF value is slightly higher than DFMF and the opposite is true for the WC samples. Similarly, PI content is higher for DFMF and lower for VCMF RC and opposite effects are shown for the WC buttermilk.

We have also described the specific fatty amide combinations as found on a sphingosine backbone for intact

sphingomyelin in our BMP samples. Molecular species of intact SM from the Mojonner-extracted lipids was separated by using a precursor scan at m/z 184, representative of phosphocholine $[\text{PO}_4(\text{CH}_2)_2\text{N}(\text{CH}_3)_3 + \text{H}]^+$ and analysis of fatty amide combinations as measured by a sphingosine LCB backbone (18:1) thereof. At least 28 different fatty amide combinations were detected covering a range of molecular masses from 647.5 to 841.7 Da (Table 4). The combinations found are similar to those described in previous studies (Karlsson et al., 1998; Byrdwell and Perry, 2007). By using several techniques in combination Karlsson et al. (1998) was able to describe intact SM from bovine milk by both the LCB and fatty amide combinations. The most common LCB were 16:1, 17:1, 18:1 and 19:1; moreover, the most common fatty amides attached to the 18:1 LCB were 16:0, 20:0, 22:0, 23:0, and 24:0. An additional study by Byrdwell and Perry (2007) described the most abundant SM species in bovine milk to be a combination of an 18:1 LCB with a 16:0 or 23:0 fatty acyl chain. Bovine milk SM are more complex with relation to other SM such as those found in bovine brain or egg yolk. They include a greater diversity of species, including odd-numbered carbon amide chains (Karlsson et al., 1998; Byrdwell and Perry, 2007).

In our study, the 6 most common saturated fatty amide species ($>5\%$ of total) for SM detected were 16:0, 20:0, 21:0, 22:0, 23:0, and 24:0. Unsaturated fatty

Table 3. The average phospholipid content (% of total) in supercritical fluid extraction-treated regular and whey buttermilk powder and whey buttermilk powder¹

Phospholipid, ² %	Regular buttermilk powder		Whey buttermilk powder	
	VCMF	DFMF	VCMF	DFMF
PE	18.6 $\pm$ 10.7	16.9 $\pm$ 12.7	10.4 $\pm$ 5.4	22.0 $\pm$ 8.8
PI	0.7 $\pm$ 0.2	4.4 $\pm$ 1.6	10.0 $\pm$ 2.2	2.4 $\pm$ 0.5
PS	6.3 $\pm$ 1.2	6.0 $\pm$ 1.5	5.1 $\pm$ 2.1	4.8 $\pm$ 2.0
PC	31.3 $\pm$ 6.0	23.1 $\pm$ 2.8	35.7 $\pm$ 3.1	23.0 $\pm$ 1.2
SM	43.1 $\pm$ 18.0	49.6 $\pm$ 9.1	38.8 $\pm$ 6.8	47.8 $\pm$ 13.3

¹VCMF = volumetric concentration; DFMF = diafiltration.

²PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; and SM = sphingomyelin.

Table 4. The fatty amide composition (% of total) of sphingomyelin found in supercritical fluid extraction-modified treatments for regular and whey buttermilk powder (% of total)

Fatty amide	Sample ¹			
	DFMF RC	VCMF RC	DFMF WC	VCMF WC
C12:0	0.16	0.39	0.14	0.14
C13:0	0.04	0.10	0.03	0.06
C14:0	1.42	3.31	1.27	1.97
C15:0	1.04	2.10	0.87	1.26
C16:0	8.29	16.91	6.81	9.04
C16:1	0.18	0.39	0.14	0.24
C17:0	0.31	0.62	0.43	0.43
C17:1	0.07	0.14	0.03	0.08
C18:0	3.10	5.67	2.58	4.81
C18:1	0.33	0.64	0.27	0.47
C19:0	0.20	0.31	0.15	0.26
C19:1	0.02	0.02	0.02	0.05
C20:0	13.26	17.61	13.36	16.34
C20:1	0.40	0.47	0.35	0.75
C21:0	11.37	11.76	11.18	10.08
C21:1	0.39	0.44	0.40	0.47
C22:0	21.42	17.21	21.12	18.63
C22:1	1.08	0.54	0.75	1.60
C22:2	0.09	0.08	0.11	0.57
C23:0	15.08	9.33	15.51	10.42
C23:1	0.23 ^a	0.21 ^a	0.28 ^a	1.18 ^b
C24:0	17.37	9.40	18.85	13.87
C24:1	1.52 ^a	1.10 ^a	2.96 ^a	4.94 ^b
C24:2	0.21	0.11	0.17	0.30
C25:0	1.90	0.82	1.69	1.33
C25:1	0.20	0.18	0.16	0.45
C26:0	0.31	0.16	0.36	0.27
C26:1	0.02	ND ²	ND	ND

^{a,b}Values in same row with different superscripts differ significantly ($P < 0.05$).

¹DFMF = diafiltration; RC = regular cream; VCMF = volumetric concentration; WC = whey cream.

²ND = not detected.

amide species were not as abundant; however, fatty amide 24:1 represented more than 1% of total species for all BMP samples. The fatty amide 26:1 was only detected in one of the BMP samples, DFMF RC. The VCMF RC BMP showed the most notable differences with respect to the other powders; an increased number of fatty amide combinations with 18 carbons or fewer are observed for this sample. For this sample, fatty amide 16:0 was rather high (16.91%) compared with the other powders (8.29, 6.81, and 9.04%), possibly explained by variability in the starting material and processing methods. There were 2 molecular species detected that had significant differences between the DFMF and VCMF BMP samples, 23:1 and 24:1. For both of these fatty amides, the VCMF WC powder had a greater amount than the other powders. The DFMF WC powder was elevated compared with the other 2; however, there was no significant difference. Because the WC powders, for both filtration processes, came from the same lot of whey cream, the high value for

these fatty amides is a result of the VCMF process. Other interesting observations include similarities between fatty amides for the different filtration modes. Fatty amides 22:0, 23:0, 24:0, and 25:0 have slightly higher amounts in both powders for DFMF compared with VCMF. Perhaps the higher fouling and flux rates observed with VCMF resulted in a lower retention of the longer chain unsaturated SM species.

BMP Composition

Results for percentage solids and ash are shown in Table 1. For both VCMF and DFMF WC samples, the total solids increased following SFE, whereas the total solids for both VCMF and DFMF RC samples decreased; however, no significant difference was observed for total solids ($P = 0.167$). Ash content increased slightly following treatment for all samples; however, there was no significant difference between the samples ($P = 0.106$). It is expected that percentage protein and percentage ash of the powder will increase and percentage fat will decrease following SFE treatment. This was found to be the case in all samples except VCMF RC, in which the percentage protein amount decreased slightly (0.20%) following treatment. The remaining dry matter in the BMP is lactose.

Results for protein content in BMP MF retentates are shown in Table 5. For all samples, the NPN and soluble protein fractions increased and the insoluble protein fraction decreased following SFE treatment. Nonprotein N was very high in all of the WC retentates, both before and after treatment (12–22% of the nitrogen fraction), and all BMP samples before and after treatment differed significantly from each other ($P = 0.000$). This may be due to the high proteolytic activity of the cheese starter (Sodini et al., 2006). It was also higher for the VCMF samples compared with the DFMF samples, possibly related to the transmission of proteins in the filtration process (Morin et al., 2006). The soluble protein fraction at pH 4.6 is mostly undenatured whey protein. This fraction was very variable among the different samples; however, the outcome is similar to the NPN results. Results were higher for the WC samples, but only the VCMF WC sample increased significantly following treatment ($P = 0.000$). A higher level for the WC is to be expected; this protein fraction includes native whey protein and some of the MFGM proteins, which do not precipitate at acidic pH (O'Connell and Fox, 2000).

Insoluble protein at pH 4.6 is mostly casein and denatured whey protein. It is observed that the regular buttermilk powder contained more insoluble protein, which is to be expected because of the tendency of cheese makers to retain caseins in the cheese; very low

Table 5. Content of the nitrogen fraction (% of DM; $\pm$ SD) of buttermilk retentate powders before and after supercritical fluid extraction (SFE) treatment

Sample ¹	Total protein	NPN	Soluble protein (pH 4.6)	Insoluble protein (pH 4.6)
BMP before SFE				
DFMF RC	45.6 $\pm$ 1.9 ^a	3.0 $\pm$ 0.3 ^a	8.9 $\pm$ 1.7 ^a	88.1 $\pm$ 2.1 ^a
VCMF RC	38.7 $\pm$ 1.3 ^a	4.7 $\pm$ 0.4 ^b	10.9 $\pm$ 0.4 ^a	84.3 $\pm$ 0.1 ^a
DFMF WC	22.6 $\pm$ 4.1 ^b	12.3 $\pm$ 0.8 ^c	24.9 $\pm$ 1.1 ^b	62.7 $\pm$ 1.9 ^b
VCMF WC	17.2 $\pm$ 1.4 ^b	20.3 $\pm$ 0.0 ^d	25.4 $\pm$ 0.0 ^b	54.3 $\pm$ 0.0 ^c
BMP after SFE				
DFMF RC	51.1 $\pm$ 2.9 ^a	3.7 $\pm$ 0.5 ^a	9.9 $\pm$ 2.1 ^a	86.4 $\pm$ 2.4 ^a
VCMF RC	38.5 $\pm$ 4.5 ^a	5.8 $\pm$ 1.1 ^b	12.2 $\pm$ 2.3 ^a	82.0 $\pm$ 3.4 ^a
DFMF WC	26.1 $\pm$ 2.1 ^b	12.8 $\pm$ 1.8 ^c	27.4 $\pm$ 4.4 ^b	59.9 $\pm$ 6.1 ^b
VCMF WC	20.8 $\pm$ 0.4 ^b	21.7 $\pm$ 1.5 ^d	33.8 $\pm$ 1.0 ^c	44.5 $\pm$ 0.5 ^c

^{a-d}Values in same column with different superscript differ significantly ($P < 0.05$).

¹BMP = buttermilk powder; DFMF = diafiltration; RC = regular cream buttermilk; VCMF = volumetric concentration; WC = whey cream buttermilk.

amounts are found in the cheese whey. For the insoluble protein content, each BMP sample did not differ significantly following treatment ($P = 0.000$). An interesting observation is that more of the insoluble protein fraction is retained in the DFMF powders compared with the VCMF powders, which is opposite for the NPN and soluble nitrogen fractions. Morin et al. (2006) previously showed higher protein transmission through the MF membrane.

In conclusion, our results show that SFE coupled with microfiltration processing helps to concentrate MFGM components in both whey cream and regular cream buttermilk powder. In addition to developing an ingredient with a concentrated amount of bioactive lipids, using whey cream as a starting material gives a product with unique composition.

ACKNOWLEDGMENTS

This work has been funded in part by the Eckelman Graduate Assistantship, the California Dairy Research Foundation, and the CSU Agriculture Research Initiative. The authors express gratitude to Pierre Morin (Université Laval), Alan Taylor (Linus Pauling Institute), the Linus Pauling Institute, and the Dairy Products and Technology Center staff.

REFERENCES

- AOAC. 1995. Official Methods of Analysis. 16th ed. Association of Official Analytical Chemists, Gaithersburg, MD.
- Astaire, J. C., R. Ward, J. B. German, and R. Jimenez-Flores. 2003. Concentration of polar MFGM lipids from buttermilk by microfiltration and supercritical fluid extraction. *J. Dairy Sci.* 86:2297–2307.
- Barenholz, Y., and T. E. Thompson. 1999. Sphingomyelin: Biophysical aspects. *Chem. Phys. Lipids* 102:29–34.
- Berra, B., I. Colombo, E. Sottocornola, and A. Giacosa. 2002. Dietary sphingolipids in colorectal cancer prevention. *Eur. J. Cancer Prev.* 11:193–197.
- Byrdwell, W., and R. Perry. 2007. Liquid chromatography with dual parallel mass spectrometry and ³¹P nuclear magnetic resonance spectroscopy for analysis of sphingomyelin and dihydrosphingomyelin II. Bovine milk sphingolipids. *J. Chromatogr. A* 146:164–185.
- Cinque, B., L. D. Marzio, C. Centi, C. Di Rocco, C. M. Riccardi, and G. Cifone. 2003. Sphingolipids and the immune system. *Pharmacol. Res.* 47:421–437.
- Dacaranhe, C., and J. Terao. 2001. A unique antioxidant activity of phosphatidylserine on iron-induced lipid peroxidation of phospholipid bilayers. *Lipids* 36:1105–1110.
- Deeth, H. C. 1997. The role of phospholipids in the stability of milk fat globules. *Aust. J. Dairy Technol.* 52:44–46.
- Fox, P. F., P. McSweeney, T. M. Cogan, and T. P. Guinee. 2000. Fundamentals of Cheese Science. Aspen Publishers Inc., Gaithersburg, Maryland.
- Hanahan, D. 1997. Signaling vascular morphogenesis and maintenance. *Science* 277:48–50.
- Huwer, A., T. Kolter, J. Pfeilschifter, and K. Sandhoff. 2000. Review: Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim. Biophys. Acta* 1485:63–99.
- Karlsson, A., P. Michélsen, and G. Odham. 1998. Molecular species of sphingomyelin: Determination by high-performance liquid chromatography/mass spectrometry with electrospray and high-performance liquid chromatography/tandem mass spectrometry with atmospheric pressure chemical ionization. *J. Mass Spectrom.* 33:1192–1198.
- Kim, M.-Y., C. Linardic, L. Obeid, and Y. Hannun. 1991. Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. *J. Biol. Chem.* 266:484–489.
- Lightle, S. A., J. I. Oakley, and M. N. Nikolova-Karakashian. 2000. Activation of sphingolipid turnover and chronic generation of ceramide and sphingosine in liver during aging. *Mech. Ageing Dev.* 120:111–125.
- Marshall, R. T. 1992. Standard Methods for the Examination of Dairy Products, 16th ed. Am. Publ. Health Association Inc., Washington, DC.
- Merrill, A. H., and E. Wang. 1986. Biosynthesis of long-chain (sphingoid) bases from serine by LM cells: Evidence for introduction of the 4-trans-double bond after de novo biosynthesis of N-acylsphinganine(s). *J. Biol. Chem.* 261:3764–3769.
- Molkentin, J. 2000. Review: Occurrence and biochemical characteristics of natural bioactive substances in bovine milk lipids. *Br. J. Nutr.* 84:S47–S53.
- Morin, P., R. Jimenez-Flores, and Y. Pouliot. 2004. Effect of temperature and pore size on the fractionation of fresh and reconstituted buttermilk by microfiltration. *J. Dairy Sci.* 87:267–273.
- Morin, P., Y. Pouliot, and R. Jimenez-Flores. 2006. A comparative study of the fractionation of regular buttermilk and whey buttermilk by microfiltration. *J. Food Eng.* 77:521–528.

- Morin, P., Y. Pouliot, R. Jimenez-Flores, and M. Britten. 2007. Microfiltration of buttermilk and washed cream buttermilk for concentration of milk fat globule membrane components. *J. Dairy Sci.* 90:2132–2140.
- Nava, V. E., O. Cuvillier, L. C. Edsall, K. Kimura, S. Milstien, E. P. Gelmann, and S. Spiegel. 2000. Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Res.* 60:4468–4474.
- O'Connell, J. E., and P. F. Fox. 2000. Heat stability of buttermilk. *J. Dairy Sci.* 83:1728–1732.
- Okazaki, T., R. M. Bell, and Y. A. Hannun. 1989. Sphingomyelin turnover induced by vitamin D₃ in HL-60 cells. *J. Biol. Chem.* 264:19076–19080.
- Perry, D. K., L. M. Obeid, and Y. A. Hannun. 1996. Ceramide and the regulation of apoptosis and the stress response. *Trends Cardiovasc. Med.* 6:158–162.
- Renner, E., G. Schaafsma, and K. J. Scott. 1989. Micronutrients in Milk. Pages 1-70 in *Micronutrients in Milk and Milk-Based Food Products*. E. Renner, ed. Elsevier Applied Science, London, UK.
- Rombaut, R., J. V. Camp, and K. Dewettinck. 2005. Analysis of phospho- and shingolipids in dairy products by a new HPLC method. *J. Dairy Sci.* 88:482–488.
- Rombaut, R., V. Dejonckheere, and K. Dewettinck. 2006. Microfiltration of butter serum upon casein micelle destabilization. *J. Dairy Sci.* 89:1915–1925.
- Rozzi, N. L., and R. K. Singh. 2002. Supercritical fluids and the food industry. *Comp. Rev. Food Sci. Food Saf.* 1:33–34.
- Sodini, I., P. Morin, A. Olabi, and R. Jimenez-Flores. 2006. Compositional and functional properties of buttermilk: A comparison between sweet, sour, and whey buttermilk. *J. Dairy Sci.* 89:525–536.
- Sullards, M. 2000. Analysis of sphingomyelin, glucosylceramide, ceramide, sphingosine, and sphingosine 1-phosphate by tandem mass spectrometry. *Methods Enzymol.* 312:32–45.
- Vesper, H., E.-M. Schmelz, M. N. Nikolova-Karakashian, D. L. Dillehay, D. V. Lynch, and J. A. H. Merrill. 1999. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* 129:1239–1250.
- Walstra, P., T. J. Geurts, A. Noomen, A. Jellma, and M. A. J. S. v. Boekel. 1999. *Dairy Technology: Principles of Milk, Properties and Processes*. Marcel Dekker Inc., New York, NY.