The influence of temperature and pressure factors in supercritical fluid extraction for optimizing nonpolar lipid extraction from buttermilk powder

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ABSTRACT

The milk fat globule membrane, readily available in buttermilk, contains complex lipids claimed to be beneficial to humans. Phospholipids, including sphingolipids, exhibit antioxidative, anticarcinogenic, and antiatherogenic properties and have essential roles in numerous cell functions. Microfiltration coupled with supercritical fluid extraction (SFE) may provide a method for removing triacylglycerols while concentrating these nutritionally valuable lipids into a novel ingredient. Therefore, SFE as a method for phospholipid concentration needs to be optimized for triacylglycerol removal in buttermilk. The SFE conditions were assessed using a general full factorial design; the experimental factors were pressure (15, 25, and 35 MPa) and temperature (40, 50, and 60°C). Particularly interesting is that only triacylglycerols were removed from buttermilk powder. Little to no protein loss or aggregation was observed compared with the untreated buttermilk powder. Calculated theoretical values showed a linear increase for lipid solubility as pressure, temperature, or both were increased; however, experimental values showed nonlinearity, as an effect of temperature. In addition, the particular SFE parameters of 35 MPa and 50°C displayed enhanced extraction efficiency (70% total lipid reduction).

Key words: supercritical fluid extraction, milk fat globule membrane, milk phospholipid

INTRODUCTION

Phospholipids (PL) account for approximately 0.2 to 1.0% of total bovine milk lipids (Molkentin, 2000). The PL are largely concentrated in the milk fat globule membrane (MFGM) and relatively small amounts are bound to caseins (Warner, 1976; Renner et al., 1989).

Some of these PL (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine) may have nutritional benefits to humans by providing antioxidative, anticarcinogenic, and antiatherogenic properties (Molkentin, 2000; Dacaranhe and Terao, 2001; Berra et al., 2002). Within eukaryotic cells, PL are primarily components of cell membranes; high concentrations are found in the membrane-rich tissues such as pancreas, liver, brain, and neural tissue (Merril et al., 1997; Berra et al., 2002). These are all impractical sources for edible lipid isolation and food ingredient development. However, because of its unique composition and concentration of these lipids, MFGM in buttermilk could be used as a PL source.

Buttermilk is readily available in large quantities from butter-producing dairy processors as a by-product and at a relatively low cost. In the United States, the average monthly production of buttermilk powder (BMP) is 4.5 million pounds with an average price of \$0.99 per pound (USDA, 2005). The composition of BMP is somewhat similar to that of skim milk powder. However, it contains a greater fat content (6% wt/wt) and lesser lactose content (49% wt/wt) compared with skim milk powder (1 and 52% wt/wt, respectively; Walstra et al., 1999). The ash components of buttermilk powder are similar to skim milk powder (approximately 8%; Carić, 1994; Walstra et al., 1999). Buttermilk contains a greater percentage of phospholipids compared with whole dairy milk (0.16 vs. 0.04%; Rombaut et al., 2006a). In addition, sphingomyelin represents approximately 19.06% of total buttermilk PL (Rombaut et al., 2006a). Therefore, using buttermilk as a concentrated source of PL is a sensible alternative. Although numerous conventional methods relying on solvents have proven to isolate PL successfully, their use may render the product inadequate unless the solvent is removed completely (Astaire et al., 2003).

Microfiltration processes are most commonly used in the dairy industry for fat and bacterial removal along with protein concentration; however, it is necessary to provide an effective separation between lipids and proteins. Morin et al. (2004) reported on the effects of temperature and pore size on microfiltration of fresh and

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reconstituted buttermilk. Greater 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 content 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. It was observed by Rombaut et al. (2006b) that separation of lipids and proteins using microfiltration and sodium citrate or ethanol addition was not possible. Although effective in allowing casein to pass through the membrane by micelle dissociation, a high degree of membrane fouling and PL loss were observed.

Supercritical fluid extraction (SFE) is an additional method that can selectively extract lipid components of a complex mixture. Carbon dioxide is a common solvent used for SFE, and is used for a variety of applications. Its properties make it an ideal solvent: it is nonexplosive, inexpensive, easily obtainable and plentiful in nature, and nontoxic. Furthermore, it has low critical temperature and pressure parameters (31.0°C, 7.38 MPa) and is easily separated from the materials extracted. These characteristics make it ideal for food processing applications (Arul et al., 1994; King, 1995; Rozzi and Singh, 2002). Several applications of SFE have been developed to extract lipid and lipid-soluble materials such as vitamins A, D, E, and K from a variety of mixtures (Turner and Mathiasson, 2000; King et al., 2001; Saldana et al., 2002).

A previous study by Astaire et al. (2003) found that by combining microfiltration and supercritical fluid extraction, the PL of the MFGM could be concentrated within reconstituted buttermilk. In this process, a cross-flow microfiltration system with a 0.8- μ m pore size was optimized for cold, reconstituted BMP for an initial concentration of lipids. Further treatment using SFE successfully increased the concentration of polar MFGM lipids (namely sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine; 9.59 to 19.74 mg of lipid/g of dry powder) in BMP by the removal of nonpolar lipids, specifically triacylglycerols (21.33 to 3.98 mg of lipid/g of dry powder). This extraction was carried out at a pressure of 37.5 MPa and a temperature of 77°C.

The goals of this work were to investigate the influence and interaction of SFE using CO₂ in relation to lipid extraction efficiency and to characterize the physical and chemical properties of polar-enriched BMP components. The specific objectives presented here were to assess the effect of SFE pressure-temperature conditions on lipid extraction efficiency and their influence on the chemical changes in BMP.

MATERIALS AND METHODS

Processing of BMP

Commercial BMP was obtained from Dairy America Inc. (Fresno, CA). For reconstitution of the dried buttermilk, tap water was added to give a 10% total solids solution and allowed to sit overnight at 4°C to hydrate fully in preparation for microfiltration.

The microfiltration procedure was described earlier (Astaire et al., 2003; Morin et al., 2004). Briefly, the microfiltration unit was an in-house manufactured stainless steel shell and tube module containing Tami Sunflower Design ceramic membranes (Tami Industries, Montreal, Canada). A membrane with a pore size of 0.80 μ m was used. All runs were carried out at low temperature (8–10°C) at a transmembrane pressure of 80 to 95 kPa. Diafiltration processes replaced the extracted permeate with water to a dilution factor of 3 $\times$. Final retentates were spray dried using a Niro Filterlab Spray Dryer (Hudson, WI). Following spray drying, the buttermilk powder was subjected to SFE treatments according to the experimental design detailed below.

SFE Processing of BMP

Lipid extraction by SFE focused on constant run conditions and powder samples, and the 2 variables to consider were pressure and temperature in the system. A general full factorial design was employed for these experiments: the pressure and temperature were modified at 3 levels (low, -1; intermediate, 0; and high, 1). Pressure levels chosen were 15, 25, and 35 MPa, respectively. The temperature levels chosen were 40, 50, and 60°C, respectively. For each experimental point (i.e., 15 MPa at 40°C), 50 g of a 3 $\times$ diafiltration/microfiltration BMP retentate (fat = 9.77% and protein = 54.10%) was mixed 1:1 with Celite 566 biosilicate (World Minerals Inc., Lompoc, CA), placed in inert bags made of Rapid-Flow milk filter tubes (Filter Fabrics Inc., Goshen, IN), and subjected to 2 $\times$ SFE. All experimental points were processed in triplicate.

The SFE system and components were acquired from Thar Designs Inc. (Pittsburgh, PA) and were described previously by Astaire et al. (2003). Circulated deionized water at 5°C was used for cooling different zones in the SFE apparatus. Fifty-pound carbon dioxide tanks were filled and inspected by Airgas West (San Luis Obispo, CA). The system conditions were controlled manually by Windows 2000-based software (Hewlett-Packard, Palo Alto, CA). The running conditions were as follows: flow rate = 20 g/min; total vessel flushes = 3.35 $\pm$ 0.40; total run time = 90.0 $\pm$ 0.11 min; and

total CO₂ used = 1,670.0 ± 200 g. Following treatment, the SFE extraction vessel was rinsed with 50 to 100 mL of chloroform:methanol (2:1 vol/vol) into a preweighed, dried aluminum pan. The solvent was evaporated on a hot plate under a ventilated hood and the residue lipids were dissolved and diluted to 10 mg/mL in chloroform-methanol (2:1, vol/vol). Lipid solubility in supercritical CO₂ was calculated by dividing the amount of lipid collected (in milligrams) from the SFE extraction vessel by the total grams of CO₂ that passed through the system.

Physical and Chemical Analysis

Color measurements were determined according to a minor modification of the Hunter Laboratory method of measuring loose powder (Nielsen et al., 1997). Treated powders were filtered through a 300-μm sieve to remove Celite. A 25-mL glass flask, top and sides covered with black tape, was packed by gravitational contraction with 3.9 g of powder. Analysis was done using a HunterLab UltraScan XE colorimeter (Hunter Laboratories, Reston, VA) and data analysis was completed using the software Universal V3.80 (Hunter Laboratories). The operating conditions were illuminant C, 10° observer value, and reflectance mode and 45/0 sensor. The CIE LAB values L* (lightness), a* (redness), and b* (yellowness) were measured, with analyses performed in triplicate.

Buttermilk powder samples were reconstituted with deionized water to 10% solids and allowed to hydrate overnight. pH was determined using an Orion Research pH meter, model 410 (Thermo Electron Corporation, Beverly, MA). Titratable acidity (TA) was determined as percentage lactic acid (AOAC, 1990; method 947.05). Treated samples were compared with the original untreated BMP (pH = 7.67 ± 0.02, TA = 0.036 ± 0.02).

Lipid Composition

Lipid Quantification. The Mojonnier ether extraction method as described by Marshall (1992) was followed. Solvents were of analytical grade, purchased from Fisher Scientific (Tustin, CA). All samples were extracted in duplicate.

Lipid Profiling by Thin Layer Chromatography. Silica gel thin layer chromatography (TLC) plates (60 F₂₅₄) were obtained from EMD Chemicals (Darmstadt, Germany), and the developing tanks were from Kontes Glass Company (Vineland, NJ). 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). Lipids were prepared in chloroform:methanol

(2:1, vol:vol) to 10 mg/mL; lipid standards were diluted to 1 mg/mL. 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 with standards (Astaïre et al., 2003). Lipid standards (sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine) were purchased from Sigma Chemical Co. (St. Louis, MO).

Supercritical CO₂ Lipid Solubility Calculations. Experimental lipid solubility in supercritical (SC)-CO₂ was calculated by dividing the amount of lipid collected in milligrams from the SFE extraction vessel by the total grams of CO₂ that passed through the system. Theoretical lipid solubility was calculated using the Hildebrand solubility parameter equation (Arul et al., 1994):

$$S = [(\Delta H_1^g - \Delta H_g - RT + CPV)/v]^{1/2},$$

where ΔH_1^g = molar enthalpy of vaporization (Jmol⁻¹), and is 0 beyond the critical point, ΔH_g = increase in enthalpy on isothermally expanding 1 mol of saturated vapor to zero pressure (Jmol⁻¹), P = pressure of SC-CO₂ (MPa), T = temperature (K), V = molar volume of SC-CO₂ (cm³mol⁻¹), R = gas constant (8.314 Jmol⁻¹K⁻¹), and C = conversion factor (1.0 Jcm⁻³MPa⁻¹).

Molar volume was found using the Van der Waals equation for a particular temperature and pressure state:

$$(P + a/Vm^2)(Vm - b) = R T,$$

where P = pressure (MPa), Vm = molar volume (cm³mol⁻¹), R = ideal gas constant (8.314 Jmol⁻¹K⁻¹), and T = temperature (K), and where a and b are estimated from the relationship of critical pressure and temperature using the equations: a = (27 R² T_c²)/64 P_c; b = (R T_c)/8 P_c; T_c = critical temperature (304.18 K); and P_c = critical pressure (7.38 MPa).

Protein Composition

Protein Determination. Percentage protein was determined by testing for total N content by the Kjeldahl method (AOAC, 1990; method 955.04). Samples were heated on a Digestion System 20, model 1015 digester (Tecator Kjeldahl System, Foss Analytical, Hillerød, Denmark). Samples were distilled with the Kjeldahl system 1026 distilling unit, and FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific) were used as the reagent. All samples were analyzed in duplicate.

Titration was done using 0.1 *N* HCl, and the percentage protein was calculated using the milk protein conversion factor, 6.38. All reagents were of Kjeldahl analysis analytical grade, purchased from Fisher Scientific.

Protein Profiling by PAGE. Samples were diluted in deionized water to yield a protein concentration of 6.6 mg/mL and added to reducing SDS sample buffer (10% SDS, 0.5 *M* Tris, pH 6.8) in a 1:1 dilution, mixed in a vortex mixer, boiled for 10 min, and centrifuged for 2 min at $12,000 \times g$. Nonreducing gel samples were prepared in the same manner with no SDS added to gel or buffers. A volume of 15 μ L (50 μ g of protein) was loaded to the Criterion 4–20% gradient Tris-HCl, 1.0-mm gels (Bio-Rad Laboratories, Hercules, CA). Gels were run at 90 V through the stacking gel, and at 120 V through the resolving gel. BlueRange Pre-stained Protein Molecular Weight Marker Mix (Pierce, Rockford, IL) was used as the molecular weight standard. Gels were visualized by staining with Coomassie Brilliant Blue (Sigma Chemical Co.). For silver stains, the Silver Stain Plus Kit (Bio-Rad) was followed according to the manufacturer's protocol.

Protein Profiling by Microfluidic Laser-Induced Fluorescence. Whey protein polymerization and glycosylation were measured as difference between reducing and non-reducing SDS microfluidic system coupled to a laser-induced fluorescence (LIF) detector using the Experion Pro260 system (BioRad). Separation of proteins from 10 to 260 kDa was obtained. Lower and upper internal alignment markers ensured clean baselines, proper molecular weight sizing, and protein mass quantitation.

Buttermilk powder samples were reconstituted with deionized water to 10% total solids and allowed to hydrate overnight. The Experion Pro260 gel stain and Pro260 sample buffer were prepared using the manufacturer's directions (Bio-Rad). Under reducing conditions, 1 μ L of β -mercaptoethanol was added to 30 μ L of sample buffer. Under nonreducing conditions, 1 μ L of deionized water was added to 30 μ L of sample buffer. Using these reagents, 2 μ L of sample buffer was added to 4 μ L of sample and 4 μ L of Pro260 ladder and mixed. The samples and ladder were heated at 95 to 100°C for 3 to 5 min, centrifuged for 1 min at 14,000 rpm, and mixed with 84 μ L of deionized water.

Before analysis, 12 μ L of gel-stain solution was added to the Pro260 microchip and the chip was primed using the Experion priming station according to the manufacturer's directions. The chip was loaded with the required volumes of sample, gel-stain, gel and ladder solutions, and placed into the electrophoresis station for protein separation. Data were analyzed using the Expe-

riion software and recorded as electrophoretograms, gel graphs, and data tables.

Protein Solubility. Protein solubility was determined as described by Wong and Kitts (2003). Protein solutions (5% total N, wt/wt) at different pH values were centrifuged at $12,000 \times g$ for 15 min at 25°C. Supernatant liquid was analyzed for total N by the Kjeldahl method. Measurements were done in triplicate.

Statistical Analysis

Graphics (main effects and interaction plots), statistics, and experimental design were performed with Minitab 14.0 software (Minitab Inc., State College, PA). The experimental design was a general full factorial design using 2 factors (pressure and temperature) at 3 different levels (low, medium, and high) in triplicate. Run orders were block randomized. Experimental points were compared with both a control (untreated) powder and to a powder treated at extreme SFE conditions (37.7 MPa and 77°C) when indicated. All comparisons were done by ANOVA with Tukey's pairwise comparison. Results were considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

The average percentage total fat reduction from the sample powders at each experimental setting is shown in Figure 1 and Table 1. The least amount of lipids was extracted at 15 MPa for all 3 temperature levels, whereas the greatest amount of lipids was extracted from the powder at a pressure of 35 MPa and a temperature of 50°C in relation to all experimental points; however, only 2 other points showed a significant difference ($P < 0.05$). This was shown in the amount of lipids extracted at the lowest pressure (15 MPa) coupled with temperature levels at 40 and 60°C. The measured lipid reduction was particularly variable for all 3 settings at 40°C, suggesting that at this temperature the extraction was variable and the results were not reproducible. In contrast, extractions performed at 60°C showed the least variation and extractions at 50°C showed variation in between that observed at 40 and 60°C.

Lipid solubility (Figure 2) was determined by measuring the amount of lipids extracted in relation to the amount of CO₂ used in the extraction. These results confirm the findings described above in which a significant difference ($P = 0.004$) of lipid solubility is observed between the powders extracted at the lowest pressure at all temperatures versus the powder with the greatest percentage fat reduced (35 MPa and 50°C). Lipid solubility was compared with a calculated solu-

Figure 1. Fat reduction (%) in supercritical fluid extraction-treated buttermilk powder for 3 different pressures (15, 25, and 35 MPa) and temperatures (40, 50, and 60°C). Bars with different letters denote significant difference ($P < 0.05$).

bility parameter, as shown in Figure 2. Although not an experimental point, theoretical solubility was calculated for 50 MPa (the pressure limit of our system) at the 3 temperature settings. In theory, the solubility parameter of CO₂ increases linearly when temperature at a constant pressure is increased; likewise, it increases linearly when pressure at a constant temperature is increased. Experimentally, however, this is not the case.

The ANOVA data indicate that pressure of the system significantly affected the percentage fat reduced in the powder determined by the low P -value (0.004) and large mean square value (929.1). The SFE system temperature and the interaction between pressure and temperature did not affect percentage fat reduced ($P = 0.445$ and 0.755 , respectively). It was observed that the underlying error distribution was normal, with one outlier having a large residual. This point was at the low

pressure and low temperature setting, which is already known to have a high standard deviation.

An interaction plot for percentage fat (Figure 3) shows that at the lowest pressure setting (15 MPa), lipid extraction efficiency was somewhat limiting to the other 2 settings, regardless of temperature. In general, for both low- and high-pressure settings, increasing the temperature from low to intermediate increased fat reduction in the powder; however, by increasing the temperature to high, the overall fat extracted was reduced. Although a high pressure tended to show high lipid extraction results, data at the low temperature indicated that the intermediate pressure setting had greater extraction efficiency, showing nonlinearity of the process. In addition, a main effects plot demonstrated that as the pressure of the system was increased, the lipid extraction efficiency increased (Figure 4). Tem-

Table 1. Chemical data on a DM basis for pressure and temperature experimental settings

Experimental setting	Fat, %	Protein, %	pH	Titrateable acidity ¹	Mean lipid solubility, mg of lipid/g of CO ₂
15 MPa/40°C	5.92 ± 2.15 ^a	57.76 ± 3.92	7.61 ± 0.04	0.041 ± 0.01	0.021 ^a
15 MPa/50°C	4.98 ± 0.83 ^{ab}	69.07 ± 0.42	7.72 ± 0.17	0.037 ± 0.02	0.027 ^{ab}
15 MPa/60°C	5.29 ± 0.48 ^a	57.14 ± 0.10	7.59 ± 0.03	0.040 ± 0.01	0.023 ^{ab}
25 MPa/40°C	3.68 ± 1.12 ^{ab}	60.31 ± 0.30	7.64 ± 0.04	0.037 ± 0.01	0.035 ^{abc}
25 MPa/50°C	3.94 ± 0.53 ^{ab}	58.31 ± 6.28	7.66 ± 0.09	0.029 ± 0.00	0.035 ^{abc}
25 MPa/60°C	3.17 ± 1.64 ^{ab}	60.00 ± 4.47	7.52 ± 0.12	0.036 ± 0.01	0.036 ^{abc}
35 MPa/40°C	4.07 ± 1.16 ^{ab}	59.50 ± 1.01	7.56 ± 0.12	0.033 ± 0.00	0.039 ^{bc}
35 MPa/50°C	2.85 ± 0.57 ^b	65.72 ± 1.21	7.66 ± 0.13	0.028 ± 0.01	0.045 ^c
35 MPa/60°C	3.64 ± 0.26 ^{ab}	60.05 ± 8.82	7.47 ± 0.13	0.043 ± 0.02	0.037 ^{bc}

^{a,b}Different superscript letters within a column denote significant difference ($P < 0.05$).

¹Titrateable acidity expressed in milligrams of lactic acid/100 mL of milk.

Figure 2. Lipid solubility properties in supercritical CO₂ for pressure (15, 25, and 35 MPa) and temperature values: 40°C (▲), 50°C (■), and 60°C (●). The top set of values (left y-axis) is for the theoretical solubility parameter and the lower set of values (right y-axis) is experimental solubility.

perature effects showed a different trend in that lipid extraction increased from 40 to 50°C, but decreased at 60°C.

Protein and lipid compositional analysis of the BMP was necessary to observe other changes taking place in the BMP during treatment. Lipids analyzed by TLC demonstrated that those present in the BMP samples following SFE treatment were mostly polar lipids, whereas the removed fraction exclusively comprised nonpolar lipids (Figure 5). There was complete retention of all polar lipids in the powder.

Visual comparison of the proteins analyzed by the reducing PAGE method showed that chemical changes were taking place during SFE treatment (Figure 6). Results were shown only for the first treatment set, because data obtained were similar throughout the triplicate analysis. Comparison of reduced protein profiles showed apparent changes in the powder samples with respect to high temperature treatment; band “fuzziness” can be used as an indicator of whey protein lactosylation because of variations in molecular mass (Morgan et al., 1997). The protein profile of the original powder (lane 2) showed a sharp band for β -LG, and following SFE treatments, bands were observed to have a smearing or greater relative molecular mass (M_r). An observed M_r increase for the caseins and whey proteins and the extremely fuzzy bands indicate that the previous SFE pa-

rameters were too intense and caused chemical changes in the powder. The SFE treatments at 60°C (lanes 4, 7, and 10), regardless of pressure, tended to show a greater whey protein M_r . The caseins also showed a greater whey protein M_r , although to a lesser extent

Figure 3. An interaction plot for percentage fat reduced shows that at the lowest pressure setting (15 MPa), lipid extraction efficiency is somewhat limiting to the other 2 settings, regardless of temperature. Temperature values: 40°C (−1), 50°C (0), and 60°C (+1); pressure values: 15 MPa (●), 25 MPa (■), and 35 MPa (◆).

Figure 4. Main effects plot for pressure and temperature on percentage fat reduction in supercritical fluid extraction-treated buttermilk powder. The values shown (-1, 0, and +1) indicate the 3 levels for pressure (15, 25, and 35 MPa, respectively) and temperature (40, 50, and 60°C, respectively).

than the whey proteins. The experimental treatment at 35 MPa and 50°C was previously noted to result in the greatest percentage fat reduction and is shown by the arrow in lane 1. This treatment had a lesser effect on proteins than the corresponding treatments at 60°C. Furthermore, nonreducing PAGE was carried out to observe any protein denaturation or polymerization that may have occurred during the SFE treatments. It was observed that there was no significant difference between the proteins before and after SFE treatment at any of the experimental points (results not shown).

Using microfluidic-LIF technology, data can be organized and analyzed in a variety of different schemes (tabular form, virtual gels, and electrophoretograms) to use information such as peak number, time, concentration, molecular weight, height, and area. Figures 7A and 7B show the electrophoretograms (fluorescence vs. molecular weight and time) of SDS-reduced and microfluidic-LIF protein profiles from the original powder and the powder that underwent the extreme SFE conditions (35 MPa/77°C). Sample peaks are those shown between the system peak (7.63 kDa) and upper marker peak (260 kDa). Results indicated that proteins from the treated BMP decreased in total concentration from 9.79 g/L in the original BMP to 4.09 g/L in the treated sample. Furthermore, the remaining peaks representing proteins in the treated BMP underwent an apparent

M_r shift (with an average of 3.1 kDa) from 23.9 to 26.4 kDa, from 40.6 to 43.7 kDa, and from 82.8 to 86.5 kDa corresponding to caseins, MFGM proteins, and lactoferrin, respectively. The observed concentration and peak loss as well as the M_r shifts can be attributed to protein denaturation, aggregation, and possible lactosylation. This result is consistent with the visual observation of SDS-PAGE.

Results showed that solubility varied from 13.1 to 20.5% according to the treatment variables (Figure 8); however, none of the treatment powders were significantly different in solubility with respect to the original nontreated powder. Powders that underwent extreme treatment conditions (37.5 MPa and 77°C) were significantly different from the original powder as well as 3 of the sample powders (15 MPa/40°C, 15 MPa/50°C, and 35 MPa/60°C). Although temperature or pressure variables alone had no effect on solubility ($P = 0.148$ and 0.075 , respectively), the interaction between temperature and pressure significantly affected powder insolubility ($P = 0.03$).

The increase of M_r of the powder proteins following treatment may be caused by the addition of lactose residues or lactosylation on the proteins, resulting in the formation of glycoproteins. Lactosylation, the covalent attachment of reducing sugars to proteins, is an early stage of the Maillard reaction, which leads to powder

Figure 5. Thin layer chromatograph showing nonpolar lipid profiles from 9 supercritical fluid extraction experimental settings with buttermilk powder (BMP) using the petroleum ether:ethyl ether:acetic acid (85:15:2, vol:vol:vol) solvent system. Lane 1 = phospholipid standard mix; lane 2 = original BMP; lanes 3 to 11, defatted samples A to I; lanes 12 to 19 = removed fat from experimental settings (A-ext to H-ext). PL = phospholipids; MAG = monoacylglycerides; DAG = diacylglycerides; Chol = cholesterol; FFA = free fatty acids; TAG = triacylglycerides; and CholEster = cholesterol esters.

Figure 6. Reducing SDS-PAGE (12%) stained with Coomassie Blue and silver stain, on supercritical fluid extraction-treated buttermilk powders (BMP) from the first trial. Lanes: 1) 35 MPa/50°C; 2) original BMP; 3) molecular weight marker; 4) 35 MPa/60°C; 5) 15 MPa/40°C; 6) 15 MPa/50°C; 7) 15 MPa/60°C; 8) 25 MPa/40°C; 9) 25 MPa/50°C; 10) 25 MPa/60°C; 11) 35 MPa/40°C. MFGM = milk fat globule membrane.

browning and solubility loss (French et al., 2002). In addition, nonenzymatic glycosylation of proteins may affect both functional and biological activities (Leonil et al., 1997; Moreno et al., 2002). The conditions that promote this reaction are medium to high temperature, intermediate water activity, and extended time; therefore, milk powder (having a low water activity) is particularly susceptible (Guyomarc'h et al., 2000). In addition to promoting glycosylation of the proteins, high heat treatments have been observed to induce interactions between MFGM components and skim milk proteins (Pappas, 1992; Ye et al., 2002). Resulting color changes in the powder were observed using Hunter

Laboratory values L^* (lightness-darkness parameter), a^* (redness-greenness parameter), and b^* (yellow-blue parameter). An increase in a^* represents an increase in red; a decrease in L^* represents an increase in darkness; and an increase in b^* indicates an increase in yellow. The powders treated at extreme conditions (37.5 MPa and 77°C) had a significant increase in darkness and redness compared with the other treatment conditions as well as the original powder ($P = 0.000$ for both L^* and a^*) as shown in Table 2. Extreme SFE treatment resulted in a significant increase in yellow ($P = 0.00$) compared with the nontreated BMP, whereas the sample powders decreased significantly in yellow color.

Figure 7. A) Reducing SDS-PAGE (12%) stained with Coomassie Blue on untreated buttermilk powder (BMP) and supercritical fluid extraction (SFE)-treated powder under extreme conditions (37.5 MPa/77°C). Lanes: 1) molecular weight marker; 2) original BMP; 3) BMP treated under extreme conditions. B) Microfluidic-laser-induced fluorescence (in relative fluorescence units) virtual electrophoretogram of reducing proteins on untreated BMP (---) and SFE-treated BMP (—) under extreme conditions (37.5 MPa/77°C). MFGM = milk fat globule membrane.

Figure 8. Protein solubility determined in a 5% protein solution for supercritical fluid extraction-treated buttermilk powders. Treatment of powders indicated by pressure (15, 25, and 35 MPa) and temperature (40, 50, and 60°C). ^{a,b}Bars with different letters denote significant difference ($P < 0.05$); error bars indicate SD.

These results indicate that the SFE conditions used for this study do not cause the powder to undergo browning as it does when treated with a greater temperature and pressure.

In conclusion, the BMP products developed by combining microfiltration and the SFE process are rich in polar lipids and protein. We have shown that decreasing the SFE pressure parameter from 37.5 to 35 MPa and the temperature from 77 to 50°C ensures adequate lipid removal of nonpolar lipids from the powder without major disruption of other components. Results show that neither protein solubility nor powder browning is affected by the optimized SFE treatment. Extraction at 40°C is shown to be very variable and unreliable perhaps

because of the instability of the supercritical nature of CO₂ under these conditions. Further knowledge into the metabolic effects and mechanisms of these products is needed before nutritional claims can be made. Certainly, a process that delivers food components with fewer modifications and no residual chemicals is more desirable.

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Table 2. Colorimetry properties (lightness, L*, redness, a*, and yellowness, b*) for pressure and temperature supercritical fluid extraction settings and the untreated buttermilk powder

Sample	L*	a*	b*
Untreated	87.98 ± 1.50 ^a	-1.9 ± 0.95 ^a	15.07 ± 1.69 ^a
37.5 MPa/77°C	81.26 ± 7.56 ^b	0.51 ± 1.94 ^b	14.48 ± 0.60 ^b
15 MPa/40°C	90.15 ± 0.92 ^a	-2.16 ± 0.04 ^a	12.06 ± 0.57 ^c
15 MPa/50°C	90.08 ± 0.76 ^a	-2.06 ± 0.09 ^a	11.72 ± 0.38 ^c
15 MPa/60°C	90.68 ± 0.61 ^a	-2.14 ± 0.06 ^a	11.90 ± 0.25 ^c
25 MPa/40°C	90.60 ± 0.65 ^a	-2.13 ± 0.02 ^a	11.87 ± 0.20 ^c
25 MPa/50°C	90.80 ± 0.59 ^a	-2.10 ± 0.05 ^a	11.51 ± 0.21 ^c
25 MPa/60°C	90.72 ± 0.38 ^a	-2.15 ± 0.03 ^a	11.56 ± 0.42 ^c
35 MPa/40°C	90.86 ± 0.52 ^a	-2.14 ± 0.07 ^a	11.66 ± 0.14 ^c
35 MPa/50°C	90.90 ± 0.55 ^a	-2.12 ± 0.04 ^a	11.95 ± 0.20 ^c
35 MPa/60°C	90.13 ± 0.83 ^a	-2.05 ± 0.12 ^a	11.70 ± 0.25 ^c

^{a-c}Means within columns with different superscript letters are statistically different ($P < 0.05$).

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