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The milk fat globule membrane, a major component in buttermilk, contains many complex lipids known to be involved in a variety of biological processes. Phospholipids, including sphingolipids, exhibit antioxidative, anticarcinogenic, and antiatherogenic properties and have essential roles in numerous cell functions. Filtration coupled with supercritical fluid extraction (SFE) may provide a method of concentrating these nutritionally valuable lipids into a novel ingredient. The objective of this dissertation was to evaluate the SFE process as a method to enrich polar lipids in buttermilk powder.

The first part of this dissertation involved the optimization of the SFE treatment for buttermilk powder. Pressure and temperature treatment factors were assessed as were the role of matrix powder additions. The parameters at 350 bar and 50°C displayed enhanced extraction efficiency of triacylglycerol removal with minimal disruption to other buttermilk components. The addition of diatomaceous

earth**,** Teflon® beads and physical vibration were shown to help reduce total lipid by 86%, 78% and 70%, respectively.

The second part of the study was to apply the optimized SFE treatment to two different sources of buttermilk powder, regular and whey cream, also passed through two different filtration modes prior to drying. The buttermilk powders were compared in terms of lipid extraction efficiency and by assessing compositional differences of initial and final products, as well as the lipids extracted from the powder. After three extractions, SFE processing reduced the total fat, namely non-polar lipids, by 38 – 55% and phospholipids were concentrated by a 5-fold factor in the powders. Specific molecular fatty acid combinations on the sphingosine backbone of sphingomyelin from the treated buttermilk powder were characterized to show unique composition.

Finally, the thermal stability of ultrafiltrated whey cream buttermilk powder prior to and following SFE treatment was observed. Salt as well as pH levels were adjusted in the reconstituted powders and protein agglomeration upon heating was assessed. Results showed that in comparison to whey protein isolate, the SFE process enhances thermal stability by reducing protein aggregation, indicating the processed whey cream buttermilk powder to be a unique product with an interesting thermal profile and composition.

Filtration followed by supercritical fluid extraction processing is an effective tool for enriching bioactive lipids in both sweet and whey cream buttermilk powder, opening the potential for novel food ingredients.

An Evaluation into the Optimization and Product Application of Supercritical Fluid Extraction and the Processing Impact on the Components in Filtrated Buttermilk Powder

by
Amy J. Spence

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APPROVED:

Co-Major Professor, representing Food Science and Technology

Co-Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Amy J. Spence, Author

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Introduction

Phospholipids account for approximately 0.2-1.0% of total milk lipids (Molkentin, 2000). They are associated with both the milk fat and proteins, largely concentrated in the milk fat globule membrane and small amounts are bound to caseins (Renner, et al., 1989, Warner, 1976). Phospholipids not only function as integral components of the membrane, but also are shown to be involved with other biological processes. Groups of phospholipids are normally found in milk and consist of about 10 fractions with the major ones being phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine (Renner, et al., 1989). Some of these have been shown to exhibit antioxidative, anticarcinogenic, and antiatherogenic properties (Berra, 2002, Dacaranhe and Terao, 2001, Molkentin, 2000). Sphingolipids, in particular, are known to have essential roles in cell to cell interactions, differentiation, proliferation, immune recognition, transmembrane signaling, and as cell receptors (Huwiler, et al., 2000, Kim, et al., 1991, Lightle, et al., 2000, Okazaki, et al., 1989). Their location on the milk fat globule membrane exterior and their interactive character suggests a major role in many different important biological interactions (Deeth, 1997).

Buttermilk has a composition somewhat similar to skim milk. However, it contains a higher percentage of phospholipids in comparison to whole dairy milk,

0.16% versus 0.04% respectively (Rombaut, et al., 2006a). In addition, sphingomyelin represents approximately 19.06% of total buttermilk phospholipids (Rombaut, et al., 2006a). Therefore, using buttermilk as an isolation source for phospholipids is a sensible alternative. Although numerous conventional methods, relying on solvents, have proven to successfully isolate phospholipids, their use may render the product inadequate for consumption, unless solvent is removed completely (Astaire, 2003). Previous studies have found that combining a concentration process, such as microfiltation, followed by supercritical fluid extraction, the bioactive lipids of the milk fat globule membrane could be concentrated within reconstituted buttermilk (Astaire, 2003; Morin, 2004; Morin, 2006). Our goals were to study this combination of processes in relation to supercritical fluid extraction optimization, description of initial and final products, examination of buttermilk source, and potential product application.

We optimized the supercritical fluid extraction process by investigating supercritical CO_2 factors in relation to lipid extraction efficiency as well as by characterizing the physical and chemical properties of polar enriched buttermilk powder components. Supercritical fluid extraction conditions were assessed using a general full factorial design; the experimental factors were pressure (150, 250, and 350 Bar) and temperature (40, 50 and 60°C). Particularly interesting is that only triglycerides were removed from the powder. Little to no protein loss or aggregation was observed in comparison to the control. Calculated theoretical values showed a linear increase for lipid solubility as pressure and/or temperature is increased; however, experimental values showed non-linearity. In addition, the particular

supercritical fluid extraction parameters of 350 bar and 50°C, displayed enhanced extraction efficiency (70% total lipid reduction) with minimal disruption to other buttermilk components.

The role of matrix additions to the buttermilk powder for extraction efficiency was evaluated. Diatomaceous earth**,** Teflon® beads and physical vibration were tested and shown to reduce total lipid by 86%, 78% and 70%, respectively. Four consecutive treatments were shown to exhaust the system; however, similar extraction efficiency was noted for one treatment with biosilicate addition, two treatments with physical vibration, or three treatments with added Teflon® beads. The extracted lipid material consisted of the non-polar fraction and protein concentration was observed to increase slightly in comparison to the control.

The initial source of buttermilk powder was studied by a comparison of the supercritical fluid extraction treatment on two different sources of buttermilk powder, regular cream and whey cream. As an additional element, each source of buttermilk was passed through two different filtration modes, specifically volumetric concentration and diafiltration, prior to drying. The treated buttermilk powder was compared in terms of lipid extraction efficiency and by assessing compositional differences of initial and final products, as well as the lipids extracted from the powder. After three extractions, processing reduced the total fat in the powders by 38 – 55% and concentrated the phospholipids in the powders to a factor of 3.92 – 4.67. Specific molecular fatty acid combinations on the sphingosine backbone of sphingomyelin were characterized with the greatest proportion being saturated. The amount of total protein, soluble nitrogen at pH 4.6 and non-protein nitrogen increased

following supercritical fluid extraction treatment whereas the fraction of insoluble nitrogen at pH 4.6 decreased. Other powder components were not altered significantly.

To give insight into potential product application of whey cream buttermilk powder, the thermal stability prior to and following supercritical fluid extraction treatment was observed. The buttermilk was ultrafiltrated prior to drying and subsequently processed using supercritical fluid extraction. The treated BMP was compared alongside a whey protein isolate powder. Salt and pH levels were adjusted in the reconstituted powders and protein agglomeration upon heating was assessed. We have shown that supercritical fluid extraction treatment, pH, and the interaction between the two have a significant effect on optical density measurements and mean particle size in the solution. Supercritical fluid extraction treatment showed no effect on the analysis of the whey protein isolate, indicating the whey cream buttermilk powder to be a unique product with interesting functionality and composition.

The buttermilk powder products developed by combining filtration and supercritical fluid extraction processes are rich in polar lipids and proteins. We believe to have developed an ingredient with an enriched amount of bioactive lipids as well as one with unique functionality. Further knowledge into the metabolic effects and mechanisms of these products is needed before nutritional claims can be made. Certainly, a deeper understanding of their biological activities may implicate that modification of the diet can lead to an increased control over health.

Literature Review

Milk Lipids

Major and Minor

Lipids are esters of fatty acids and related components that are soluble in nonpolar organic solvents and insoluble in water (Walstra et al., 1999). The terms "fat" and "lipid" are used interchangeably; however, "fat" is usually considered to consist of a triglyceride mixture (Walstra et al., 1999). Fats mainly occur in the form of small droplets, having a high interfacial tension with water (Mulder and Walstra, 1974). Bovine milk fat includes many different classes of lipids. Not only is it comprised of mono-,di-, and triacylglycerols, but also free fatty acids, phospholipids, glycolipids, glycerine, and steroids. Minor constituents consist of waxes, alcohols, carotinoids, vitamins and lipoproteins (Molkentin, 2000, Renner et al., 1989, Warner, 1976). Located in the fat globule, 98-99% of the lipids are triglycerides; however, the fatty acid distribution among the triglycerides present enables milk fat to be one of the most complex natural fats (Renner et al., 1989, Warner, 1976). Milk fat composition is largely influenced by the stage of lactation, season and breed (Palmquist and Beaulieu, 1993). Regardless, milk fat has enough constant characteristics that it is discernible from other fats and oils by chemical and physical tests (Warner, 1976). The remaining 1-2% of the fat globule are minor lipids, the specific amounts are listed in Table 2.1 (Renner et al., 1989).

Free fatty acids represent only 0.1-0.4% of total milk lipids and they can vary with species, breed, feed, season, and other factors. The diversity of the free fatty acids depends upon their volatility, solubility, degree of unsaturation, saponification number, and iodine number (Warner, 1976). Similar fatty acids can differ

considerably in terms of chemical reactivity and nutritional properties due to their chain length and unsaturation of the acid residues. Melting range as well as crystallization behavior are determined largely from fatty acid composition including branching, incorporation of groups (-OH, =O), and cis-trans isomerism (Mulder and Walstra, 1974). Some of the free fatty acids are combined with glycerine in milk fat to form mono-, di-, and triglycerides (Warner, 1976). Although triglycerides are the most inactive and apolar lipids, their fatty acid composition does not solely determine their physical properties. The individual triglycerides and the fatty acid pattern is the ultimate determinant (Mulder and Walstra, 1974). Within milk fat, 70% of the fatty acids are saturated with palmitic, myristic, and stearic acids being the most common. These types of fatty acids are reported to increase human plasma levels of cholesterol (total and LDL), further increasing a risk factor for artherosclerosis (Kromhout, 2001, Molkentin, 2000). The remaining 30% are unsaturated, including oleic, linoleic, and linolenic acids (Warner, 1976). Diets high in unsaturated fatty acids are reported to decrease plasma LDL-cholesterol levels, thus having an antiatherogenic effect (Kromhout, 2001, Molkentin, 2000). Conjugated linoleic acids (CLA) comprise a group of conjugated or nonmethylene interrupted dienes; those CLA with one trans bond occurs mainly in milk fat, derived from linoleic, and possibly, linolenic acids (Jensen, 2002; Molkentin, 2000). CLA has been found, in a number of studies, to have antioxidant, antiatherogenic, and immunomodulating properties (Molkentin, 2000). Despite the favorable aspect of these, unsaturated fatty acids are unstable and may react with other elements, ultimately causing an adverse effect to milk flavor (Warner, 1976).

Another type of fatty acid found in milk fat is trans fatty acids (TFA). They are presumed to be caused by the hydrogenation of unsaturated fatty acids by rumen bacteria. The amount of TFA found in milk tends to depend upon seasonal feeding. A diet high in TFA from partially hydrogenated vegetable oils has been reported to have numerous biological effects. TFAs are thought to increase LDL-cholesterol, leading to an increase in atherosclerosis and coronary heart disease as well as causing a variety of further physiological and toxicological aberrations. However, correlations between atherosclerosis increase and TFAs found in milk fat have not been founded (Molkentin, 2000).

The major sterol in milk is cholesterol, comprising 0.25 – 0.45% of total lipids. Milk fat also contains small amounts of lanosterol, dihydrolanosterol and 7-dehydrocholesterol (provitamin D_3) (Molkentin, 2000). Sterols show some surface activity, are highly insoluble in water, and easily associate with phospholipids (Mulder and Walstra, 1974). Dietary cholesterol is important for fat absorption via bile acid synthesis and steroid hormone synthesis (Molkentin, 2000). However, it has been shown that elevated plasma cholesterol levels can have a negative health effect with regard to atherosclerosis and coronary heart disease (Kromhout, 2001, Molkentin, 2000).

The glycolipids (cerebrosides and gangliosides) constitute about 0.01 – 0.7% of bovine milk fat. This fraction includes the following components: glucosylceramide, lactosylceramide and monosialogangliosides (GM_3, GM_2, GD_3, GD_2, and GD_{1b}). Glycolipids have been found to mediate important metabolic and cellular events and they are largely concentrated in the milk fat globule membrane

(MFGM) (Jensen, 2002). Other lipids are present in very small quantities and include xanthophyll, squalene and waxes. These lipids may have uses as vitamins (mainly A – (ß-carotenoid), D, and E (α-tocopherol)), antioxidants (tocopherols) or pigments (carotenoids). Volatile trace constituents found in milk fat are also responsible for flavour, including several kinds of lactones, unsaturated aldehydes and ketones (Mulder and Walstra, 1974).

Phospholipids (PL) account for approximately 0.2-1.0% of total milk lipids (Molkentin, 2000). The type of feed and season will determine the PL content (Renner et al., 1989). Several PL groups are normally found in milk and consist of about 10 fractions. The predominant fraction is phosphatidylethanolamine (PE), followed by phosphatidylcholine or lecithin (PC) and sphingomyelin (SM). Phosphatidylinositol (PI) and phosphatidylserine (PS) comprise a small portion. In small amounts, lysophosphatidylcholine and ethanolamine are found while diphosphatidylglycerol is found only in traces (Renner et al., 1989). PLs are associated with both the milk fat, largely concentrated in the milk fat globule membrane (MFGM), and the milk proteins, small amounts are bound to caseins (Renner et al., 1989, Warner, 1976). PLs are destroyed by heat and contribute to the oxidized flavors in milk and milk products (Warner, 1976).

Milk Fat Globule Membrane

Milk fat consists as small globules surrounded by a surface layer or membrane, called a Milk Fat Globule Membrane (MFGM) (Walstra et al., 1999). The membrane is composed of a complex composition and structure and it functions to prevent coalescence of the fat globules; in other words, to assist the distribution of the fat

globules in the milk's aqueous phase (Deeth, 1997, Walstra et al., 1999). The MFGM contains only 2-6% of the total mass of the fat globules (Deeth, 1997). The formation of milk fat begins in the secretary cells of the mammary gland; within the endoplasmic reticulum, fat droplets coalesce and migrate from the basal to the apical region of the cell. The fat globules are eventually excreted from the cell through the apical region of the plasma membrane into the lumen (Figure 2.1). As they are excreted, the globule is enclosed by a typical bilayer membrane which has a similar composition to the plasma membrane. The MFGM provides the phospholipids, cholesterol, glycolipids, proteins, enzymes, and glycoproteins associated with milk fat (Deeth, 1997, Hamosh et al., 1984). Table 2.2 lists the approximate composition of the MFGM.

The MFGM components are associated with one another existing as complexes, frequently non-covalently linked. The approximate ratio of proteins to lipids within the membrane is 1:1 (Kanno, 1990). Neutral lipids, mostly triglycerides, and complex lipids, phospholipids and glycolipids, have an approximate ratio of 2:1 (Walstra et al., 1999). A study by Fong et al. (2007) found that 56% of total MFGM lipid was composed of triglycerides and their fatty acid composition contained higher proportions of palmitic and stearic acids and fewer unsaturated fatty acids, such as C14:1, C16:1, C18:1 and conjugated C18:2, as compared to milk fat triglycerides. PL content, determined by mass balance, represented 40.6% of the lipid fraction.

Several other substances (Fe, Cu, and carotenoids) are contained in trace quantities (Table 2.2). The skim milk portion of milk contains membrane material in these same concentrations; it does not originate from the milk fat globules, but rather

due to material sloughed from the mammary cells surface during milk secretion. However, a variety of treatments, such as agitation, can cause portions of the MFGM to be dislodged as well (Deeth, 1997, Walstra et al., 1999).

An integral role to the stability and composition of the MFGM are the PLs. The major types and their estimated percentages are: PC, 31%; PE, 30.5%; SM, 19.9%, PI, 7.1%; PS, 5%; lactosyl-cerebroside, 3.4% and glucosyl-cerebroside, 0.3% (Fong et al., 2007). Having both lipophilic and hydrophilic properties, the PLs contribute to the emulsification stability of the MFGM, an important functional characteristic. The membrane arrangement is rather asymmetric; the outer and inner surfaces differ in composition. The choline-containing PL (PC and SM) and the glycolipids (cerebrosides and gangliosides) are located primarily on the outer surface while the neutral lipids (PE, PS, and PI) are primarily concentrated on the inside. Of the proteins, glycoproteins are concentrated on the outer surface while other proteins, such as xanthine oxidase, strongly bind lipid material and are situated on the inner surface. Other proteins, such as butyrophilin, are transmembrane proteins and are imbedded within the membrane (Deeth, 1997, Jensen, 2002). Figure 2.2 illustrates a typical bilayer membrane.

The MFGM plays an important role in fat globule protection and, in effect, milk flavor. Damage to the PL bilayer, generally accomplished by the enzyme phospholipase C, can lead to flavor defects in milk and milk products. A specific defect, known as “bitty” or “broken” cream is caused by degradation of membrane PLs by the enzyme. This damage can be visualized on the surface of hot tea or coffee when the fat globules coalesce to form visible flecks (Deeth, 1997).

Phospholipids not only function as integral components of the membrane, but have been shown to be involved with other biological processes. Some PLs have been shown to exhibit antioxidative, anticarcinogenic, and antiatherogenic properties (Berra, 2002, Dacaranhe and Terao, 2001, Molkentin, 2000). Sphingolipids, in particular, are known to have essential roles in cell-to-cell interactions, differentiation, proliferation, immune recognition, transmembrane signaling, and as cell receptors. In addition, in vitro results suggest that milk membrane lipids have bacteriocidal activities and may protect against gastrointestinal infections (Huwiler et al., 2000, Kim et al., 1991, Lightle et al., 2000, Okazaki et al., 1989). A recent study by Noh and Koo (2004) demonstrated that sphingomyelin is an effective inhibitor of intestinal absorption of cholesterol in rats. The highly saturated long chains of fatty acyl groups were explained to inhibit absorption by decreasing the rate of luminal lipolysis, micellar solubilization, and transfer of micellar lipids to the enterocyte. Their location on the MFGM exterior and their interactive character suggests a major role in many different important biological interactions (Deeth, 1997).

Sphingolipids and Cell Signaling

Sphingolipids (SL) and their breakdown products not only play a structural role in lipid bilayers, but are of interest because they are an emerging class of bioactive molecules that have profound effects on cell regulation (Barenholz and Thompson, 1999, Cinque et al., 2003, Vesper et al., 1999). The sphingolipids include sphingomyelin (SM), sphingosine (S), sphingosine 1-phosphate, ceramide, and ceramide 1-phosphate and are metabolites of one another. Within recent years, they have been discovered as important signaling molecules (Cutler and Mattson, 2001,

Huwiler et al., 2000, Signorelli and Ghidoni, 2005, Vesper et al., 1999). They have been implicated in a variety of biological functions including cell growth regulation, cell development, adhesion, cross membrane trafficking, stress responses, roles in ageing and age-related disease, and apoptosis (Cinque et al., 2003, Kim et al., 1991, Lightle et al., 2000, Okazaki et al., 1989, Vesper et al., 1999). Although there are no known nutritional requirements for sphingolipids, evidence is suggesting that they are 'functional components' of food (Berra, 2002, Lemonnier et al., 2003).

Sphingolipids consist of a long-chained sphingoid backbone and all together, may represent the most structurally diverse, as well as complex, group of lipids in nature (Berra, 2002, Vesper et al., 1999). Along with PCs, SMs form one of the major classes of PLs and comprise more than 50% of membrane PLs (Barenholz and Thompson, 1999). Up 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 budding uses as anticancer agents or for regulating disorders in which apoptosis plays a crucial role (Nava et al., 2000, Perry et al., 1996). Many of their roles in 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).

The molecular structure of the commonly named 'sphingosine' backbone, an aminodiol, is shown in Figure 2.3. Its proper chemical name is D-*erythro*-1,3-dihydroxy-4,5-*trans*-octadecene and all naturally occurring SL contain only the D-*erythro* form (Hanahan, 1997, Merril et al., 1997, Vesper et al., 1999). This backbone

differentiates the SLs from the glycerol-containing lipids, such as triglycerides. Ceramide, a sphingosine acylated at the 2-amino position, is the next building block in SL structure (Huwiler et al., 2000). This structure is shown in Figure 2.4. Ceramide is the basic frame for most SLs; however, differences in the lipid backbone structure and substituents at the 1-hydroxyl position create the complexities of SL structures (Huwiler et al., 2000). Linking the 1-hydroxy group of ceramide to a phosphorylcholine head group produces sphingomyelin; this is induced by the enzyme sphingomyelin synthase (Huwiler et al., 2000). Milk SM contains more longer-chain saturated fatty acids, 22:0, 23:0 and 24:0, which account for 66% of its total fatty acid content (Noh and Koo, 2004). The naturally occurring form of SM is the D-*erythro*-(2S,3R) form; however, its stereoisomer, L-*threo*-(2S,3S), has been separated through normal-phase HPLC. This results in semi-synthetic preparations of SM (Ramstedt and Slotte, 2000). The stereoisomer structures are shown in Figure 2.5.

Within eukaryotic cells, SLs are primarily components of cell membranes; however, they also occur in lipoproteins, the lamellar barrier of skin, the Golgi apparatus and the liposomes. The membrane-rich tissues such as pancreas, liver, brain and neural contain large concentrations of SLs as well (Berra, 2002, Merril et al., 1997). Evidence suggest that SL structure and their interaction with other phospholipids, as well as cholesterol and proteins enable the formation of rafts, located in cellular membranes, including the plasma membrane, late secretory pathway and endocytic components. These highly organized rafts have been studied to deduce their functional roles in hematopoietic cell signaling. Raft location and their associations with proteins have been implicated as being important in their

functionality (Brown and London, 2000). Mutagenesis has been used to suggest that two proteins Lck and LAT must be in SL rafts in order to function. Janes et al. (1999) iscovered the signaling stimulation of T cells was abolished in cells with a non-associating mutant form of Lck. However, when the same mutant was bridged back to the rafts via antibodies, signaling was replenished. This suggests that mutation did not inactivate the proteins function, only its raft association. Additional studies suggest that a mutant non-palmitoylated LAT, localized on the plasma membrane, but not present in the rafts is unable to serve as a substrate for tyrosine phosphorylation or function in signaling (Lin et al., 1999, Zhang et al., 1998).

Within SL-rich plasma membrane domains, glycosylphosphatidylinositol (GPI)-anchored glycoproteins are often found. Recent evidence suggests that the association of these proteins with membrane lipids may be an inherent property of their cell functions. These proteins are known to function in interactions between adjacent cells and other extracellular substrates (Brown and Waneck, 1992). Ilangurmaran (1997) investigated this association using a mutant cell line from mouse melanoma cells. A complete removal of sphingolipids from the plasma membrane showed defects in cell-substratum adhesion mechanisms. Upon addition of either glycosylated sphingolipids or sphingomyelin, normal cell adhesion occurred. Furthermore, an analysis by Lefèvre (2000) showed that binding could occur between β-lactoglobulin protein and SM on the lipid bilayer, ultimately causing conformational changes. Because SM structure, long and highly saturated hydrocarbon chains, allow for incorporation within the lipid bilayer of the cell membrane and SM has a Tm near 37°C (body temperature) it is believed that these lipids play important roles in protein-

membrane attachment, allowing specialized protein interactions (Brown and Waneck, 1992, Cutler and Mattson, 2001).

Although not typically found in the prokaryotic cell, some bacterial taxa contain high concentrations of sphingolipids. Two bacterial genera known to contain and synthesize SLs are *Sphingobacterium* and *Sphingomonas*. In other anaerobic bacteria, a high level (up to 70%) of the total extractable lipids was found to be SLs. A specific gram-negative bacterial species, *Flectobacillus major*, was observed to have even higher levels (90% of total extractable lipids) of polar lipids (Batrakov et al., 2000).

During the past 30 years, evidence has accrued linking sphingomyelin and sphingolipids in general to serving prominent roles in cellular signal transduction and regulation of cell functions. Upon cell stimulation, which includes a variety of stimuli, enzymatic cleavage of the membrane phospholipids can cause release of bioactive messenger molecules within the cell. For example, an innovative signaling pathway identified SM to be cleaved by a phospholipase C-like enzyme, generating ceramide and phosphorylcholine. This pathway was termed the "Sphingomyelin Cycle" and is the first step in the formation of second lipid messengers involved in controlling immune cell development, differentiation, activation, proliferation and function (Cinque et al., 2003, Cutler and Mattson, 2001, Levade and Jaffrézou, 1999, Parodi, 1997, Signorelli and Ghidoni, 2005). Throughout this cycle, occurring from minutes to hours after cell stimulation, up to 30-40% of the total cellular sphingomyelin is hydrolyzed. Nevertheless, SM levels will shortly return to baseline levels (Levade and Jaffrézou, 1999).

The existence of the SM cycle was established by Okazaki et al. (1989) in HL-60 human myelocytic leukemia cells. It was observed that vitamin D_3 caused the activation of a neutral sphingomyelinase, resulting in the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Consequently, a study by Kim et al. (1991) in HL-60 cells showed that sphingomyelin hydrolysis is induced specifically in response to inducers of monocytic differentiation (Vitamin D_3, TNFα, and γ-IFN) but not to neutrophilic differentiation inducers, suggesting important functions in cell differentiation. The hydrolysis of SM to ceramide is an important event because ceramide is believed to play a key role in the regulation of cell functions (Levade and Jaffrézou, 1999). In addition to SM hydrolysis, ceramide can be formed directly from sphingosine by the enzyme ceramide synthase (Huwiler et al., 2000). Ceramide undergoes a cycle of phophorylation and dephosphorylation by the enzymes ceramide kinase and lipid phosphomonoesterase, respectively. When phosporylated, the lipid ceramide 1-phosphate is produced; it is hypothesized that this process induces a unique signaling molecule (Bajjalieh and Bachelor, 1999). Hydrolysis of ceramide by the enzyme ceramidase produces sphingosine. Phosphorylation of sphingosine by sphingosine kinase will result in sphingosine 1-phosphate; both sphingosine and sphingosine 1-phosphate are involved in intracellular signaling pathways (Bajjalieh and Bachelor, 1999, Cutler and Mattson, 2001, Huwiler et al., 2000). Figure 2.6 explains the sphingolipid signaling cascade.

The metabolites formed by this cascade are involved in a number of intracellular signaling mechanisms including the activation and regulation of kinases, protein kinase A and tyrosine kinase A; inhibition of protein kinase C; protein

phosphatases; oncogenes; transcription factors; cellular differentiation and proliferation; sterol homeostasis; and apoptosis (Bajjalieh and Bachelor, 1999, Chatterjee, 1999, Levade and Jaffrézou, 1999, Signorelli and Ghidoni, 2005).

At least seven different sphingomyelinases (SMase) have been distinguished in mammalian cells, tissues and biological fluids. The best characterized of these enzymes are the acid sphingomyelinase (aSMase) and the neutral sphingomyelinase (nSMase) (Levade and Jaffrézou, 1999). Membrane bound nSMase is responsible for the hydrolysis of SM to ceramide and phosphorylcholine as described above; this event is optimized at a neutral pH (~7.4) and requires magnesium (Mg^{2+}) for optimal activation (Chatterjee, 1999). Studies have shown that nSMase is activated by many stimuli, including cytokines and growth factors, neurotransmitters, hormones, reactive oxygen species, and ceramide as well (Huwiler et al., 2000). This sphingomyelinase is reported to have a higher activity in isolated plasma membranes (Lightle et al., 2000). The acid sphingomyelinase is cation independent, has an acidic pH optimum, and is located in the lysosomes (Gatt, 1999). Although this enzyme was identified more than 30 years ago and is one of the best characterized, its specific hydrolase activity is still under investigation (Levade and Jaffrézou, 1999). A third enzyme, alkaline SMase has been located in the intestinal tract, specifically at high levels in the middle and lower small intestine and lower levels in the colon. Studies have shown that it has a direct inhibitory effect on the growth of HT-29 human colon cancer cells suggesting that this SMase has a role in the regulation of cell proliferation in the colon (Hertervig et al., 2003).

The binding of an extracellular ligand to its respective receptor activates the sphingomyelin cycle. Some of the known sphingomyelin cycle activators include apoptosis inducers (TNFα, nitric oxide, dexamethasone), differentiation inducers (vitamin D3, NGF, TNFα, retinoic acid, progesterone), inflammatory cytokines (INFγ, interleukin 1α, interleukin 1β), and cell damaging agents (ionizing radiation, UV light, osmotic or heat shock, serum withdrawal, oxidative stress, chemotherapeutics, oxidized low-density lipoproteins). However, the specific action of these inducers on sphingomyelinase activation or the type of sphingomyelinase that is activated is still not known. In addition, cycle regulation and termination is not yet clear (Berra, 2002). Studies have shown that sphingomyelinase deficiencies can lead to sphingolipids storage diseases characterized by sphingolipid accumulation (Ferlinz et al., 1999).

Apoptosis or programmed cell death is a biochemically regulated process, which is initiated by internal or external stimuli and causes a cell to cease functioning. This process is thought to play an important role in a wide variety of biological and pathological conditions including development and tissue remodeling, cancer, ischemia and infarction, immune disorders, and neurodegenerative diseases. Cancer pathogenesis and autoimmune disorders are believed to be a condition of defective apoptosis. Advances in cell biology have lead to important insights into apoptosis pathways and the components involved with regulation. Recent studies have observed that a signal transduction pathway involving sphingolipids plays a crucial role in apoptosis regulation (Hertervig et al., 2003, Perry et al., 1996, Signorelli and Ghidoni, 2005).

Because the stimuli involved in sphingomyelin cycle regulation are thought of as stress inducers for cells, it is believed that the metabolites of this cycle may be crucial intermediates involved in cell apoptosis. In response to the stress inducers described above, ceramide levels have been observed to be elevated (Sawai and Hannun, 1999). Changes in ceramide levels are believed to have an effect on cell regulation. At low levels, cell survival and division is promoted, at higher levels, cell division and differentiation is inhibited, and at even higher levels, cellular dysfunction and apoptosis is induced (Cutler and Mattson, 2001). Ceramide is thought to regulate cell processes by targeting a variety of cellular components. Recent studies have identified a number of ceramide-activated protein kinases and phosphatases. Specifically, a member of the protein kinase C family can be stimulated by ceramide, leading to an activation of NF-κB, a transcription factor that is stimulated by oxidative and metabolic cellular stress (Cutler and Mattson, 2001). Moreover, the addition of a ceramide analog into pro-myeloid U937 cells caused cell death; this mechanism mimicked the effects of cytokine TNF-α, a known inducer of cellular apoptosis (Huwiler et al., 2000). Studies have shown that the wide variety of cellular targets that exist for ceramide and the range of outcomes upon ceramide generation are highly cell specific (Perry et al., 1996).

It is hypothesized that tumor cells, i.e. cancer, are a direct effect of hindered ceramide generation. Modrak et al. (2002) observed the effect of increased sphingomyelin content and effects of chemotherapeutics for the treatment of HT-29, HCT15 and GW-39 human colonic tumor xenografts. It was found that upon administration of exogenous sphingomyelin, the tumor cells were more responsive to

the anticancer drugs. This effect is believed to be attributed to the tumor cells possessing insufficient sphingomyelin or sphingomyelinase levels. In any case, the limiting factor for apoptosis is thought to be decreased ceramide production.

The roles of the sphingolipid metabolites, ceramide, sphingosine, and sphingosine 1-phosphate in radiation induced apoptosis in cancer cell lines were examined by Nava et al. (2000). The prostate cancer cells that were studied had different sensitivities to γ-irradiation. It was observed that increased amounts of the sphingolipid metabolites increased the susceptibility of the resistant cancer cells by inducing apoptosis. These results suggest that sphingomyelin metabolites are critical components in radiation-induced apoptosis in human cancer cells, and cancer cells may be induced to cell death by preventing metabolite generation. This may have a therapeutic value to cancer patient treatment.

Because sphingolipids have been shown to have the potential of inhibiting carcinogenesis, it is hypothesized that the consumption of sphingomyelin may provide bioactive compounds to inhibit the production of tumors. A study by Dillehay et al. (1994) involved feeding sphingomyelin to CF1 mice, initiating colon cancer by the carcinogen 1,2-dimethylhydrazine, and finally, determining colonic cell proliferation and formation of colonic tumors. It was determined that feeding sphingomyelin to the mice reduced the number of abberant colonic crypts and decreased the incidence of tumors by half. These observations suggest that sphingolipids may be a significant class of chemopreventive agents.

Further studies involving feeding female CF1 mice buttermilk extracted glycosphingolipids by Schmelz et al. (2000) observed the same trend; consumption of

sphingolipids suppresses colonic cell proliferation. It was established that a diet composed of 0.025 to 0.1% of glucosylceramide, lactosylceramide and gangliosides inhibits colon cancer formation in the tumor-induced mice by 50 to 60%, comparable to earlier studies involving sphingomyelin feeding. Although the number of apoptotic cells was not altered in the mice, it is believe that the released metabolites of the sphingomyelin cycle are the cause of the reduced proliferation.

Because dietary SLs are hydrolyzed throughout the intestinal tract there is thought that supplementation of the diet with SL can be used for both chemopreventative and chemotherapeutic outcomes. A study by Lemonnier et al. (2003) found that supplementing DMH-treated CF1 mice with non-pharmacological amounts of SM both prior to and after tumor initiation significantly reduced tumor formation. Although the apoptosis rate was not seen to be above normal levels, a more feasible account for tumor suppression is that exogenous SLs may restore normal regulation of cell growth and death.

Increasing evidence shows that the detrimental effects of aging may be accompanied by a decrease in sphingomyelin synthesis and an elevation in sphingomyelin hydrolysis, resulting in ceramide accumulation. An evaluation performed by Lightle et al. (2000) was accomplished in order to determine the effects of aging to the liver, specifically related to sphingolipid analysis. It was found that the levels of ceramide and sphingosine were elevated in the livers of old male Fisher 344 rats in comparison to young rats. In addition, the levels of aSMase and nSMase were elevated and SM synthase activity was lower in the older animals, resulting in a buildup of ceramide levels. This study suggests that increased ceramide levels may be

a feature of the aging process. Moreover it is observed that SL accumulate in the membranes linearly with age, hypothesized to be due to the long-chain sphingolipids (particularly ceramides with 18 carbons and greater) that cannot transport themselves across lipid bilayers (Cutler and Mattson, 2001). Sphingolipid accumulation has been shown to slow the efflux of sterols, a necessity for growth and development, but after adult maturation may promote atherosclerosis and cancer (Cutler and Mattson, 2001).

It is likely that nSMase may contribute to plaque rupture in advanced atherosclerosis. The aortic smooth muscle cells in atherosclerosis undergo proliferation at a higher rate than normal vascular wall cells due to increased amounts of growth factors, leading to plaque formation and rupture, and ultimately to death. It is observed that nSMase activity is higher in atherosclerotic plaques; thus leading scientists to hypothesize that nSMase may be involved in plaque rupture by means of apoptosis. Further research into nSMase-induced apoptosis may contribute significantly to drug design and other applications toward combating premature plaque rupture in advanced atherosclerosis (Chatterjee, 1999).

Proper sphingolipid metabolism is necessary, as defects can lead to serious diseases known as sphingolipidoses. These diseases are characterized by the accumulation of sphingolipids corresponding to defects in specific enzymes involved in hydrolysis. Ten different types of Neimann-Pick diseases have been characterized; two examples are Niemann-Pick disease and Farber disease. Niemann-Pick disease is a recessively inherited metabolic disorder resulting in acid sphingomyelinase deficiencies. Insufficient activity of this enzyme leads to accumulation of sphingomyelin in the spleen, liver, lungs, bone marrow, and, in some people, the

brain. There are two types of the aSMase-deficient Niemann-Pick disease, typed A and B (infantile and juvenile, respectively). Children born with the type A form of the disease are characterized by jaundice, enlargement of the liver, and profound brain damage; they rarely live beyond 18 months of age. Type B, with an onset during the pre-teens, is characterized by enlargement of the liver and the spleen, and may live a comparatively long time. Because of the natural aSMase deficiency of this disease, cells from Neimann-Pick patients may provide a valuable tool to explain the biochemical roles of sphingolipids in cell signaling (Lansmann et al., 2003).

The accumulation of ceramide, caused by a deficiency of lysosomal acid ceramidase, is the basis for Farber disease, a rare autosomal recessive inherited disease. This enzyme hydrolyzes ceramide to sphingosine and free fatty acids. Hoarseness, painful and swollen joints, nodules under the skin, and growths in the lungs and other parts of the body characterize this disease. Farber's disease typically manifests itself a few months after birth and typically ends with an early death, although a few patients survive to their teens (Park and Schuchman, 2006, Zhang et al., 2000). Recent research into genetic mapping of the ceramidase gene may provide insight into this disease (Zhang et al., 2000). A "knock-out" mouse model of acid ceramidase has been produced, gene encoding has been obtained, gene mutations causing Farber disease have been identified, and the recombinant enzyme has been produced and characterized. Although many important advances have been made, more information is required to fully understand the enzyme's role in SL-mediated signal transduction and human disease (Park and Schuchman, 2006).

A challenging aspect into the treatment of sphingolipidoses is the diagnosis of the disease. In addition to the measurement of enzyme activities, analysis of sphingolipids in tissues has been accomplished using TLC and HPLC. Recently, a new method using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF-MS) was shown to directly detect sphingolipids in human tissues and cultured skin fibroblasts. Both Neimann-Pick and Farber disease were detected by autopsied liver, spleen, and brain tissue; however, this type of biopsying is too invasive. Although DE MALDI-TOF-MS using cultured skin fibroblasts was useful in determining Farber disease, this method was not able to distinguish Neimann-Pick disease. Further studies with DE MALDI-TOF-MS, such as improvement of lipid preparation methods, may prove this method a reliable diagnostic tool on living patients (Fujiwaki et al., 2002, Samuelsson et al., 1997).

Sphingomyelin signaling and the levels of its metabolites are of importance to cellular metabolism. Increases in ceramide levels are associated with cellular responses to stress, aging, and atherosclerosis; this implies that reducing sphingomyelin signaling may be beneficial. Conversely, decreases in ceramide levels may serve to suppress certain cancers. Controlling sphingomyelin and ceramide levels by way of dietary manipulations may lead to an increased control over ones health.

Lipid Analysis

Thin Layer Chromatography (TLC)

Chromatography methods are the most widely used for the analysis and identification of both simple and complex lipids (Christie, 1987). Thin Layer Chromatography (TLC) has served lipid analysts well for many years being

inexpensive and versatile. Consisting of a strip of glass, plastic, or aluminum as a backing to a plate with a thin layer of silica gel (sometimes mixed with other substances) as the adsorbent, TLC can separate mixtures of compounds. This method remains a powerful and rapid technique, enabling qualitative comparisons of large numbers of samples (Hammond, 1993).

TLC is particularly useful for rapid qualitative sample examination, method development, and compound screening (Christie, 1987). It is simple, quick, inexpensive, and requires only small amounts of sample (Christie, 1987). Component separation depends upon the interaction of the components and the adsorbent silica. Those components that interact strongly with the silica will tend to move slowly on the plate relative to those that do so weakly (Hammond, 1993). Silica gel has adsorptive properties due to hydroxyl groups which interact with polar functional groups (for example, ester bonds, phosphate, hydroxyl, and amine groups) in compounds (Christie, 1987). Highly polar compounds do not migrate far whereas the more non-polar a compound is, the further it will travel up the plate during analysis. Movement on the TLC plate is also dependant upon the mobile phase, consisting of a variety of solvent systems. Mobile phase selection for a particular analysis depends on what compounds are present in the sample (Hammond, 1993). A common solvent system for the analysis of nonpolar lipids is a mixture of petroleum ether, ethyl ether, and acetic acid (95:15:2 v:v:v). The sample spots resulting on the plate when using this solvent system are in the following order: cholesterol esters at the solvent front, followed by triacylglycerides, free fatty acids, cholesterol, diacylglycerides (1,3-

followed by 1,2-), monoacylglycerides, and phospholipids (remaining at the point of application) (Hammond, 1993).

In one-dimensional TLC systems, the most commonly used mobile phase for polar lipid analysis consists of chloroform-methanol-aqueous mixtures of different polarities (Christie, 1987, Hammond, 1993). A solvent system consisting of chloroform, methanol, and water (65:25:4 v:v) has been used successfully in the analysis of complex lipids in membrane systems; the major classes are phospholipids, sphingolipids, and cholesterol (Gomes-Quitana et al., 1998). Using this system, a complex misture (consisting of SM, cholesterol, nonpolar lipids, PE, PS, and PC) of lipids would separate in the following order: nonpolar lipids at the solvent front, followed by cholesterol, cardiolipin, PE, PS, PC, then SM closest to the point of application (Hammond, 1993).

Complex lipid separations can be achieved as well, however, a wide variety of solvent systems exists and no single TLC system can separate all complex lipids in one dimension. In order to have complete separation of all polar lipids, a two dimension system can be used; this may resolve the components that appear as one compound in the first dimension. When needed, it offers considerable advantages; however, two dimensional TLC is time-consuming and sensitive to external environmental stimuli (Hammond, 1993).

In order to visualize the compounds separated by TLC, the plate is subjected to a variety of treatments. These treatments can be nondestructive or destructive, where the sample spots are converted chemically to carbon or colors. Nondestructive methods include the use of fluorescing agents, UV light, and exposure to iodine

vapors. Spots containing organic samples will show a bluish or yellow fluorescence when exposed to UV. Samples are not destroyed with this method, making it the choice for preparative TLC; however, background fluorescence may interfere with sample visibility. There are several types of destructive techniques including exposure to ferric chloride and sulfuric acid. For identification of a wide range of organic molecules, exposure to iodine vapors or sulfuric acid charring are frequently used. Specific chemical reagents react with specific compounds, so one method many only reveal one type of compound. For example, ferric chloride spray identifies phenols and dinitrophenylhydrazine identifies neutral plasmalogens. A wide variety of spray reagents can be used to identify functional groups, such as amine, phosphate or carbohydrate moieties, in lipids. Therefore, as a preparative and qualitative method, TLC remains a powerful and rapid technique (Christie, 1987, Hammond, 1993).

Although simple and inexpensive, TLC has a number of disadvantages; quantitative errors can be as high as 20% and it is a relatively tedious process. The advent of high-pressure liquid chromatography (HPLC) has somewhat overshadowed this method. Many separations of lipids by means of HPLC have proven to be more efficient in reproducibility and quantification than TLC (Christie, 1987, Hammond, 1993).

High-Pressure Liquid Chromatography (HPLC)

High-pressure liquid chromatography (HPLC) is a compound separation technique that can be used for both sample justification and purification. It is a form of “closed column” chromatography in which a mobile liquid phase is forced under controlled high pressure through a narrow bore column and analytes are detected as

they exit the column. Analyte separation is dependant upon their distribution between the mobile and stationary phases. Separation mechanisms commonly used in HPLC are adsorption chromatography, partition chromatography, ion exchange chromatography, ion pairing chromatography, and size exclusion chromatography. Adsorption chromatography is the most commonly used method for phospholipid separations. Using this method, the stationary phase is the liquid-solid interface and molecules are reversibly bound to the surface by dipole-dipole interactions. Depending on the compound, the strength of interaction is different, leading to variable residence times at the stationary phase enabling substance separation (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993).

Parameters that affect HPLC separation are explained in the following paragraphs and in Figure 2.7. Between the mobile and stationary phases, a dynamic equilibrium exists and an analyte will move from one end of the column to the other dependant upon their specific equilibrium coefficient or capacity factor, k' (Figure 2.7a). The capacity factor is defined as the ratio of the amount or concentration of a given component in the stationary phase to that in the mobile phase; it is defined in terms of the solute's retention time and the elution time of the solvent. The rate of movement for a certain compound is inversely proportional to the capacity factor. Because this is compound specific, different compounds will migrate at different rates, so separation is possible. The relative retention α, also known as the separation factor, is a ratio between two capacity factors (Figure 2.7b). This is a measure to describe the ability of a specific mobile and stationary phase system to discriminate between two compounds where the reference compound is the denominator. The relative retention

is dependant on the temperature and the properties of the phases (i.e. impurities in the mobile phase) and is independent of column manufacture (length, quality of packing) and flow velocity.

Column packing and mass transfer phenomena are characterized by the number of theoretical plates, n, associated with a specific column (Figure 2.7c). With an increased number of theoretical plates, more complex sample mixtures can be separated. This is a measurement depending on the ratio of the retention time of a specific component to the band width of the component peak. The chromatographic equilibrium between the mobile and stationary phase is established as the height equivalent of a theoretical plate, h (Figure 2.7d). It is measured as a ratio of column length to the number of theoretical plates. Since a large number of theoretical plates is desired, the height should be as small as possible and is dependant upon the particle size, the flow velocity, the mobile phase (viscosity) and the packing quality.

A typical HPLC system is comprised of six basic components: solvent storage and delivery system, sample injection device, column, detector, and waste recovery (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993). A schematic diagram of this system is shown in Figure 2.8. The solvent storage and delivery system is comprised of storage bottle space and a pulse-free pump. Typically, up to four solvent bottles are available for mobile phase creation and are capped to prevent evaporative loss; caps should allow a tubing inlet to pass through. Solvents are normally degassed before use because small gas bubbles can collect in other components, such as the pump heads and the detector, and ruin the analysis. The pump is utilized to ensure a precise, reproducible, constant and pulse-free delivery and flow of the mobile phase.

The pump should be capable of delivery solvents under pressures of up to 6000 psi; pressure of delivery is dependent upon the resistance to flow created by the column system. Many separations can be achieved using an isocratic elution, where a single-solvent system remains constant during the analysis. More complex analyses utilize a gradient elution system, where the mobile phase composition is gradually strengthened throughout the separation. During this type of elution, highly retained compounds are eluted more quickly and the compounds that elute earlier remain well resolved. Generally, the rate and amount of solvent delivery is controlled by the pump (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993).

There are a variety of sample injection devices that exist and they can be controlled either manually or automatically. Overall precision is improved with an autosampler due to digital control and this is controlled by a valve which allows line switching. Loops that have a calibrated volume are attached to the line and when the valve is in the load position, the sample loop is filled (generally 10 to 100μL in volume). Turning the valve from the load to inject position connects the sample loop to the mobile phase flow and the sample is sent to the column (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993).

Because separation occurs here, the column is thought to be the heart of the HPLC system. The column is made of stainless steel, which is relatively inert to chemical corrosion and is packed with a micro-particulate adsorbent or other phase depending on the type of separation. The stationary phase is a porous solid and the surface area is inversely related to the pore size in the particles. In normal phase chromatography, the stationary phase consists of highly polar silica along with a

relatively nonpolar mobile phase (hexane or *i*-propylether); the separation properties for HPLC are the same as TLC. During the analysis, the least polar component is eluted first with the most polar component eluting last; mobile phase polarity can be increased to decrease elution time. Conversely, reversed phase chromatography consists of a nonpolar stationary phase, often a hydrocarbon, coupled with a relatively polar mobile phase, such as water or acetonitrile. In this method, the most polar compound is eluted first and increasing the mobile phase polarity will increase the retention time (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993). Figure 2.9 depicts the relationship between polarity and elution times for normal and reversed phase chromatography.

Once the analyte elutes the column it is transported to the detector, which generates an electrical signal that is proportional to the level of some property of the analytes, generally compound mass. Typically connected to a computer or integrator, the signal is measured and plotted as a chromatogram to give qualitative and quantitative results. The characteristics of a good detector are sensitivity, linearity, predictability, reliability, nondestructiveness, ease of use, and low dead volume. Some common types of HPLC detectors are ultraviolet absorbance (UV); evaporative light scattering (ELSD); and mass spectrometry (MS). Ultraviolet absorption detectors are the most commonly used because of their cost and simplicity. The mobile phase is passed through a flow cell, where an UV/visible photometer radiation beam is located. When a UV-absorbing analyte passes through the cell, a signal is generated that is proportional to the solute concentration. UV detectors can give high selectivity and sensitivity to compound analysis and quantitative analysis can be

performed with a high degree of precision. However, only compounds with certain functional groups, such as alkenes, aromatics, and compounds that have multiple bonds between C and O, N, or S, will absorb wavelengths in this range of the spectrum. A few lipids have functional groups with high extinction coefficients in the UV range and they exhibit a weak absorbance in the range 200 to 210 nm; this is predominately due to the presence of isolated double bonds. There are some disadvantages to using this type of detector. Many of the valuable solvents for lipid chromatography absorb strongly in this UV range as well. The few solvents that are transparent in this region are water, hexane, isopropanol, acetonitrile and methanol. Because of the types of functional groups that absorb UV are varied in lipids, quantification is not easy and saturated fats may be overlooked (Brown and DeAntonis, 1997, Christie, 1987, Hammond, 1993).

An evaporative light scattering detector (ELSD) is commonly termed a "mass detector" because the analyte is detected and the amount of material or mass that is detected is measured. With this instrument, the solvent containing the analyte emerges from the end of the column, passes through a heating chamber where it is nebulized by a stream of air or nitrogen to the form of minute droplets. The droplets then pass through a light beam which is reflected and refracted. The amount of scattered light is measured and is related to the amount of analyte in the solvent. The ELSD is considered to be universal in their applicability, highly sensitive, inexpensive, and simple to use. Solvent selection is important because they must be sufficiently volatile to evaporate in the heating chamber. The disadvantages associated with ELSD are sample recovery, detector linearity, and sample refractive

index differences. Because the solute and solvent are evaporated in the light beam, the detector is destructive to the sample and it is lost; however, there is the option of inserting a stream splitter between the column and the detector to collect sample. To optimize the detector to resolve linearity problems, the flow-rate of the nebulizer gas and the evaporation temperature can be adjusted. Finally, detector response is dependent on the refractive index of the sample. If instrumental parameters are adjusted and calibrated correctly, good quantitative results can be obtained. Because of the simplicity, selectivity and cost of this detector, it is used fairly often in HPLC lipid analysis. Recent detection experiments using ELSD have proven that this method allows rapid, repeatable base-line separation of all PL classes. Along with separation, ELSD improved detection sensitivity by tenfold or more, and solvent consumption was reduced by 80% (Christie, 1987, Hammond, 1993).

This technique is an improvement over UV detection because chain-length and degree of unsaturation of the fatty acid compounds have little effect on the response of the detector. Therefore, ELSD is useful for quantitative analysis by not requiring a positive identification of the fatty acid composition. Numerous phospholipid applications have been presented, including analyses with both polar and neutral species and those with simple and complex solvent systems (Ramstedt and Slotte, 2000, Silversand and Haux, 1997, Vaghela and Kilara, 1995).

Mass spectrometry (MS) is a powerful analytical tool that can supply both qualitative and quantitative data. Following the success of gas chromatography-mass spectrometry (GC-MS) systems, interfacing MS with HPLC systems have been developed (Christie, 1985). MS has the ability to provide compound molecular

weight, empirical formula, and complete structure (Christie, 1985). It is used for a variety of applications including identification of unknown compounds, quantification of known compounds, and chemical and molecular structure elucidation. The combination of MS with HPLC allows for both separation and identification in the same step (Brown and DeAntonis, 1997). MS is a very sensitive technique; detection of compounds can be accomplished with very minute quantities (as little as 10^{-12}g or 10^{-15} moles for a compound of mass 1000 Daltons) (Crews et al., 1998). Further, compounds can be identified at very low concentrations (one part in 10^{12}) in chemically complex mixtures. MS allows a user to observe all isotopes present in a compound simultaneously and to visualize ions of different masses (Crews et al., 1998). Previously, the advent of HPLC-MS was hindered by interface design; flow rate differences and sample vaporization problems are among the interfacing difficulties (Brown and DeAntonis, 1997). However, these problems have been overcome and many interfaces are now available, the most common being electron impact or ionization (EIMS) and chemical ionization (CIMS) (Christie, 1985).

EIMS is also known as a hard ionization method and has been in use since the advent of MS. In a typical EIMS analysis, the compound or mixture of compounds of interest are vaporized in a vacuum chamber, and then bombarded with electrons having energy of 25-80 eV. The high energy electron stream causes a valence electron to be ejected from the compound and generates a cation-radical molecular ion $[M]^+$ which has a high energy, creating ion fragments. These fragments are ultimately detected and plotted according to their mass-to-charge ratio; the resulting spectrum is a linear plot of m/z compared with relative abundance. Although both positive and

negative ions are generated in the ion source at the same time, only one polarity is recorded at a time; hence any given mass spectrum consists of either positve or negative ions (Crews et al., 1998).

CIMS is a soft ionization procedure and was developed most recently. In this method, molecular ions that are generated are less likely to undergo fragmentation because the excess energy transferred to the analyte during ionization is much less than EIMS. In contrast, CIMS involves an ion molecule reaction between a substrate and a charged reagent gas to produce a low energy even ion $[MH]^+$ species. CIMS allows an extended ability to obtain molecular weight information in comparison to EIMS. However, both methods have limitations. For example, nonvolatile substances or thermally labile compounds do not give useful spectra and substances with functionality prone to fragment display weak or nonexistent molecular ions. Other MS fragmentation methods include fast atom bombardment, secondary ion mass spectrometry, electrospray, thermospray, field ionization, and matrix assisted laser desorption/ionization (Crews et al., 1998).

Phospholipids have low volatility and are unstable to heat give no parent ion peak by conventional MS; however, they can be analyzed by techniques such as CIMS, where ionization and volatilization techniques are less drastic (Hemming and Hawthorne, 1996). Since many lipids occur as families of molecules differing only in their fatty acid composition, MS is useful in analyzing the individual molecular species (Hemming and Hawthorne, 1996).

A number of studies have been performed on phospholipids separation using both normal and reversed phase HPLC with a variety of mobile phases and detectors.

Early experiments showed HPLC coupled with UV could purify phospholipids better than conventional chromatography methods (Geurts Van Kessel et al., 1977, Kiuchi et al., 1977). However, these earlier methods found difficulty in complete lipid separation and, in many cases, only partial separation was accomplished. An improved normal-phase HPLC method for separation and quantification of polar lipids was described by Silversand and Haux (1997). Their methods developed were highly reproducible and allowed base-line separation of all investigated polar lipid classes (phosphatidic acid, diphosphatidylclycerol, PE, PC, SM, PS, PI, and lysophosphatidylcholine) from fish liver and egg extracts.

Using an ELSD detector, Ramstedt and Slotte (2000) presented an HPLC method for separation and purification of sphingomyelin stereoisomers. A binary gradient of solvent mixtures (hexane, isopropanol, water, and acetic acid) was optimized for maximal separation of the D-*erythro* and L-*threo* isomers. Normal phase HPLC coupled with reversed phase HPLC was employed by Řezanka and Mareš (1990); six lipid fractions from water alga were separated by preparative normal-phase and up to twelve fractions were obtained by further analysis using reversed-phase. Good separations occurred however, separation was not possible when the ceramides differed only in the position of the amide bond. Smith et al. (1981) describes a convenient method for beef brain complex lipid separation by argentation HPLC coupled with reversed-phase HPLC and detected using UV. It was observed that the lipids were separated based upon the number of double bonds independent of the number of carbon atoms. Phospholipids from whey protein concentrates were separated and quantified by HPLC and ELSD by Vaghela and

Kilara (1995) using a binary solvent system consisting of chloroform, methanol, water, and ammonium hydroxide. The ammonium hydroxide was used as a solvent modifier and it was found that in combination with ELSD, a rapid, repeatable base-line separation of whey phospholipids was achieved. In recent years, Rombaut et al. (2005) developed a new HPLC-ELSD method for separation of glucosylceramide, lactosylceramide, phosphatidic acid, PE, PI, PS, PC, SM and lysophosphatidylcholine from dairy products. This method utilizes an acid buffer to maintain column performance and total separation, including column regeneration, is facilitated in less than 21 minutes.

Several studies of phospholipid separation utilizing reversed-phase HPLC have been presented. Jungalwala et al. (1979) showed that molecular species of SM from bovine brain and sheep and pig erythrocytes could be separated in a convenient method. It was observed that sphingomyelin species had a retention time that increased progressively as the number of carbon atoms in the hydrophobic chains increased. This method operated under UV detection at 203 nm. PC species from egg, bovine brain, and porcine liver were successfully separated by reversed-phase HPLC and described by Smith and Jungalwala (1981). Although not specific for phospholipids, Ioffe et al. (Ioffe et al., 2002) developed a reversed-phase HPLC method for a lecithin-based prodrug and discusses the wide category of general uses for the procedure, including phospholipids analysis. The method is adaptable for ELSD and MS detection.

Lipid Sources

Buttermilk

History and Economics.

The history of producing butter dates back to as early as 2000 B.C. In this time period, the process of making butter consisted of placing cream into goatskin and churning by swinging the goatskin from rafters or placing it on the back of a horse's saddle (IDEA, 2007). Throughout history, the consumption of butter and milk cream has been associated with a high standard of living and are coveted as a delicacy in developing countries (Jimenez-Flores, 1997). The price and perceived function of butter, as with any food product, determines its demand; and US sales volume of butter is largely established by its price (Jimenez-Flores, 1997).

Butter production in the US during 2006 totaled 1.45 billion pounds, an increase of 7.5% from 2005. California and Wisconsin led in butter production at 31% and 26.2%, respectively. Domestic butter consumption has steadily increased over the past five years (2002-2006) from 581,000 to 665,000 metric tons. The amount of butter that is exported out of the US annually has varied slightly over the past 5 years increasing from 3,000 metric tons in 2002 to 12,000 metric tons in 2006. The Russian Federation receives a bulk of international imports with an average of 134,000 metric tons per year (USDA, 2007). Butter consumption worldwide has steadily increased from 4 million metric tons in 1997 to over 6.5 million metric tons in 2006 and non-fat milk solids have also increased from 2 million metric tons to 3 million metric tons for the same time period (USDA, 2007). In the U.S., the average

monthly production of buttermilk powder is 4.5 million pounds with an average price of 99 cents per pound (ERS:USDA, 2005).

Buttermilk: Processing.

Buttermilk is a by-product of the butter-making process. Generally, butter is prepared by the churning and working of cream. During churning, air is beaten into the cream, resulting in small bubbles. This causes new air-water interfaces to be continually formed. When the fat globules touch these bubbles, the MFGM along with some of the liquid fat is spread over this interface. As the churning proceeds, air bubbles collide with each other and the adhering fat globules eventually coalesce to make small clumps. The small clumps will become larger because of direct collision between them during churning. During this process, the liquid fat sticks together and the surrounding MFGM is left as a deflated membranous sack free in the aqueous buttermilk fluid (Figure 2.10). The resulting butter is eventually separated from the buttermilk. The churning process is completed rapidly and efficiently so that the formed butter is of proper consistency and the buttermilk has a low fat content. Continuing the churning process for various lengths of time after the butter grains are formed can alter their size. However, the size of the grains will affect their separation from the buttermilk (Walstra et al., 1999).

Liquid buttermilk can be used as a food ingredient however it is usually dried for stability and convenience. Similar to the production of skim milk powder, buttermilk is first pasteurized, concentrated and finally spray or roller dried. Buttermilk powder has a shelf life of approximately 3 months, limited primarily because of its increased acidity, the presence of added microorganisms, and of fat

oxidation. Buttermilk is often evaporated to 16% or 45% solids before roller or spray drying, respectively (Carić, 1994).

Buttermilk: Composition.

Buttermilk is a solution of residual fat, protein, lactose and minerals in water (Table 2.3). The composition of buttermilk powder is similar to skim milk powder (SMP). However, it contains a higher fat content (6% w/w) and lower lactose content (49% w/w) in comparison with SMP (1% and 52% w/w, respectively). The carbohydrate and ash components of buttermilk are similar to skim milk. Buttermilk contains a higher percentage of phospholipids in comparison to whole dairy milk, 0.13% versus 0.035% respectively (Walstra et al., 1999). In addition, sphingomyelin represents roughly one-third of total buttermilk phospholipids, with phosphatidylcholine and phosphatidylethanolamine representing about a third as well (Astaire, 2003).

Numerous other bovine lipids, some possessing bioactive properties, may also be found in buttermilk. These include saturated, monounsaturated, polyunsaturated, and trans fatty acids, conjugated lineoleic acids (CLA), steroids, and ether lipids (Parodi, 1997). Free fatty acids consist of only 0.1-0.4% of total milk lipids and are associated with lipid metabolism regulation as well as promoting healthy cholesterol levels (Warner, 1976). CLA has been shown to have antiatherogenic, and immunomodulating properties in addition to reducing adipose fat (Parodi, 1997). Steroids are important for the resorption of fats and ether lipids are believed to influence biochemical and biophysical processes (Parodi, 1997).

Dried buttermilk contains approximately 34% of proteins, many of which are associated with the MFGM. The stage of lactation in the cow will determine the amounts of the specific protein components of the MFGM (Palmquist and Beaulieu, 1993, Ye et al., 2002). The major MFGM proteins included in buttermilk (in order from heaviest molecular weight to lightest) are Mucin I (MUC1), Xanthine dehydrogenase/oxidase (XDH/XO), Periodic acid Schiff III (Pas III), Cluster of Differentiation (CD36), Butyrophilin (BTN), Periodic acid Schiff 6/7 (PAS 6/7), Adipophilin (ADPH), and Fatty-acid binding protein (FABP). Many MFGM proteins are not easily purified and their functions are not all clearly defined (Mather, 2000). Caseins (α, β, γ, and κ-caseins), whey proteins (α-Lactalbumin, β-Lactoglobulin, albumin and immunoglobulins) and other minor proteins are included in buttermilk along with MFGM associated proteins (Walstra et al., 1999).

Of the major MFGM associated proteins, six will stain strongly with Coomassie blue reagent whereas the two glycoproteins, MUCI and PAS III can only be detected by staining with PAS reagent or a modified silver-staining procedure. MUCI is a glycoprotein whose biological functions are uncertain. It contains heavily glycosylated (up to 50% of its weight) exoplasmic domains and is expressed on the apical plasma membrane of epithelial cells in many tissues besides the MFGM. MUCI has an approximate molecular weight of 254.3 KD and is presumed to protect exposed surfaces from physical damage and pathogens. It may also play immunoprotective and signaling roles as well (Mather, 2000).

XDH/XO has an approximate molecular weight of 155 KD and is a complex molybdenum-containing redox enzyme. Although it is a widely studied protein, the

full range of its physiological functions remain uncertain. Some presumed functions of XDH/XO include signaling, compound oxidation, immunoprotection as well as antibacterial roles. It is also believed to play a structural and functional role in the formation of the MFGM by forming a protein complex that interacts with lipid components at the apical cell surface (Mather, 2000).

PAS III has a molecular weight of 95 KD and is poorly characterized. It contains large amounts of carbohydrate and is visualized by staining with the PAS reagent. This protein is located on apical surfaces of mammary epithelial cells and it's function is uncertain (Mather, 2000).

CD36 stains faintly in Coomassie blue reagent and has an approximate size of 77 KD. It contains roughly 24% of carbohydrates and comprised 5% or less of the total protein associated with the MFGM. Mostly, it is recovered from buttermilk in the MFGM fraction after centrifugation. This protein has been shown to have many diverse functions including thrombospondin and collagen receptor, platelet activation and aggregation, intercellular adhesion, thrombospondin-mediated inhibition of angiogenesis, and cell scavenger functions. Although it is not clear why this protein is expressed on the MFGM, some hypotheses on its specific function include thrombospondin and long-chain fatty acid receptor for transcytosis across mammary secretory cells (Mather, 2000).

BTN has a molecular weight of roughly 66 KD and is the most abundant protein in the MFGM (34-43% of total). This protein is an integral protein with a firm association for the membrane. The function of BTN is not clear (Mather, 2000).

ADPH is a chief component of the MFGM insoluble fraction and was formerly overlooked because its molecular weight, 52 KD, is similar to that of PAS 6/7. It is associated with lipid droplets and has a restricted distribution in tissues. The function of ADPH remains unclear; however, possible roles may include the cellular uptake of long-chained fatty acids and in the accretion and transport of intracellular lipids (Mather, 2000).

PAS 6/7 proteins have molecular weights that range from 43 to 59 KD because of posttranslational modifications. They are major components of the MFGM and are the products of a single gene. PAS 6/7 are believed to function as adhesive proteins although their specific role in the MFGM is unknown (Mather, 2000)

FABP has the lightest molecular weight of all the MFGM proteins at 13 KD. This protein was found to be an isoform of the mammary-derived growth inhibitor (MDGI) protein found in heart tissue. Although the physiological functions of FABP are not entirely clear, FABP may function in the control of lipid metabolism, the accretion of lipid droplets in the cytoplasm or in the intracellular transport of fatty acids (Mather, 2000).

Buttermilk: Products and Uses.

Buttermilk is typically an undesirable by-product because of insufficient demand. It is highly susceptible to spoilage due to rancidity (Walstra, 1999). However, it is frequently dried and sold as a highly functional food ingredient, often to companies looking for a cheap alternative to milk solids, used in a variety of foodstuffs. It is incorporated into baked goods in order to impart desireable flavor, aid in the development of browning, incorporation of air into the product and prevent

staling by binding water. Buttermilk powder is included in ice cream as a non-fat milk solid source and to aid in the air-water interface stabilization. It is often used as an ingredient in dry mixes such as pancakes, waffles or biscuit to impart brownness upon heating and dairy flavor. Buttermilk powder is used in processed cheese slices for increased viscosity and structure, and in chocolate production for flavor and emulsifying properties (Sodini et al., 2006). Fryksdale (2001) extensively reviewed buttermilk's diverse functionality. Among the functional properties of buttermilk powder include browning, emulsification, foaming and water binding.

An investigation into the emulsion properties and the effects of proteolysis was performed. It was shown that the emulsion properties of MFGM isolates improved after treatment with trypsin and chymotrypsin. MFGM isolates from industrial buttermilk demonstrated a greater improvement than did MFGM from unheated cream. This study indicates that the protein and lipid associations within the MFGM are disrupted during buttermilk processing (Corredig and Dalgleish, 1997).

Because of its unique composition and concentration of MFGM, buttermilk could be used as a source of bioactive lipids. Buttermilk is readily available in large quantities from butter-producing dairies as a waste product and has a relatively low cost (Fryksdale, 2000, Walstra et al., 1999). A previous study by Astaire (2003) found that by using microfiltration and supercritical fluid extraction, the bioactive lipids of the MFGM could be enriched within reconstituted buttermilk.

Whey Cream

History and Economics.

The history of producing cheese from milk dates back thousands of years, possibly since before 3000 B.C. (Warner, 1976). Most likely, a fortuitous chain of events produced the first fermented dairy foods; however, with recent increased knowledge about the chemistry and microbiology of dairy processing, cheese making has evolved into a standardized process to include hundreds of different varieties (Fox et al., 2000). In 2006, world production of cheese was 14.1 million metric tons (FAS-USDA, 2006). Cheese production, as seen in Figure 2.12 has increased at an average annual rate of roughly 4% over the past 30 years (Fox et al., 2000). Within the United States, total cheese production, excluding cottage cheese, was 4.3 million metric tons, approximately 30.5% of the worlds supply (USDA, 2007).

Cheese consumption varies widely between countries (Fox et al., 2000). In 2006, the total world consumption of cheese was 13.9 million metric tons, with the U.S. and the European Union, having the largest consumption at 4.4 and 6.2 million metric tons, respectively (USDA, 2007). Cheese consumption has steadily increased over many years for many reasons, including a positive dietary image, convenience and flexibility in use, and the great diversity of flavors and textures (Fox et al., 2000). There are now over 300 different cheese varieties available in the U.S. marketplace with cheddar and Italian type cheeses (mozzarella, provolone, ricotta, etc) being the major types produced, at 41% and 41.8%, respectively (Figure 2.11) (IDFA, 2006, USDA, 2007).

Since whey is the liquid byproduct of making cheese, the amount produced is determined by cheese production (Goggin, 1998). Every 10 units of full-fat milk will yield about 1 unit of cheese and 9 units of fluid whey. Whey can be further processed into several different products, including whey protein concentrate, whey protein isolate, and dried lactose. In 2005, the U.S. manufactured over 2.1 billion pounds of total whey products, of which, 1.05 billion pounds was dry whey and 324 million pounds was whey protein concentrate, and exported 592 million pounds (39.6% of total production) of whey products (IDFA, 2006, USDA, 2007). Whey products may not compete with cheese and ice cream for recognition; however, no other dairy product has had a steady annual increase in export value (Goggin, 1998).

Cheese Production.

The production of all types of cheeses involves a similar protocol (Figure 2.12). In order to produce quality cheeses, milk used for manufacture must be selected, pre-treated, and standardized. Milk selected for cheese production should be absent of chemical taints and free fatty acids, and should be of good microbial quality. The milk is standardized to have a certain fat-in-dry matter content and pre-treated for reducing microbial numbers (i.e. pasteurization). The cheese milk may be treated with $CaCl_2$, to aid in coagulation, and pigments, to color the cheese. Starter cultures are added to the cheese milk 30-60 minutes before rennet addition; this step generally reduces the pH of the milk by about 0.1 unit, either by ripening or pre-acidification.

After the initial steps, the milk is converted to cheese curd, and involves three basic operations: acidification, coagulation, and dehydration. Acidification may be carried out through the fermentation of lactic acid bacteria or direct acidification and

affects many aspects of cheese manufacture. Coagulation is the essential step in production and involves coagulation of the casein component of the milk protein system to form a gel that entraps the fat, if present. Coagulation is achieved by adding rennet, acidification to pH 4.6, or acidification to a pH greater than 4.6 in combination with a heat treatment. Once coagulated, the gel is cut to expel the whey and dehydrated. The remaining curds may be salted, molded, pressed, and ripened to form a mature cheese (Fox et al., 2000). Depending on the type of cheese being produced and the type of coagulant used, two types of whey may result: sweet and acid. Sweet whey is derived from rennet coagulation in the manufacture of cheddar, swiss, mozzarella, and similar types of cheeses. Acid whey is derived from direct acidification in the manufacture of cottage and ricotta cheeses (Jelen, 2000). Sweet whey contains less acid and calcium, and more lactose than acid whey. Most of the U.S. products come from sweet whey (Jelen, 2000). Products manufactured using whey include whey powder, whey protein concentrate (WPC), whey protein isolate (WPI), reduced-lactose whey, and demineralized or reduced-mineral whey, as well as additional products using other components of whey (i.e. lactose) (Goggin, 1998).

Whey: Composition.

Roughly 50% of the solids in milk are included into cheese; the remainder (90% of the lactose, ~20% of the protein, and ~10% of the fat) is present in the whey (Fox et al., 2000). Table 2.4 lists the solids composition of sweet cheese whey. It is clear that the bulk of whey is water (Johnson and Law, 1999). Whey contains 50% of the total solids from the original milk, including essentially all of the lactose and whey proteins, 50%-100% of the milk salts and some fat (Fox et al., 2000). Lactose, a sugar

unique to milk, is the principal component of the whey apart from water, representing 4.6% of the sweet whey (Fox et al., 2000). The most valuable whey component is whey protein, representing 0.8% of the whey. Whey protein, being neither acid nor rennet coagulable, will pass from the milk into the whey during the cheesemaking process (Jensen, 2002). Bovine whey contains two main proteins, β-lactoglobulin and α-lactalbumin, and is also rich in the non-casein components of milk; serum proteins, two iron binding proteins lactoferrin and transferrin, as well as vitamin B_{12} and folic acid bound to whey protein (Renner et al., 1989).

After lactose and whey protein, minerals and fat make up the third major component, 0.5%, of whey dry matter. The minerals include calcium, magnesium, phosphate, citrate, sodium, and potassium (Jensen, 2002). Bovine milk fat consists of fatty acids, glycerine, phospholipids, and other minor material (Warner, 1976). Of the lipids, 98-99% are triglycerides located in the fat globule. The remaining 1-2% are minor lipids with the following components: diglycerides 0.3-1.6%, monoglycerides 0.002-0.1%, phospholipids 0.2-1.0%, cerebrosides 0.01-0.07%, sterols 0.2-0.4%, and free fatty acids 0.1-0.4% (Renner et al., 1989).

Whey: Processing.

The most common method of whey processing is the concentration of proteins into whey protein isolates (WPI) and whey protein concentrates (WPC). Proteins dissolved in whey can be collected in various ways, including ultrafiltration, gel filtration, ion exchange, evaporation and precipitation. Ultrafiltration of whey is commonly applied, resulting in separation as well as concentration; the spray-dried ultrafiltered product is WPC. WPI is obtained by separation of the proteins utilizing

ion exchange processes. This product, mainly comprised of β-lactoglobulin and α-lactalbumin, is often ultrafiltered to concentrate the protein resulting in a very pure product (Walstra et al., 1999).

Whey: Products and Uses.

No longer regarded as a waste product, cheese whey is now used in animal feeds and many food products, including dairy products, baked goods, candies, snack foods, dry mixes, processed meats, infant formulas, nutritional beverages, and in other dried, frozen and prepared foods. The use of whey products as ingredients is not only based on nutritional qualities, but also the functionality of the product. Other non-food uses include production of neutraceuticals and pharmaceuticals. Whey cream may also prove to be a unique source of bioactive lipids (Jelen, 2000).

Microfiltration

Filtration processes are used in a variety of industrial food systems, ranging form dairy product processing to water, wastewater and sewage treatments. Although filtration applications are diverse, the main principles of the filtration process are the same; generally, filtration processes select for the concentration of specific components in a mixture by passage through a semipermeable membrane, which acts as a selective barrier. The driving force for this process may be an electric potential difference (electrodialysis) or a pressure difference over the membrane. In the end, the permeating or retaining phase will become enriched in one or more components of the mixture. The degree of component selectivity is dependant on membrane (pore size and reactivity), temperature, pressure, velocity, flow schematic, and other

conditions (Akhtar et al., 1995, Astaire, 2003, Boyd et al., 1999, Karleskind et al., 1995, Rinn et al., 1990, Walstra et al., 1999).

There are four general categories for filtration processes based upon membrane selectivity; in order from the least selective to the most, they are microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Figure 2.13 depicts these categories in dairy processing along their selectivity ranges and what types of components are separated with their application. Microfiltration processes are most commonly used in the dairy industry for fat and bacterial removal along with casein protein concentration. Filtration processes are extending possibilities of making dairy products with unique properties and functionality (Karleskind et al., 1995, Samuelsson et al., 1997, Walstra et al., 1999).

For microfiltration processes, the membrane pores are fairly wide, and the pressure difference is small (Walstra et al., 1999). The most widely used microfiltration membranes are composed of polyvinylidene fluoride (PVDF), polyacrylonitril, polysulfone, polypropylene, and ceramic materials (Akhtar et al., 1995, Samuelsson et al., 1997). Ceramic membranes are popular because of their long life and extreme temperature resistance. Microfiltration membranes are usually cylindrical with a series of semipermeable tubes that run in channels down its length. During the process, the batch is fed into the channels within the cylinder and permeable substances exit the channels through the pores. The liquid passing the membrane is called permeate; the retained solution is termed the retentate (Walstra et al., 1999).

The major problem affecting the efficiency of microfiltration is the build-up of foulants (or fouling) at the membrane surface and within the pores. Fouling can lead to reduced permeate flux and membrane selectivity. Although frequent cleaning of the membranes can extend longevity, all systems eventually decline in performance due to a gradual foulant build-up. Various design features, such as low, uniform transmembrane pressure, cross-flow of the stream, high flow rates, and increasing circulation velocity may lessen the degree of fouling (Akhtar et al., 1995, Samuelsson et al., 1997). Generally, cross-flow systems are utilized to minimize fouling. This design utilizes a pump to pass the feed stream tangentially to the membrane rather than perpendicularly. The retentate is recycled back to the feed tank continuously until the desired concentration is obtained. As the retentate is concentrated, volume is lost due to the exiting permeate; addition of water is used to compensate for this loss. This process is termed diafiltration, and is referred to in terms of a volume of water added back into the system, i.e. in 1x diafiltration, 100% of the starting volume has been added back in (Akhtar et al., 1995, Samuelsson et al., 1997, Walstra et al., 1999). The development of adding coatings to the membrane surface has been studied as an additional way to reduce membrane fouling. A phospholipids bilayer applied to both PVDF and cellulose acetate membranes was found to reduce membrane fouling of proteins. This coating was applied to mimic natural cellular plasma membranes. An increase in performance and a decrease in membrane fouling were observed (Akhtar et al., 1995).

An investigation by Rombaut et al. (2006) determined the effects of MFGM separation from butter serum via microfiltration upon addition of sodium citrate for

casein micelle disassociation. Destabilization of the micelle is thought to enable transmission of the protein through the membrane in order 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 content was increased in the retentate when using a 0.1μm membrane. In addition, improved separation between lipids and proteins was shown using fresh rather than reconstituted buttermilk.

Supercritical Fluid Extraction

Introduced in the mid-1980s, supercritical fluid extraction (SFE) is a technique that utilizes the solvent power of substances at temperatures and pressures near their critical point (Williams, 1981). In this state, the supercritical fluid has high diffusivity and low density and viscosity, enabling it to easily penetrate a wide range of sample matrices for the extraction of analytes (Fuoco et al., 1997, Taylor et al., 1997, Williams, 1981). This type of extraction is significantly effective for the extraction of substances with low polarity and average molecular weight and extraction can be accomplished at moderate temperatures (Williams, 1981). Often referred to as “green chemistry”, supercritical fluids are an environmentally benign alternative to other

solvents and, among their benefits, possess opportunities for reduction of reactor volumes and accelerated chemical processes (Hauthal, 2001). Recently, solvent databases have been developed to aid in solvent selection, listing valuable information regarding solubility parameters and critical points (Hauthal, 2001).

Carbon dioxide is a common solvent used for SFE, used for a variety of applications including the extraction of nonpolar lipid material. Its properties make it an ideal solvent: non-explosive, inexpensive, easily obtainable and plentiful in nature, and non-toxic. Furthermore, it has low critical temperature and pressure parameters (31.1°C, 73.8 bar) and is easily separated from the substances extracted. These characteristics make it ideal for food processing applications. Used with a polar co-solvent (such as alcohols or other organic chemicals), its extraction efficiency can be increased, widening the range of solvent extraction (Arul et al., 1994, Astaire, 2003, Fuoco et al., 1997, Hauthal, 2001, Hierro and Santa-Maria, 1992, King, 1995, Rozzi and Singh, 2002, Williams, 1981). However, when used alone, CO_2 can only extract nonpolar materials (Boselli and Caboni, 2000).

A wide range of solvents has been utilized for scientific as well as commercial, developmental and pharmaceutical applications. Table 2.5 lists the commonly used solvents and their critical temperatures and pressures, many of which are used as cosolvents with carbon dioxide (Williams, 1981). Most of the solvents listed in Table 2.5 besides carbon dioxide are disadvantageous for use in SFE because of the difficulty to handle and to obtain in a pure form (Rozzi and Singh, 2002).

Substances dissolved in supercritical fluids can be extracted from a matrix in three ways: heating at a constant pressure, depressurization at a constant pressure, or

adsorption at a constant pressure and temperature utilizing an adsorbing agent (Hierro and Santa-Maria, 1992, Turner and Mathiasson, 2000). When introduced to a sample, the supercritical fluid solubilizes certain compounds, depending on numerous conditions: solvent and solute polarities, pressure and temperature conditions, and the complexity of the phase (Hauthal, 2001, King, 1995, Turner et al., 2001, Williams, 1981). Van der Waals forces are the main contributors to substance solubility in supercritical fluids; this leads to an increased solubility during constant temperature as pressure is increased. Temperature changes affect solubility in a more complicated manner; increasing temperature under isochoric conditions will lead to an increase in solute vapour pressure, and in turn, increased solubility (Arul et al., 1994). For supercritical CO_2, a temperature increase will, in turn, increase solubility, provided the pressure is higher than 345 bar; this manner is most likely related to its density (Hierro and Santa-Maria, 1992).

SFE is accomplished in four steps: diffusion of the supercritical fluid into the sample matrix, release of soluble analytes, flow of solutes out of the sample matrix, and removal of solutes out of sample. Upon cooling to ambient temperatures, the solutes can be separated from the solvent and the supercritical fluid is returned to its normal state and discarded as waste or recycled for further use (King, 1995, Taylor et al., 1997, Turner et al., 2001). Figure 2.14 is a phase diagram for carbon dioxide, showing the effects of pressure and temperature on its state. Four main phases, directed by environmental pressure and temperature, of carbon dioxide exist: solid, liquid, gas, and supercritical. At the triple point, a substance can exist as a solid, liquid, and gas, simultaneously. CO_2 reaches its triple point when the pressure is at

5.07 bar and the temperature is at –56.6°C. This is also the lowest point that CO_2 can exist as a liquid. Below this pressure point at any temperature, carbon dioxide will persist and behave as a gas. Increasing both the pressure and the temperature to 73.8 bar and 31.06°C, respectively, will cause the solvent to reach its supercritical point. Above this point, if the temperature is increased, the only phase that exists is the supercritical state. In this phase, supercritical fluids are neither liquids nor gases and possess one uniform density. If the pressure is increased once the supercritical state is achieved, the density will change and be equivalent to that of a liquid solvent; in turn, giving it liquid-like solvent properties. The supercritical fluid also has gas-like viscosities, allowing analyte mass transfer to accelerate. Moreover, the density and solvent strength can be modified in a number of ways by varying pressure and temperature (Taylor et al., 1997, Turner et al., 2001).

A solubility parameter describes the solubility of a certain substance in supercritical fluids; it is described by calculating variables at the time of extraction. The Hildebrand Solubility Parameter equation (Figure 2.15) gives a rough estimate of the ability of a solvent to dissolve a solute. The solvent strength is calculated as a function of the reduced density of the supercritical fluid (ρ) with respect to the reduced density of the solvent in liquid state (ρ_{liq}), and the critical pressure of the solvent (P_c). At atmospheric pressure, the solubility parameter is zero; as pressure increases, solubility also increases. Theoretically, when the densities of the supercritical fluid and the target analyte are equal, then maximum solubility is achieved (Rozzi and Singh, 2002, Turner et al., 2001). Although successful with a variety of solutes in

supercritical fluids, the equation is not employed for esters, ketones, alcohols and other polar liquids (Rozzi and Singh, 2002).

Carbon dioxide demonstrates a polarity in between that of dicholoromethane and ethyl ether, dissolving non-polar compounds such as fats, oils and aroma components, such as terpenes, quite readily (Hierro and Santa-Maria, 1992). A number of applications of SFE have been developed to extract lipid and lipid soluble materials from a variety of mixtures, including the extraction of lipid soluble vitamins (A, D, E, and K); various seed, nut, bean, palm, and wood oils; essential oils; cocoa butter; meat fats; milk fats; phospholipids; pesticides; cholesterol; and pharmaceutical components (Arul et al., 1994, Berg et al., 1997, Boselli and Caboni, 2000, Ferreira de Franca et al., 1999, Gonzalez-Vila et al., 2000, Hierro and Santa-Maria, 1992, King, 1995, King et al., 2001, Lehotay, 1997, Markom et al., 2001, Ronyai et al., 1998, Saldana et al., 2002, Spricigo et al., 1999, Taylor et al., 1997, Williams, 1981).

Advantages of SFE over conventional organic solvents include sample preparation, minimal consumption of solvents, oxygen elimination and heat reduction (Taylor et al., 1997, Turner et al., 2001). As in the case of fat-soluble vitamins, degradation may occur with extreme pH, oxygen, light and heat exposure; thus, conventional extraction methods are not always ideal (Turner et al., 2001). SFE technology is no longer just a laboratory technique as it is successfully utilized in large commercial scale plants in both the USA and Europe for caffeine, tea, spices, hops and flavor extraction processes (Huang et al., 2004).

A comparison of SFE with conventional methods has been investigated by Berg et al. (1997). In this study, they compared SFE using ethanol-modified carbon

dioxide with the Bligh and Dyer method (chloroform-methanol extraction) and the Schmid, Bondyznski, and Ratzlaff method (acid hydrolysis extraction). Following method optimization, it was found that there was no significant difference between the methods for total fat extraction and lipid classes from meat. In addition, their extraction time, in comparison to the Bligh and Dyer method, was reduced. Another investigation comparing SFE with AOCS/AOAC official methods for determining total fat content of oilseed and ground beef was presented by Taylor et al. (1997). It was found that ethanol-modified carbon dioxide exhibited higher oil recoveries for soybean, safflower, sunflower and cottonseed oils as well as from ground beef samples.

The solubility of cholesterol and its esters (cholesteryl acetate, cholesteryl butyrate and cholesteryl benzoate) in SFE was studied by Huang et al. (2004). Temperature and pressure of carbon dioxide ranged from 308.15K to 328.15K and 90 bar to 270 bar, respectively. The effects of adding a polar cosolvent, methanol and acetone, was also examined. Results indicated that cholesterol solubility in SC-CO_2 is a linear function of pure solvent density as well as having an impact from the vapor pressure effect. It was evident that solubility increased in the presence of a cosolvent and acetone has a greater affect on cholesterol solubility than methanol.

A number of studies involving the use of SFE for lipid extraction from milk and milk products have been presented. There are a number of reasons for interest into milk fat treatment including the creation of new products low in cholesterol or in saturated fats by fractionation, improved butter spreadability, fat-soluble vitamin extraction, milk pollutant analysis, and concentration of polar MFGM lipids (Arul et

al., 1994, Astaire, 2003, Hierro et al., 1995, Hierro and Santa-Maria, 1992, Kaylegian, 1995, Ramos et al., 2000, Turner and Mathiasson, 2000). It has been shown by Arul et al. (1994) that milk fat triglycerides are soluble in supercritical carbon dioxide and the solubility parameter increases with increased pressure. Five distinct fractions of triglycerides were obtained over a range of temperatures (50-80°C) and pressures (120-330 bar); each specific extraction parameter for the fractions was dependant on the chain length of the triglycerides. Differences in solubility were explained by solute-solvent interactions and vapor pressure. An examination into SFE extracted lipids from ewe's milk was accomplished by Hierro et al. (1995). It was observed that as the pressure increased, the concentrations of short-chained triglycerides decreased and concentrations of long-chained triglycerides increased in the extracts. Moreover, operating the SFE at pressures above 190 bar and at a temperature of 40°C was shown to obtain an extract concentrated in long-chained triglycerides and cholesterol enriched.

Astaire et al. (2003) studied SFE coupled with microfiltration as a method for polar MFGM lipid concentration within buttermilk powder. In this process, a cross flow microfiltration system with 0.8μ pore size was optimized for cold, reconstituted buttermilk powder for an initial concentration of lipid material. Further treatment using SFE successfully increased the polar MFGM lipids (namely sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine) concentration in buttermilk powder by the removal of nonpolar lipid materials. This extraction was optimized at a pressure of 375 bar and temperature of 77°C. The properties of the lipids recovered after SFE indicated that supercritical carbon dioxide interacts preferentially on lipid

polarity rather than molecular weight differences of the lipids, and that nonpolar lipids in the powder have different solubility. Results also showed that the presence of lactose could interfere with the SFE process; this observation was previously demonstrated by Turner and Mathiasson (2000). Although not encountered here, it has previously been reported that water content in the sample can have an effect on SFE; it can either hinder it by blocking sample contact with solvent or facilitate it by acting as a modifier (Turner et al., 2001). Furthermore, absorption of CO2 by the water can lead to increased acidity, and, in turn, lead to hydrolysis and degradation of the analytes (Turner et al., 2001). Therefore, samples with large amounts of water, such as milk and milk products, may require drying before SFE treatment (Turner et al., 2001). Perhaps the substantial amount of nonpolar lipid material extracted with SFE in the above study can be attributed to drying of the buttermilk before SFE treatment.

Another benefit that SFE may offer to the food industry is its ability to eliminate pesticides from food products. Traditional methods using organic solvents are often undesireable because no method can extract all pesticides with a single approach and solvents are often combined. SFE has been found to reduce the time, cost, manual labor, glassware, and lab space as well as eliminating the use of hazardous solvents. Although limitations do exist in the range of pesticides that can be extracted, SFE is an emerging method for pesticide removal (Lehotay, 1997). Specifically in milk products, Polychlorinated biphenyls (PCBs), a known health risk, have been shown to be removed from full-fat milk powder using SFE. The objective of the study was to characterize PCB distribution in the milk fat globule, not to

necessarily purify the fraction. The PCBs were extracted along with cholesterol and a variety of glycerides under the same range of conditions (136-233 bar at 50°C) (Ramos et al., 2000) .

The use of supercritical carbon dioxide and high pressure is receiving much attention not only as a lipid extraction method, but as a non-thermal processing method to eliminate vegetative microorganisms, endospores and undesireable enzymes (Enomoto et al., 1997, Spilimbergo et al., 2002). SFE treatment for this purpose is advantageous in the food industry because it does not cause loss of color, nutrients, or flavor of the food, as well as being easily controlled, environmentally safe, and more economically convenient. High-pressure treatments (2000-7000 bar) are currently employed for microorganism inactivation in foodstuffs, however, supercritical CO_2 treatment may become a relevant alternative (Enomoto et al., 1997, Spilimbergo et al., 2002). Studies have shown that although useful for killing vegetative cells, endospores can endure high-pressure treatments. Spilimbergo et al. (2002) verified that supercritical CO_2 allowed complete deactivation of *Bacillus subtilis* and *Pseudomonas aeruginosa* endospores when the conditions were cycled (30 cycles per hour) at 80 bar and 75°C; when all other conditions were the same, partial deactivation occurred at 35°C. Another study by Enomoto et al. (1997) demonstrated that endospore cells of *Bacillus megaterium* were completely deactivated by supercritical CO_2 at 580 bar and 60°C with a treatment more than 30h. Supercritical fluid extraction offers many benefits as an alternative to traditional solvent extractions. Shown to have excellent lipid extraction properties as well as antimicrobial uses, this

method may prove to have significant uses in both food development and food quality issues.

The goals of this dissertation were to study the combination of filtration and SFE processes in relation to the enrichment of phospholipids in buttermilk powder. This was accomplished by optimization of the SFE system, description of initial and final products, examination of buttermilk source, and potential product application.

Table 2.1. The approximate amounts of minor lipids found in the milk fat globule (Renner et al., 1989).

Lipid	*Amount (% of globule)*
Diglycerides	0.3 - 1.6
Monoglycerides	0.002 – 0.1
Phospholipids	0.2 – 1.0
Glycolipids	0.01 – 0.0.7
Sterols	0.2 – 0.4
Free fatty acids	0.1 – 0.4

Table 2.2. The approximate average composition of the MFGM (Walstra et al., 1999).

Component	**mg per 100g fat globules**	**mg per m^2 fat surface**	**% of membrane material**
Protein	1800	9.0	70
Phospholipids	650	3.2	25
Cerebrosides	80	0.4	3
Cholesterol	40	0.2	2
Carotenoids + vit. A	0.04	2×10^{-4}	--
Fe	0.3	1.5×10^{-3}	--
Cu	0.01	5×10^{-5}	--

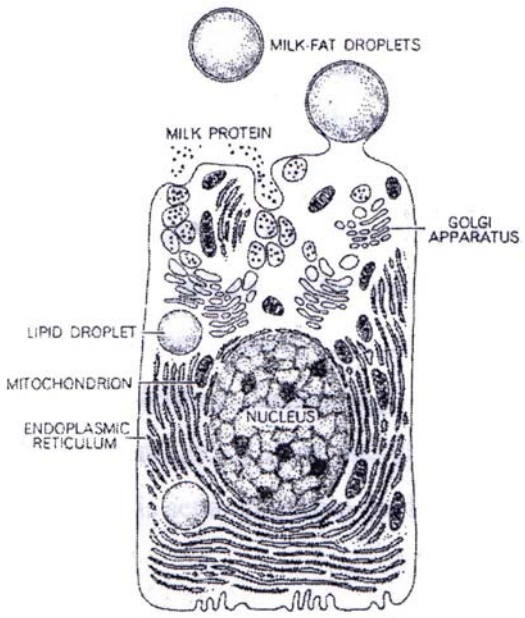

Figure 2.1. The manufacture of milk fat globules inside the mammary gland and their release into the lumen. Adopted from (Jenness and Patton, 1969).

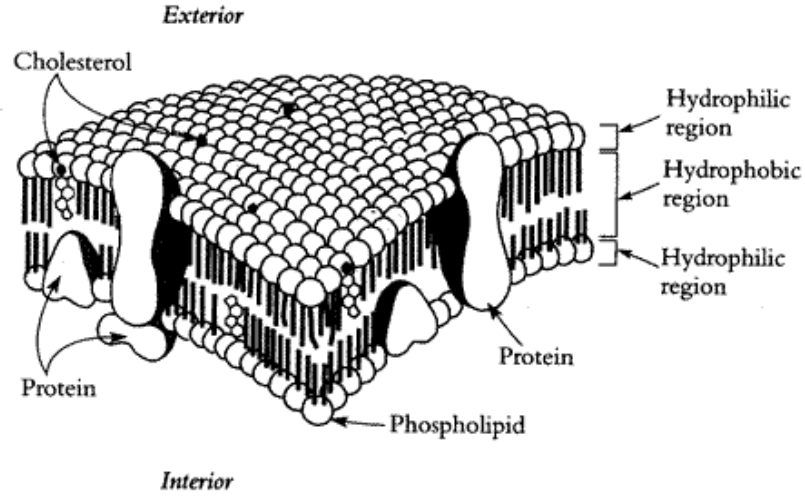

Figure 2.2. Typical bilayer membrane (Sahelian, 2000).

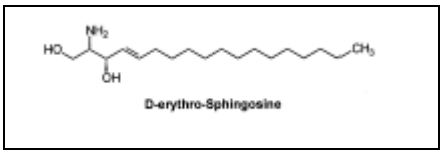

Figure 2.3. The molecular structures of sphingosine, which is the backbone of all Sphingolipids (Huwiler et al., 2000).

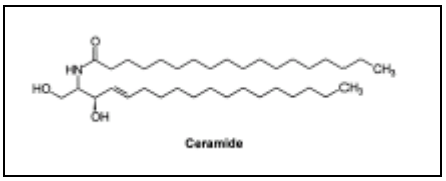

Figure 2.4. Molecular structure of ceramide in its naturally occurring form, D-*erythro*-(2S,3R). The R chain of the fatty acid linked to the carboxamide group can vary in length (Huwiler et al., 2000).

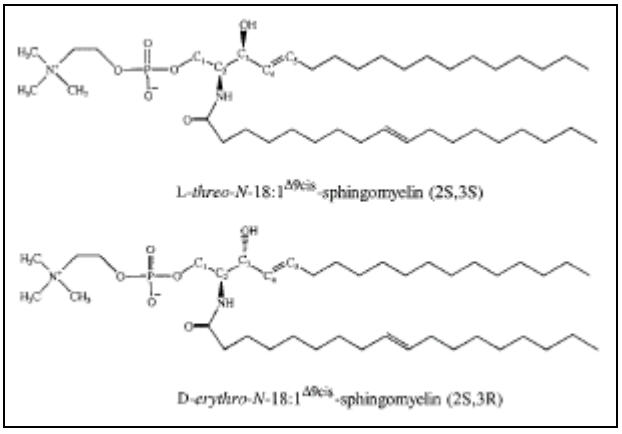

Figure 2.5. The molecular structures of the two SM stereoisomers: D-*erythro*-*N*-18:1 (2S,3R) and L-*threo*-*N*-18:1-(2S,3S) (Ramstedt and Slotte, 2000).

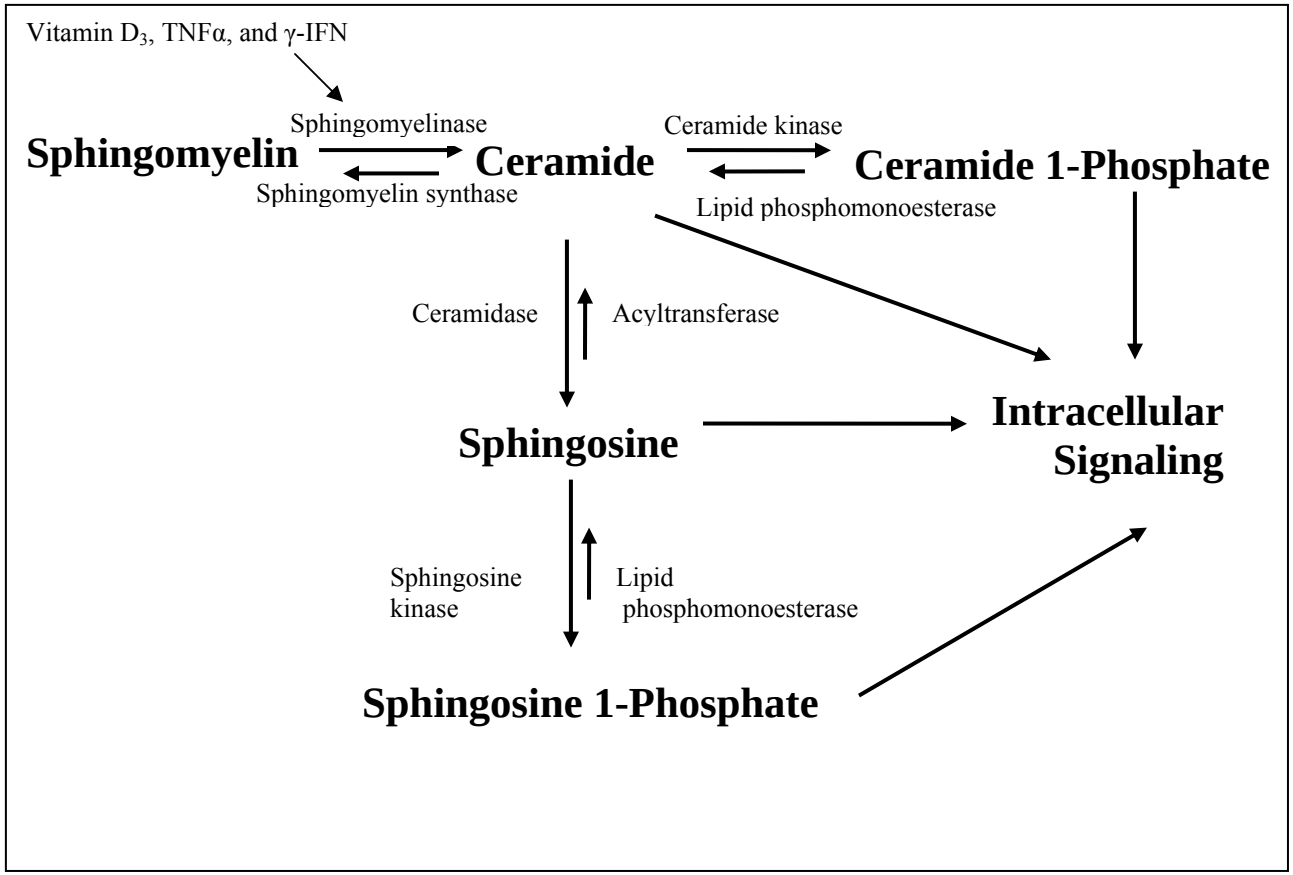

Figure 2.6. The sphingolipid signaling cascade produces many metabolites, shown here. These metabolites are involved in intracellular signaling to regulate cell function. The hydrolysis of sphingomyelin produces the second messenger ceramide, which can be modified further to produce two other intracellular signaling lipids, sphingosine and sphingosine 1-phosphate. Ceramide may be phosphorylated by ceramide kinase to generates ceramide 1-phosphate. Adopted from Bajjalieh and Batchelor (1999).

a) $k' = (t-t_0)/t_0$

b) $\alpha = k'_2/ k'_1$

c) $n = 16(t/w)^2$

d) $h = L/n$

Figure 2.7. Parameters which affect HPLC separation (Christie, 1987).

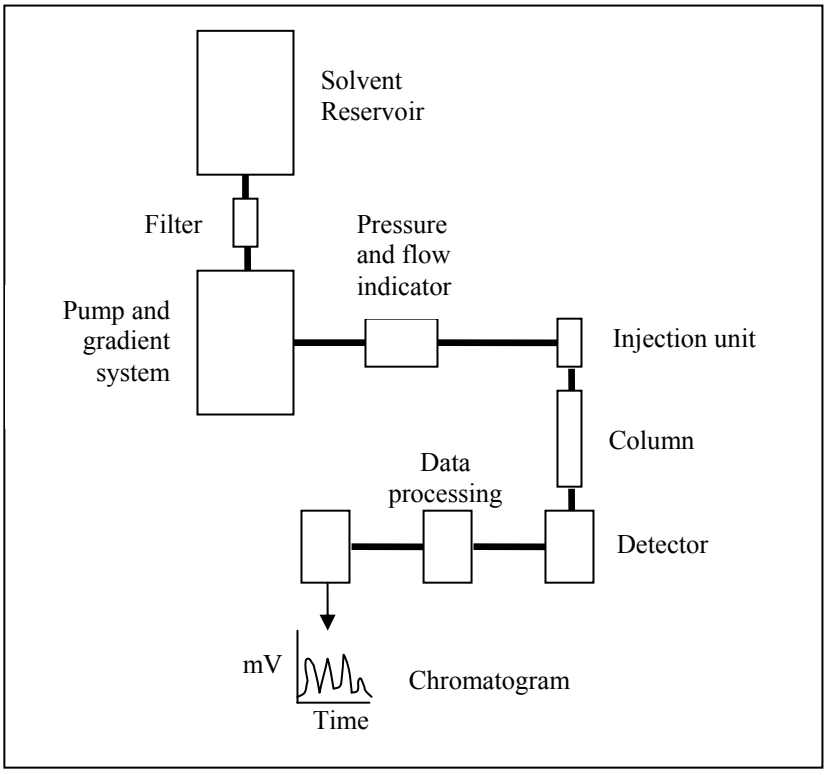

Figure 2.8. Block diagram of a general HPLC system - adapted from Brown and DeAntonis (1997).

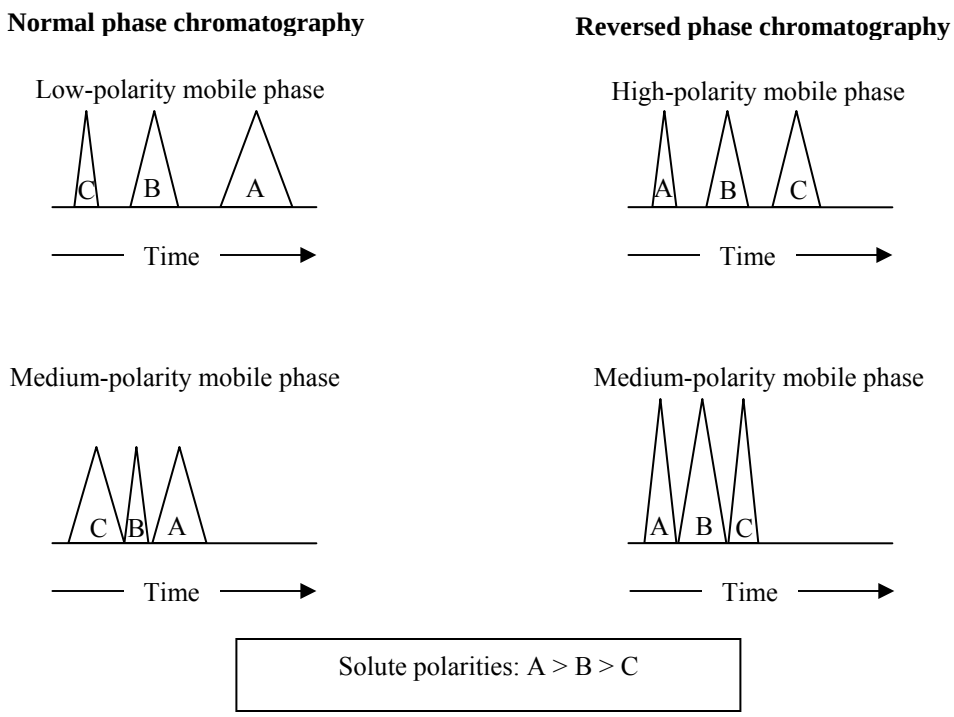

Figure 2.9. Relationship between polarity and elution times for normal and reversed phase chromatography - adapted from Brown and DeAntonis (1997).

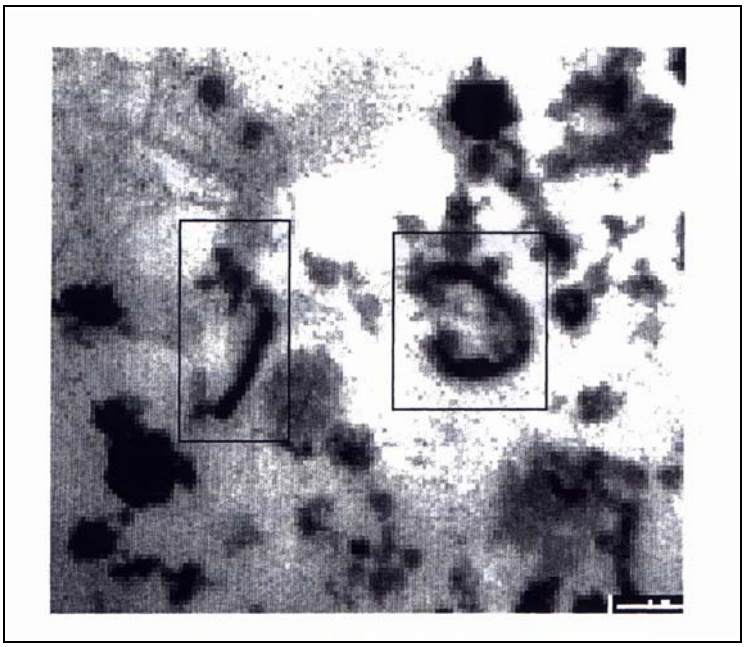

Figure 2.10. A scanning electron micrograph of buttermilk shows the MFGM. The deflated membranous sacks are easily distinguished from the proteins and fat in the surrounding fluid as the crescent shaped objects (photo compliments of Beth Fryksdale, Cal Poly State University, San Luis Obispo).

Table 2.3. Typical composition of buttermilk powder (Carić, 1994).

Component	**% Buttermilk powder**
Protein	34%
Fat	6%
Carbohydrate	49%
Moisture	3%
Ash	8%

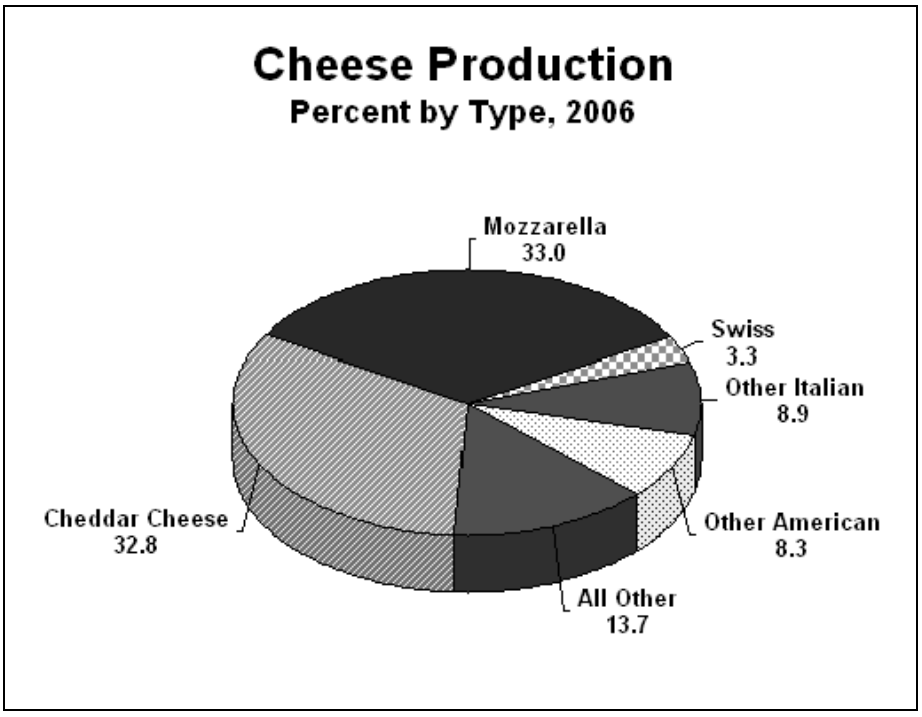

Figure 2.11. U.S. Cheese production percent by type (USDA, 2007).

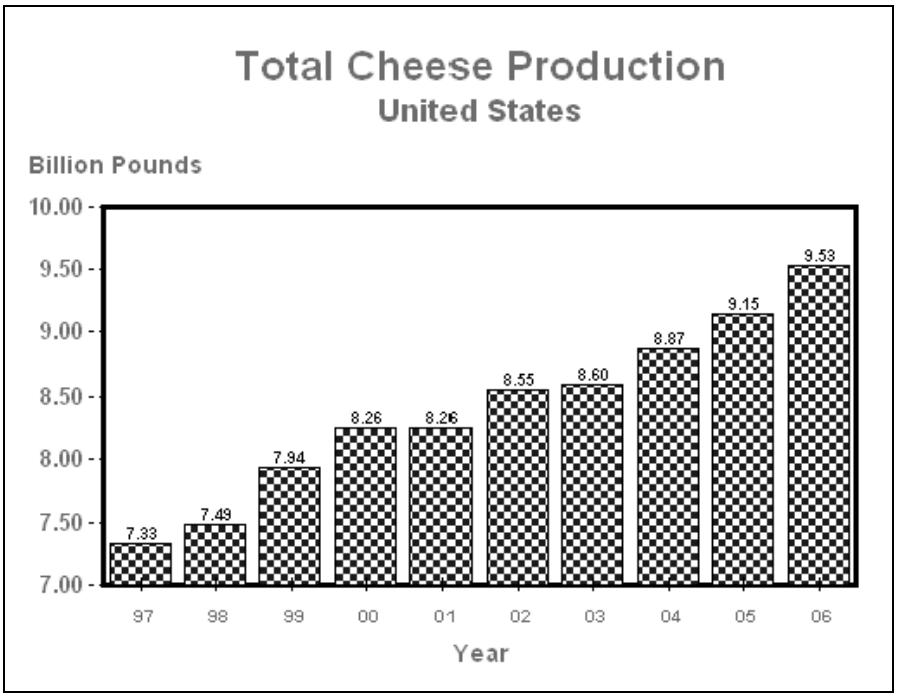

Figure 2.12. U.S. total cheese production from 1997 to 2006 in billion pounds (USDA, 2007).

Table 2.4. Composition of sweet whey from cheesemaking (as % of total by weight) (Johnson and Law, 1999).

Component	**%**
Water	93.2
Total solids	6.8
Protein	0.8
Fat	0.5
Lactose	4.6
Minerals (ash)	0.5
Lactic Acid	<0.1
Cheese fines	0.1-0.3

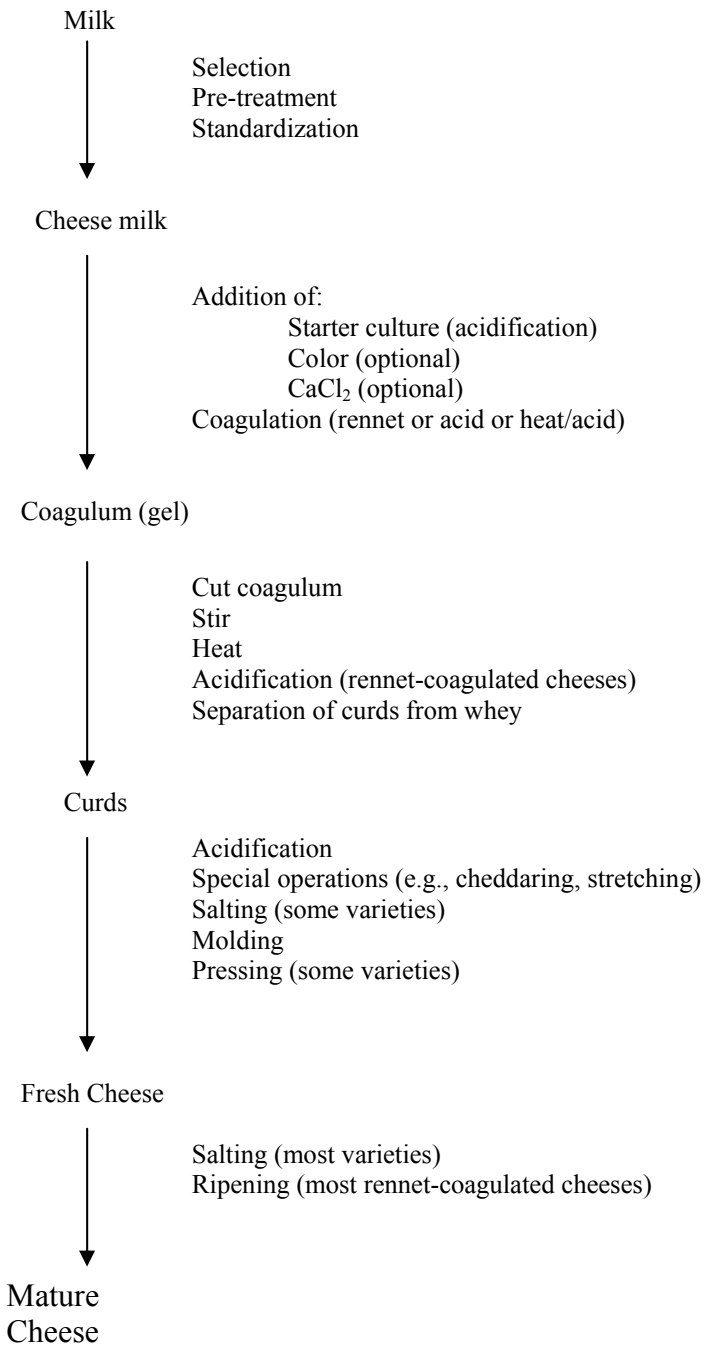

Figure 2.13. General protocol for cheese manufacture (Fox et al., 2000).

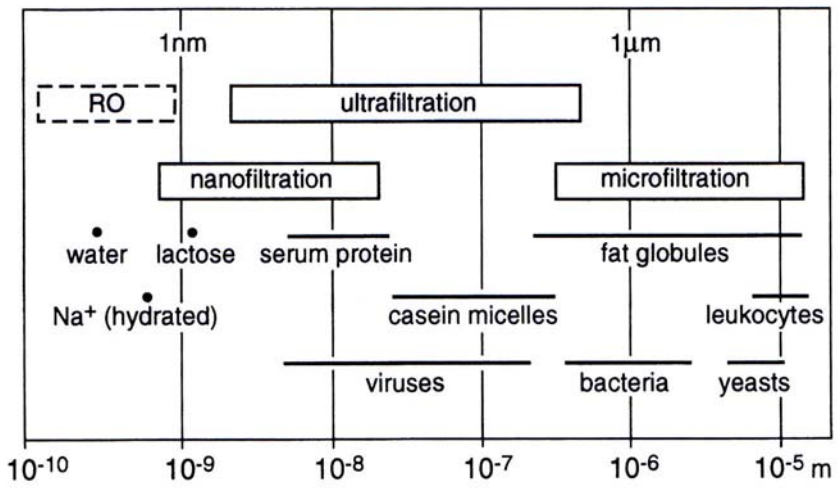

Figure 2.14. Approximate particle sizes for the four classifications of membrane processes. The size of some molecules and particles in milk is also indicated (Walstra et al., 1999).

Table 2.5. The critical temperature and pressure points of some commonly used supercritical fluid extraction solvents (adapted from Williams, 1981).

Substance	**Critical Temperature K**	Critical Pressure **bar**
Acetone	508	47.0
Ammonia	406	113.5
Benzene	562	48.9
Carbon dioxide*	304	73.8
Diethyl ether	467	36.4
Ethanol*	514.15	61.8
Ethylene	282	50.3
Methane	191	46.0
Methanol	513	80.9
n. Pentane	470	33.7
Propane	370	42.4
Propylene	365	46.2
Toluene	593	41.1
Water*	647	221.2

*Carbon dioxide, ethanol, and water are generally recognized as safe (GRAS) substances.

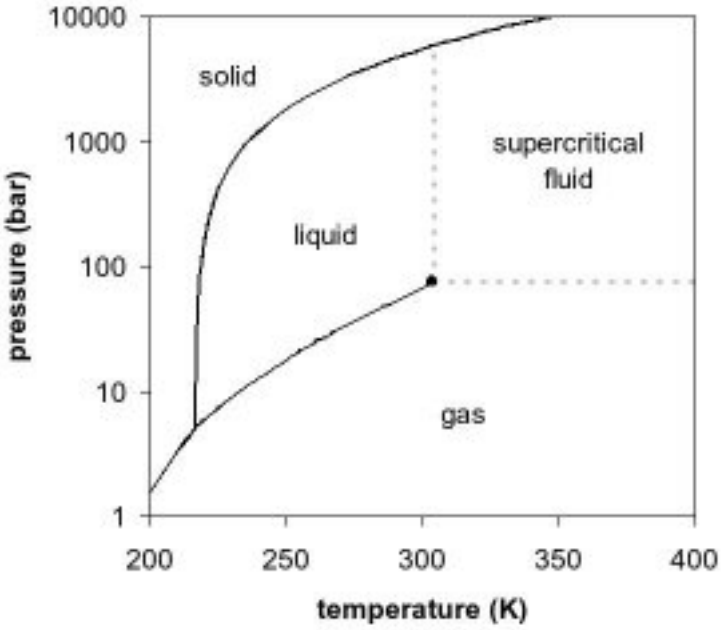

Figure 2.15. The carbon dioxide phase diagram describes the pressure and temperature parameters between phases. Above the critical point, the solvent exists as a supercritical fluid. Image adopted from (Reid et al., 1987).

$$\delta = 1.25 P_c^{1/2} [\rho/\rho_{liq}]$$

Figure 2.16. The Hildebrand Solubility Parameter describes the solvating capability of a compressed gas, where δ is the solubility parameter, Pc is the critical pressure, ρ is the gas density and ρ_{liq} is the density of the liquid (Rozzi and Singh, 2002, Turner et al., 2001).

The Influence of SCO_2 Factors in Supercritical Fluid Extraction for Optimizing Non-Polar Lipid Extraction from Buttermilk Powder

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

[1]Rafael Jimenez-Flores

Tel: (805) 756-6103

Fax: (805) 756-2998

rjimenez@calpoly.edu

[*]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

The milk fat globule membrane, readily available in buttermilk, contains complex lipids known to be involved in a variety of biological processes. 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 removing triglycerides 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. SFE conditions were assessed using a general full factorial design; the experimental factors were pressure (150, 250, and 350 bar) 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 in comparison to the untreated buttermilk powder. Calculated theoretical values showed a linear increase for lipid solubility as pressure and/or temperature is increased; however, experimental values showed non-linearity, as an effect of temperature. In addition, the particular SFE parameters of 350 bar 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 0.2-1.0% of total milk lipids (Molkentin, 2000). PLs are associated with both milk fat and proteins; they are largely concentrated in the milk fat globule membrane (**MFGM**) and small amounts are bound to caseins (Renner, et al., 1989, Warner, 1976). Phospholipids not only function as integral components of the membrane, but also are shown to be involved with other biological processes. PL groups are normally found in milk and consist of about 10 fractions with the major ones being phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine (Renner, et al., 1989). Some of these PLs have been shown to exhibit antioxidative, anticarcinogenic, and antiatherogenic properties (Berra, 2002, Dacaranhe and Terao, 2001, Molkentin, 2000). Sphingolipids, in particular, are known to have essential roles in cell to cell interactions, differentiation, proliferation, immune recognition, transmembrane signaling, and as cell receptors (Huwiler, et al., 2000, Kim, et al., 1991, Lightle, et al., 2000). Their location on the MFGM exterior and their interactive character suggests a major role in many different important biological interactions (Deeth, 1997).

Within eukaryotic cells, PLs are primarily components of cell membranes; large concentrations are found in the membrane-rich tissues such as pancreas, liver, brain and neural tissue (Berra, 2002, Merril, et al., 1997). 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 source of PLs.

Buttermilk is readily available in large quantities from butter-producing dairy processors as a by-product and at a relatively low cost. In the U.S., the average monthly production of buttermilk powder (**BMP**) is 4.5 million pounds with an average price of 99 cents per pound (USDA, 2005). The composition of BMP is somewhat similar to skim milk powder (roughly 8%) (Walstra, et al., 1999). However, it contains a higher fat content (6% w/w) and lower lactose content (49% w/w) in comparison with skim milk powder (1% and 52% w/w, respectively (Walstra, et al., 1999). The ash components of buttermilk are similar to skim milk. Buttermilk contains a higher percentage of phospholipids in comparison to whole dairy milk, 0.16% versus 0.04% respectively (Rombaut, et al., 2006a). In addition, sphingomyelin (SM) represents approximately 19.06% of total buttermilk PLs (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 successfully isolate PLs, their use may render the product inadequate, unless solvent is removed completely (Astaire, 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 in microfiltration 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 content was increased in the retentate when using a 0.1μm 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, high membrane fouling along with PL loss was 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, utilized for a variety of applications. Its properties make it an ideal solvent: non-explosive, inexpensive, easily obtainable and plentiful in nature, and non-toxic. Furthermore, it has low critical temperature and pressure parameters (31.1°C, 73.8 bar) 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). A number of 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 (King, et al., 2001, Saldana, et al., 2002, Turner and Mathiasson, 2000).

A previous study by Astaire et al. (2003) found that by combining microfiltration and supercritical fluid extraction, the bioactive lipids of the MFGM could be concentrated within reconstituted buttermilk. In this process, a cross flow microfiltration system with 0.8μm pore size was optimized for cold, reconstituted buttermilk powder for an initial concentration of lipids. Further treatment using SFE successfully increased the polar MFGM lipids (namely SM, PC, and PE) concentration (9.59 to 19.74 mg lipid/g dry powder) in BMP by the removal of nonpolar lipids,

specifically triacylglycerols (21.33 to 3.98 mg lipid/g dry powder). This extraction was carried out at a pressure of 375 bar and a temperature of 77°C.

The goals of this work were to investigate the influence and interaction of SFE using CO_2 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 buttermilk powder.

MATERIALS AND METHODS

Processing of Buttermilk Powder

Commercial buttermilk powder 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 let sit overnight at 4°C to fully hydrate 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 and France). A membrane with a pore size of 0.80 μm was used. All runs were carried at low temperature (8-10°C) at a transmembrane pressure of 80 to 95 kPa. Diafiltration processes (**DFMF**) replaced the extracted permeate with water to a dilution factor of 3x. 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.

Supercritical Fluid Extraction Processing of Buttermilk Powder

Lipid extraction by SFE focused on constant run conditions and powder samples and the two 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 were chosen at 150, 250, and 350 bar, respectively. Temperature levels were chosen at 40, 50, and 60°C, respectively. For each experimental point (i.e. 150 bar at 40˚C), 50g of a 3x DFMF 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 2x SFE. All experimental points were processed in triplicate. The SFE system and components were acquired from Thar Designs, Inc. (Pittsburgh, PA) and are previously described by Astaire et al. (2003). Briefly, the in-house unit includes the following: 500 ml vessel, model P-50 high-pressure pump, automated back pressure regulator model BPR-A-200B, and PolyScience brand water bath and pump unit (model 9505). Circulated deionized water at 5°C was used for cooling different zones in the SFE apparatus. Fifty lb. 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). The running conditions were as follows: flow rate, 20g/min; total vessel flushes, 3.35 ± 0.40; total run time, 90.0 ± 0.11 min; and total CO_2 used, 1670.0 ± 200 grams.

Following treatment, the SFE extraction vessel was rinsed with 50 mL – 100 mL of chloroform:methanol (2:1 v/v) into a pre-weighed, 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, v:v). Lipid solubility in supercritical CO_2 was calculated by dividing the amount of lipid collected (in mg) from the SFE extraction vessel by the total grams of CO_2 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 300μm sieve to remove Celite™. A 25 mL glass flask, top and sides covered with black tape, was packed by gravitational contraction with 3.9g of powder. Analysis was done using a HunterLab UltraScan XE colorimeter and data analysis was completed using the software Universal V3.80. The operating conditions were: illuminant C, 10° observer value, and reflectance mode and 45/0 sensor. The CIE LAB values L*, a*, and b* were measured, with analyses performed in triplicate.

BMP samples were reconstituted with deionized water to 10% solids and let hydrate overnight. pH was determined using an Orion Research pH meter, model 410 (Thermo Electron Corporation, Beverly, MA). Titratable Acidity was determined as % lactic acid (AOAC Official Method 947.05). Treated samples were compared with respect to the original untreated BMP (pH = 7.67 ± 0.02, TA = 0.036 ± 0.02) in order to determine any acidic changes in the BMP upon processing.

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 TLC. Silica gel TLC plates (60 F_{254}) were by EMD Chemicals (Darmstadt, Germany), and the developing tanks by Kontes Glass Company (New Jersey). The polar lipids were separated using chloroform-methanol-water (65:25:4, v:v); nonpolar lipids were separated using petroleum ether-ethyl ether-acetic acid (85:15:2, v:v). Lipids were prepared in chloroform-methanol (2:1, v:v) to 10 mg/ml; lipid standards were diluted to 1 mg/ml. 100 µg 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 (Astaire, 2003). Lipid Standards were purchased from Sigma Chemical Co.: sphingomyelin (**SM**), phosphatidylcholine (**PC**), and phosphatidylethanolamine (**PE**).

SC-CO_2 Lipid Solubility Calculations. Experimental lipid solubility in SC-CO_2 was calculated by dividing the amount of lipid collected (in mg) from the SFE extraction vessel by the total grams of CO_2 that passed through the system.
Theoretical lipid solubility was calculated using the Hildebrand solubility parameter equation (Arul et al., 1994):

$$S = [(\Delta H^{g}_{l} - \Delta H_{g} - RT + CPV)/v]^{1/2}$$

ΔH^{g}_{l} = 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-CO_2 (MPa)

T = temperature (K)

V = molar volume of SC-CO_2 (cm^3mol^{-1})

R = gas constant (8.314 $Jmol^{-1}K^{-1}$)

C = conversion factor (1.0 $Jcm^{-3}MPa^{-1}$)

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

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

P = pressure (MPa)

Vm = molar volume (cm^3mol^{-1})

R = ideal gas constant (8.314 $Jmol^{-1}K^{-1}$)

T = temperature (K)

Where a and b are estimated from the relationship of critical pressure and temperature using the equations:

$$a = (27 R^2 T_c^2) / 64 \ P_c$$

$$b = (R T_c) / 8 P_c$$

T_c = critical temperature (304.18 K)

P_c = critical pressure (7.38 MPa)

Protein Composition.

Protein Determination. Percent protein was determined by testing for total Nitrogen content by the Kjeldahl method (AOAC Official Method 955.04). Samples

were heated on a Digestion System 20, model 1015 digester. Samples were distilled with the Kjeldahl System, 1026 Distilling Unit, and FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific, Tustin, CA) were used as reagent. All samples were analyzed in duplicate. Titrations were done using 0.1 N HCl, and the percent protein was calculated using the milk protein conversion factor, 6.38. All reagents were of Kjeldahl analysis analytical grade**.**

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, 0.5M Tris, pH 6.8) in a 1:1 dilution, mixed in a vortex, boiled for 10 minutes, and centrifuged for 2 minutes at 12,000 x g. Non-reducing gel samples were prepared in the same manner with no SDS added to gel or buffers. A volume of 15µl (50 µg protein) was loaded to the Criterion™ 4-20% gradient TRIS-HCL, 1.0mm gels (Bio-Rad Laboratories, Hercules, CA). Gels were run at 90 V through the stacking gel, and 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 Company, St. Louis, MO). For silver stains, the Silver Stain Plus Kit (Bio-Rad, Hercules, CA) was followed according to manufacturer's protocol.

Protein Profiling by Microfluidic Laser-Induced Flurescence (Experion^{TM}). 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^{TM} Pro260 system (BioRad, Hercules, Ca). Separation of proteins from 10kD to 260kD was obtained. Lower and upper

internal alignment markers ensured clean baselines, proper molecular weight sizing and protein mass quantitation.

BMP samples were reconstituted with deionized water to 10% total solids and let hydrate overnight. The Experion Pro260 gel stain and Pro260 sample buffer were prepared using the manufacture's directions. Under reducing conditions, 1 µl of β-mercaptoethanol was added to 30 µl of sample buffer. Under non-reducing conditions, 1 µl of deionized water was added to 30 µl of sample buffer. Using these reagents, 2 µl of sample buffer was added to 4 µl of sample and 4 µl of Pro260 ladder and mixed. The samples and ladder were heated at 95-100°C for 3-5 minutes, centrifuged briefly, and mixed with 84 µl of deionized water.

Prior to analysis, 12 µl of gel-stain solution was added to the Pro260 microchip and the chip was primed using the Experion priming station according to the manufacture'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 was analyzed using the Experion software and recorded as electropherograms, gel graphs, and data tables.

Protein Solubility. Protein solubility was determined as described by Wong and Kitts (2003). Protein solutions (5% TN, wt/wt) at different pH values were centrifuged at 12,000 x *g* for 15 min at 25°C. Supernatant liquid was analyzed for total nitrogen by the Kjeldahl method. Measures 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., PA). The experimental design was a general full factorial design using two factors (pressure and temperature) at three different levels (low, medium, and high) in triplicate. Run orders were block randomized. Experimental points were compared to both a control (untreated) powder and to a powder treated at extreme SFE conditions (377 bar 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 % total fat reduction from the sample powders at each experimental setting is shown in Figure 3.1 and Table 3.1. The lowest amount of lipids was extracted at 150 bar for all three temperature levels, and it is observed that at a pressure of 350 Bar coupled with a temperature of 50ºC, the greatest amount of lipids were extracted from the powder in relation to all experimental points; however only two other points showed a significant difference ($P < 0.05$). This was shown in the amount of lipids extracted at the lowest pressure (150 bar) coupled with temperature levels at 40ºC and 60ºC. The measured lipid reduction was particularly variable for all three settings at 40ºC; suggesting that at this temperature the extraction was highly variable and results were not reproducible. In contrast, extractions performed at 60ºC show the least variation and at 50 ºC the variation fits somewhere between the two.

Lipid solubility (Figure 3.2) was determined by measuring the amount of lipids extracted in relation to the amount of CO_2 used in the extraction. These results confirm the findings described above where a significant difference (P = 0.004) of lipid solubility is observed between the powders extracted at the lowest pressure at all temperature values versus the powder with the greatest % fat reduced, 350/50. Lipid solubility was compared with a calculated solubility parameter, also shown in Figure 3.2. Although not an experimental point, theoretical solubility was calculated for 500 bar, the pressure limits of our system, at the three temperature settings. In theory, the solubility parameter of CO_2 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.

Analysis of variance data indicates that pressure of the system significantly affects the % fat reduced in the powder; determined by the low P-value (0.004) and large mean square value (929.1). SFE system temperature and the interaction between pressure and temperature do not affect % fat reduced (P = 0.445 and 0.755, respectively). It was observed that the underlying error distribution is normal, with one outlier having a large residual. This point is at the low pressure and low temperature setting, already known to have a high standard deviation.

An interaction plot for % fat (Figure 3.3) shows that at the lowest pressure setting (150 bar), lipid extraction efficiency is somewhat limiting to the other two settings, regardless of temperature. In general, for both low and high pressure settings, increasing the temperature from low to intermediate increases fat reduction in the powder; however, by increasing the temperature to high, the overall fat extracted is

reduced. Although a high pressure tends to show high lipid extraction results, data at the low temperature indicates that the intermediate pressure setting has greater extraction efficiency, showing non-linearity 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 3.4). Temperature effects showed a different trend in that lipid extraction increased from 40ºC to 50ºC but decreased at 60ºC.

Protein and lipid compositional analysis of the BMP was necessary to observe any other changes taking place in the BMP during treatment. Lipids analyzed by TLC demonstrate that those present in the BMP samples following SFE treatment were in its majority polar lipids while the removed fraction were composed exclusively of non-polar lipids (Figure 3.5). There is complete retention of all polar lipids in the powder.

Visual comparison of the proteins analyzed by the reducing PAGE method show that chemical changes are taking place during SFE treatment (Figure 3.6). Results were shown only for the first treatment set, as data obtained was similar throughout the triplicate analysis. Comparison of reduced protein profiles show apparent changes in the powder samples with respect to high temperature treatment; band "fuzziness" can be used as an indicator of whey protein lactosylation due to variations in molecular mass (Morgan, et al., 1997). The protein profile of the original powder (lane 2) shows a sharp band for β-lactoglobulin, and following SFE treatments, bands are observed to have a smearing or larger Mr (relative molecular mass). An observed Mr increase for the caseins and whey proteins and the extremely fuzzy bands shows that the previous SFE parameters were too intense and cause chemical changes in the powder. SFE treatments, regardless of pressure, at 60°C

(lanes 4, 7, and 10) tend to show a higher whey protein Mr, as well as the caseins to a lesser extent, than the 40°C and 50°C treatments. The experimental point having a treatment at 350/50 was previously mentioned to offer the greatest percent in fat reduction and is shown by the arrow in lane 1. This treatment had a lower effect on proteins than the corresponding treatments at 60°C. Further, non-reducing PAGE was carried out in order 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 electropherograms) in order to utilize information such as peak number, time, concentration, molecular weight, height and area. Figures 3.7A and 3.7B show the electropherogram (florescence vs. molecular weight and time) of SDS-reduced and microfluidic-LIF protein profiless from both the original powder and the powder that underwent the extreme SFE conditions (350 bar/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 have decreased in total concentration from 9.79 g/l in the original BMP to 4.09 g/l in the treated sample, possibly due to solubility loss. Furthermore, the remaining peaks represent proteins in the treated BMP have undergone an apparent Mr shift, an average of 3.1 kDa; 23.9 to 26.4 kDa, 40.6 to 43.7 kDa, and 82.8 to 86.5 kDa corresponding to caseins, MFGM proteins and lactoferrin respectively. The observed concentration and peak loss as well as Mr shifts can be

attributed to protein denaturation, aggregation, and possible lactosylation. This result is consistent with the SDS-PAGE visual observation.

Results showed that protein solubility varied from 13.1% to 20.5% according to the treatment variables (Figure 3.8), however none of the treatment powders were significantly different in solubility with respect to the original non-treated powder. Powders that underwent extreme treatment conditions (375 bar and 77ºC) showed a significant difference from the original powder as well as three of the sample powder: 150/40, 150/50, and 350/60 ($P < 0.05$). 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 Mr 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 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 (a_w), and extended time; therefore, milk powder, having a low a_w is particularly susceptible (Guyomarc'h, et al., 2000). Not only 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 was 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 (375/77) had a significant increase in darkness and redness in comparison to the other treatment conditions as well as the original powder (P = 0.00 for both L* and a*) as shown in Table 3. Extreme SFE treatment had a significant increase in yellow (P = 0.00) in comparison with the non-treated BMP while the sample powders decreased significantly in yellow color. 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 higher temperature and pressure.

In conclusion, the buttermilk powder products developed by combining microfiltration and SFE process are rich in polar lipids. We have shown that by decreasing the SFE pressure parameter from 375 to 350 bar and temperature from 77°C 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 due to instability of the supercritical nature of CO_2 at 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 the food components with lesser modifications and no residual chemicals is more desirable.

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Table 3.1. Compositional data on a dry matter basis for pressure and temperature experimental settings and the untreated BMP.

Sample [Pressure (bar) / Temperature (°C)]	% Fat	% Protein	pH	TA	Mean Lipid Solubility (mg lipid/g CO_2)
150 / 40	5.92 ± 2.15[a]	57.76 ± 3.92	7.61 ± 0.04	0.041 ± 0.01	0.021[a]
150 / 50	4.98 ± 0.83	69.07 ± 0.42	7.72 ± 0.17	0.037 ± 0.02	0.027[ab]
150 / 60	5.29 ± 0.48[a]	57.14 ± 0.10	7.59 ± 0.03	0.040 ± 0.01	0.023[ab]
250 / 40	3.68 ± 1.12	60.31 ± 0.30	7.64 ± 0.04	0.037 ± 0.01	0.035[abc]
250 / 50	3.94 ± 0.53	58.31 ± 6.28	7.66 ± 0.09	0.029 ± 0.00	0.035[abc]
250 / 60	3.17 ± 1.64	60.00 ± 4.47	7.52 ± 0.12	0.036 ± 0.01	0.036[abc]
350 / 40	4.07 ± 1.16	59.50 ± 1.01	7.56 ± 0.12	0.033 ± 0.00	0.039[bc]
350 / 50	2.85 ± 0.57[b]	65.72 ± 1.21	7.66 ± 0.13	0.028 ± 0.01	0.045[c]
350 / 60	3.64 ± 0.26	60.05 ± 8.82	7.47 ± 0.13	0.043 ± 0.02	0.037[bc]

[a,b]different letters in a single column denote significant difference ($P < 0.05$)

Table 3.2. Colorimetry properties for pressure and temperature SFE settings and the untreated BMP

Sample	L*	a*	b*
Untreated	87.98 ± 1.50[a]	-1.9 ± 0.95[a]	15.07 ± 1.69[a]
375 / 77	81.26 ± 7.56[b]	0.51 ± 1.94[b]	14.48 ± 0.60[b]
150 / 40	90.15 ± 0.92[a]	-2.16 ± 0.04[a]	12.06 ± 0.57[c]
150 / 50	90.08 ± 0.76[a]	-2.06 ± 0.09[a]	11.72 ± 0.38[c]
150 / 60	90.68 ± 0.61[a]	-2.14 ± 0.06[a]	11.90 ± 0.25[c]
250 / 40	90.60 ± 0.65[a]	-2.13 ± 0.02[a]	11.87 ± 0.20[c]
250 / 50	90.80 ± 0.59[a]	-2.10 ± 0.05[a]	11.51 ± 0.21[c]
250 / 60	90.72 ± 0.38[a]	-2.15 ± 0.03[a]	11.56 ± 0.42[c]
350 / 40	90.86 ± 0.52[a]	-2.14 ± 0.07[a]	11.66 ± 0.14[c]
350 / 50	90.90 ± 0.55[a]	-2.12 ± 0.04[a]	11.95 ± 0.20[c]
350 / 60	90.13 ± 0.83[a]	-2.05 ± 0.12[a]	11.70 ± 0.25[c]

[a-c]Means within columns with different letters are statistically different ($P < 0.05$).

Sample names indicate treatment parameters used: Pressure (bar) / Temperature (°C)

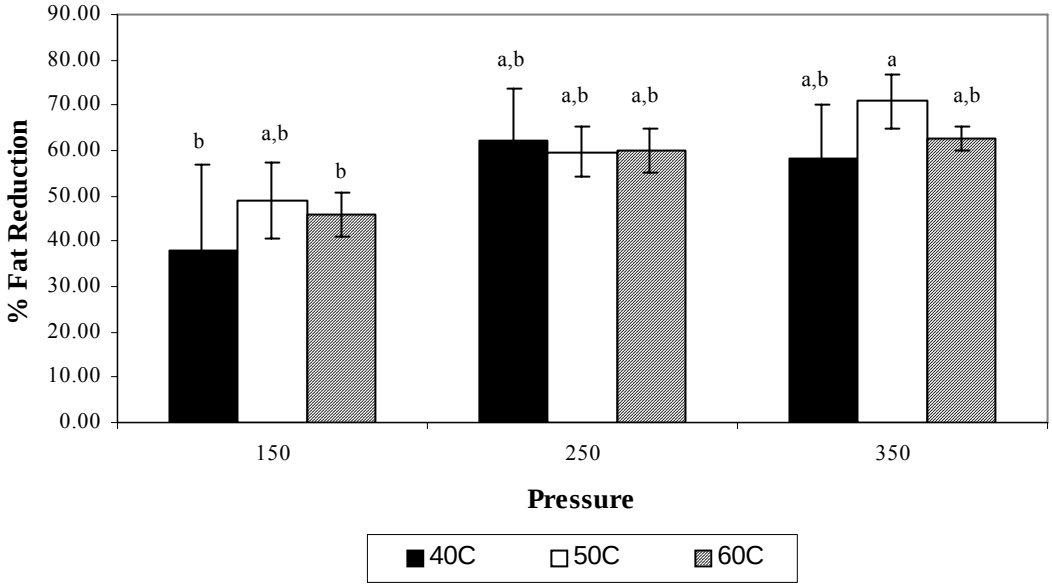

Figure 3.1. Percent fat reduction in SFE treated buttermilk powder for three different pressures (150, 250, and 350 bar) and temperatures (40°C, 50°C, and 60°C). Results marked with a different letter denote a significant difference between means ($P < 0.05$).

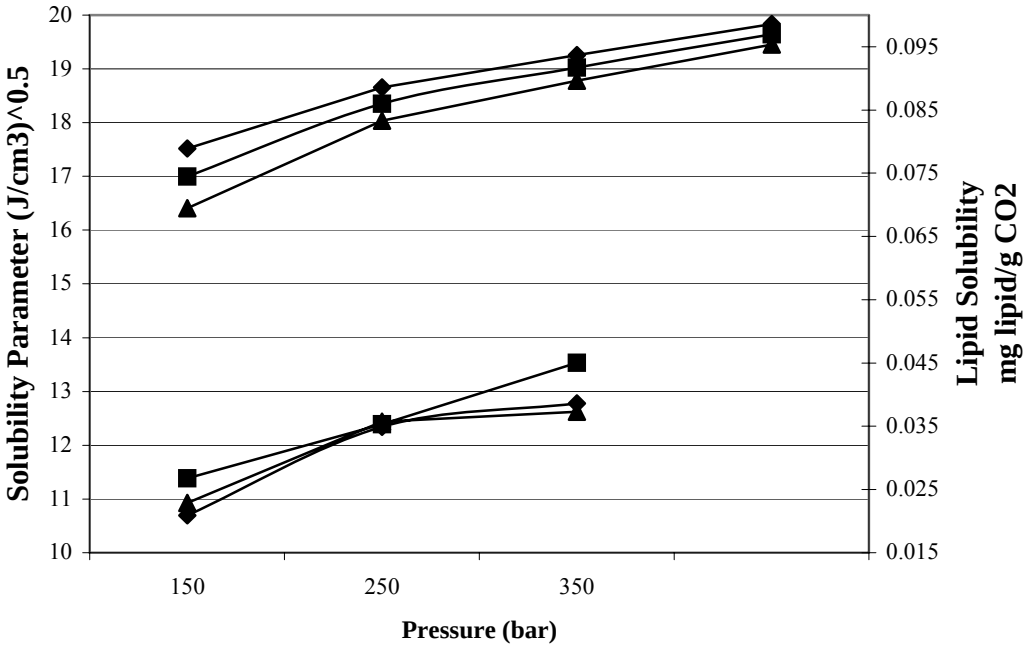

Figure 3.2. Lipid solubility properties in SC-CO2 for pressure (150, 250 and 350 bar) and temperature values: 40°C (▲), 50°C (■), and 60°C (♦). The top chart is for the theoretical solubility parameter and the lower chart is experimental solubility.

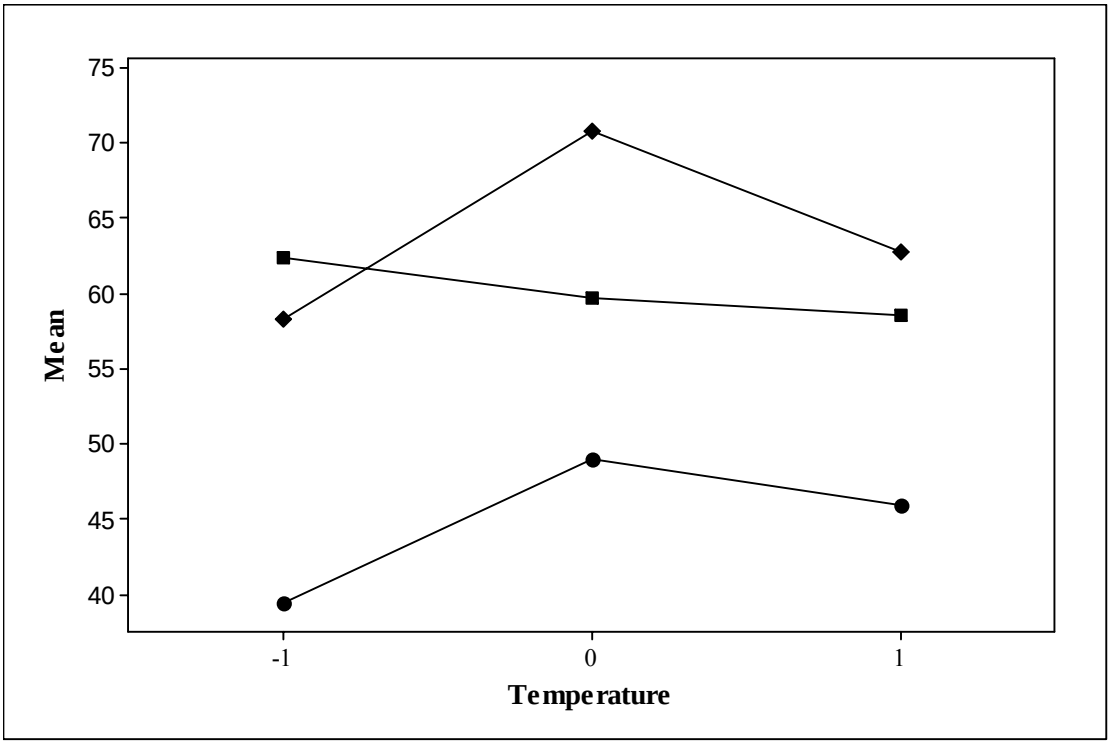

Figure 3.3. An interaction plot for % fat reduced shows that at the lowest pressure setting (150 bar), lipid extraction efficiency is somewhat limiting to the other two settings, regardless of temperature. Temperature values: 40°C (-1), 50°C (0), and 60°C (+1). Pressure values: 150 bar (●), 250 bar (■), and 350 bar (♦).

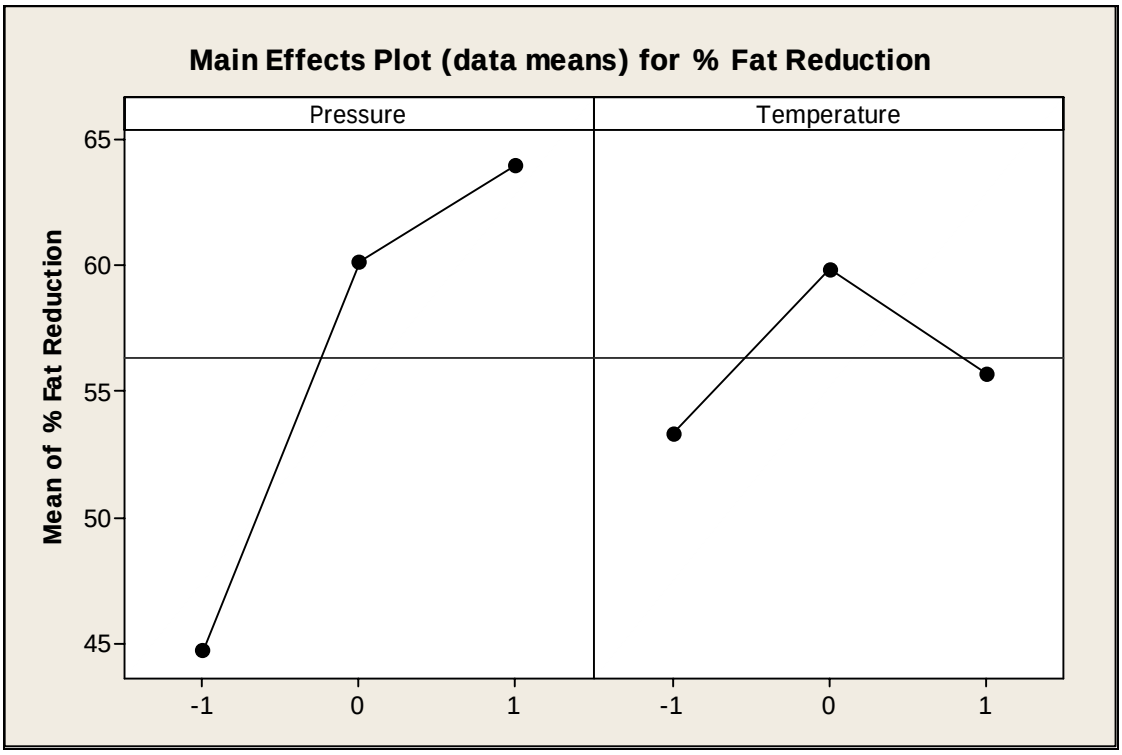

Figure 3.4. Main effects plot for pressure and temperature on % fat reduction in SFE treated buttermilk powder. The values shown (-1, 0, and +1) indicate the three levels for pressure (150, 250, and 350 bar, respectively) and temperature (40°C, 50°C, and 60°C, respectively).

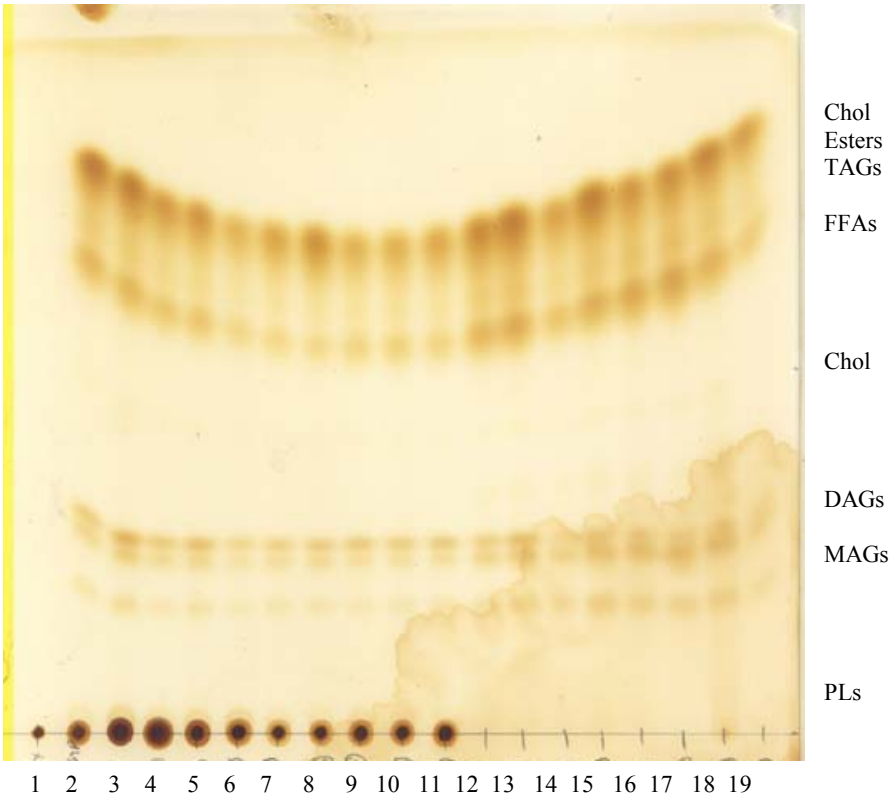

Figure 3.5. Thin layer chromatograph showing nonpolar lipid profiles from nine SFE experimental settings with BMP powder using the petroleum ether-ethyl ether-acetic acid (85:15:2, v:v) solvent system. Sample order is as follows: lane 1, phospholipid standard mix; lane 2, original BMP; lanes 3-11, defatted samples A-I; lanes 12-19, removed fat from experimental settings (A-EXT to H-EXT). Abbreviations are as follows in order from bottom to top: PLs = phospholipids, MAGs = monoacylglycerides, DAGs = diacylglycerides, Chol = Cholesterol, FFAs = free fatty acids, TAGs = triacylglycerides, Chol Esters = cholesterol esters.

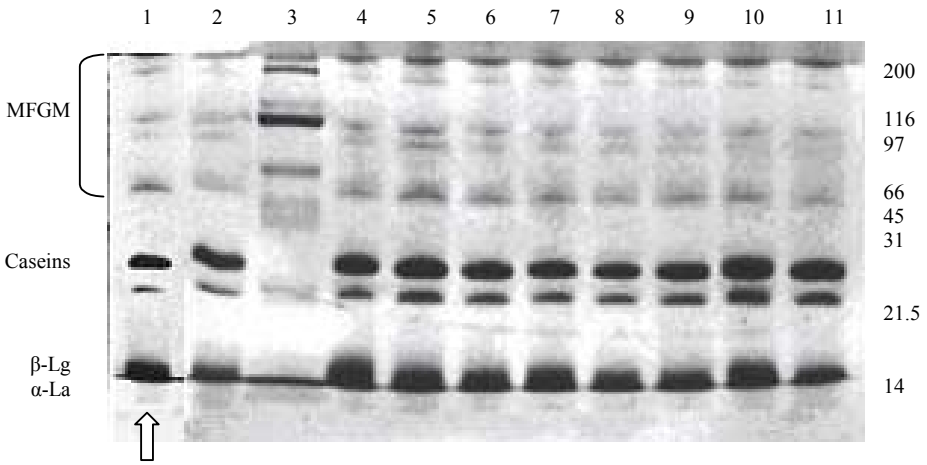

Figure 3.6. 12% Reducing SDS-PAGE, stained with Commassie blue and silver stain, on SFE treated buttermilk powders from the first trial. Lanes are as follows (indicates pressure/temperature SFE settings): 1) 350/50; 2) Original BMP; 3) MW marker; 4) 350/60; 5) 150/40; 6) 150/50; 7) 150/60; 8) 250/40; 9) 250/50; 10) 250/60; 11) 350/40.

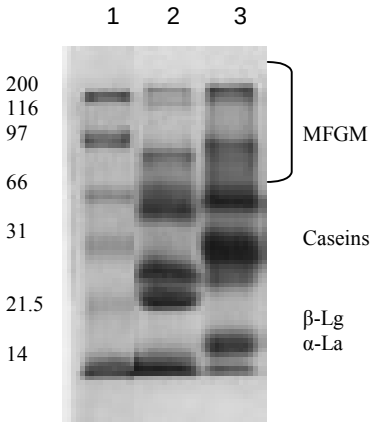

Figure 3.7A. 12% Reducing SDS-PAGE, stained with Commassie blue, on untreated buttermilk powder and SFE-treated powder under extreme conditions, 375/77. Lanes are as follows: 1) MW marker; 2) Original BMP; 3) BMP treated under extreme conditions.

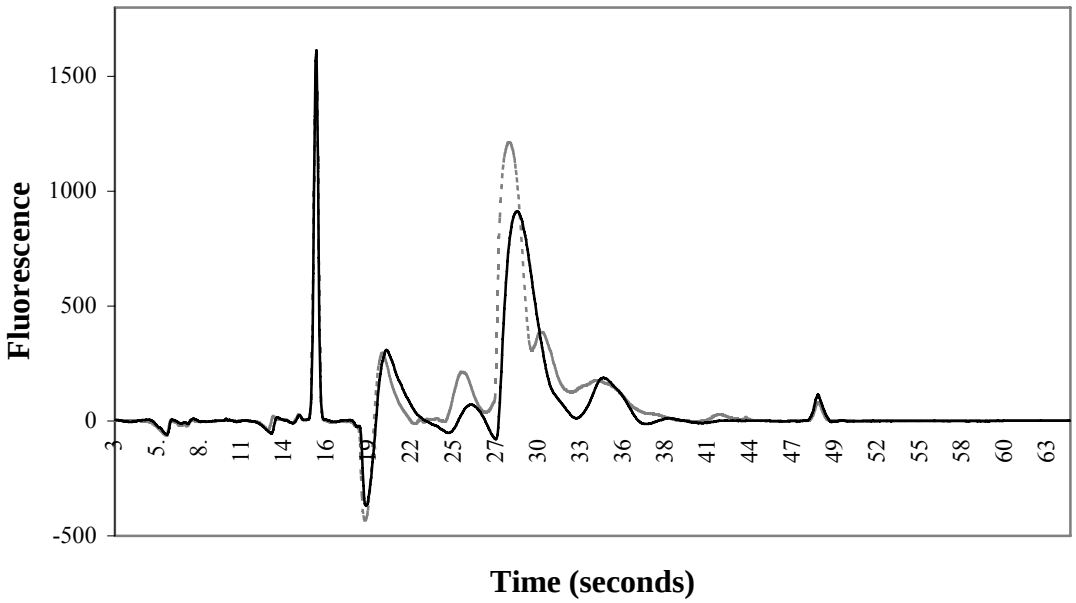

Figure 3.7B. Microfluidic-LIF virtual electropherogram of reducing proteins on untreated BMP (- - -) and SFE-treated BMP (──) under extreme conditions (375/77).

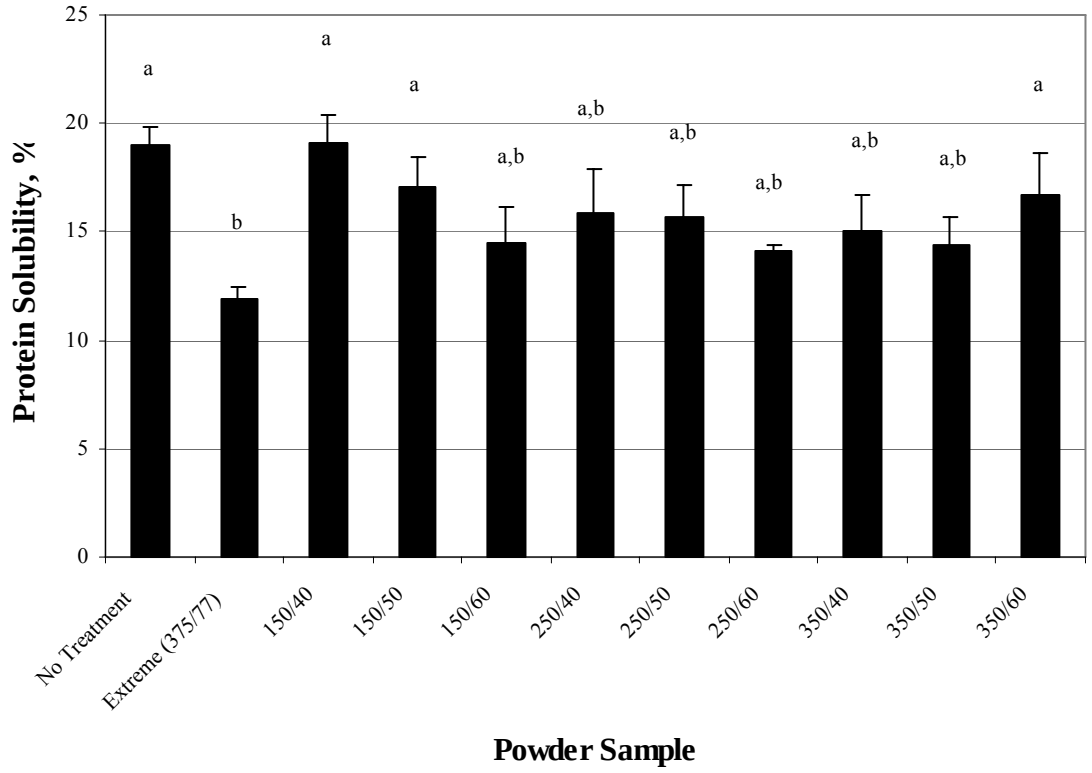

Figure 3.8. Protein solubility determined in a 5% protein solution for SFE treated buttermilk powders. Results marked with a different letter denote significant difference ($P < 0.05$). Powder sample names indicate treatment parameters used: Pressure (bar) / Temperature (°C)

The Evaluation of Supercritical Fluid Extraction Aids for Optimizing Non-Polar Lipid Extraction from Buttermilk Powder

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

[1]Rafael Jimenez-Flores

Tel: (805) 756-6103

Fax: (805) 756-2998

rjimenez@calpoly.edu

[*]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

The milk fat globule membrane, present in buttermilk, contains complex lipids such as phospholipids. These lipids, including sphingolipids, are known to be involved in a variety of biological processes exhibiting antioxidative, anticarcinogenic, and antiatherogenic properties and have essential roles in numerous cell functions. Microfiltration coupled with supercritical fluid extraction (SFE) may provide a method of enriching these nutritionally valuable lipids into a novel ingredient. Therefore, SFE as a method for phospholipid enrichment needs to be optimized for lipid removal effectiveness. The role of matrix additions to the buttermilk powder for extraction efficiency was evaluated. Diatomaceous earth**,** Teflon® beads and physical vibration were tested and shown to reduce total lipid by 86%, 78% and 70%, respectively. Four consecutive treatments were shown to exhaust the system; however, similar extraction efficiency was noted for one treatment with biosilicate addition, two treatments with physical vibration, or three treatments with added Teflon® beads. The extracted lipid material consisted of the non-polar fraction and protein concentration was observed to increase slightly in comparison to the control. Although there is higher lipid extraction from the powder with addition of diatomaceous earth, a removable aid is ideal for an edible product.

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

INTRODUCTION

Phospholipids (**PL**) are associated with both the milk fat and proteins, and are largely concentrated in the milk fat globule membrane (**MFGM**) (Renner et al., 1989, Warner, 1976). Phospholipids function as integral components of the membrane, and are also shown to be involved with other biological processes. Sphingolipids (**SL**), in particular, are known to have essential roles in cell survival, growth, and death (Huwiler et al., 2000, Kim et al., 1991, Lightle et al., 2000, Okazaki et al., 1989). Their interactive character and their location on the MFGM exterior suggests a major role in many different important biological interactions (Deeth, 1997). 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, 2002). Because of its unique composition and concentration of these lipids, MFGM in buttermilk could be used as a source of PLs. Buttermilk has unique properties as a functional ingredient and is readily available in large quantities from butter-producing dairy processors as a by-product and at a relatively low cost (Sodini et al., 2006).

Several attempts in fractionating buttermilk components have been made, primarily using filtration processes. Morin et al. (2004) reported on the effects of temperature and pore size in microfiltration of fresh and reconstituted buttermilk. When using a temperature of 25°C, higher amounts of retained fat coupled with a large amount of protein transmission were observed. Improved separation between lipids and proteins was shown using fresh rather than reconstituted buttermilk. Pore size also affected buttermilk fractionation, both protein and lipid content was

increased in the retentate when using a 0.1 μm membrane. Rombaut et al. (2006) showed that using microfiltration and sodium citrate or ethanol addition for the separation of lipids and proteins was not possible. Although micelle dissociation allowed effective transmission of casein through the membrane, high membrane fouling along with PL loss was observed.

Supercritical fluid extraction (**SFE**) is an additional method utilizing carbon dioxide that can selectively extract lipid components of a complex mixture. The properties of CO_2 make it an ideal solvent: non-explosive, inexpensive, easily obtainable and plentiful in nature, and non-toxic. Furthermore, its low critical temperature and pressure parameters (31.1°C, 73.8 bar) and ease of separation from the materials extracted makes it ideal for food processing applications (Arul et al., 1994, Hauthal, 2001, King, 1995, Rozzi and Singh, 2002). SFE is currently used to extract lipid and lipid soluble materials such as vitamins A, D, E, and K from a variety of mixtures (King et al., 2001, Markom et al., 2001, Saldana et al., 2002, Turner and Mathiasson, 2000).

A previous study by Astaire et al. (2003) found that the bioactive lipids of the MFGM could be concentrated within reconstituted buttermilk by combining microfiltration and SFE. A cross flow microfiltration system (0.8 μm pore size) was optimized for cold, reconstituted buttermilk powder initially concentrating the lipids. Further treatment using SFE successfully removed nonpolar lipids, specifically triacylglycerides (21 to 4 mg lipid/g dry powder) to increase the polar MFGM lipid (namely SM, PC, and PE) concentrations (9.6 to 19.7 mg lipid/g dry powder) in BMP. In previous studies, the addition of biosilicate material, specifically Celite, served to

increase efficiency by providing space between the powder particles, enabling better penetration of solvent, and in turn, increasing lipid extraction (Astaire, 2003, Berg et al., 1997). However, the addition of biosilicate material leaves the product inedible due to sandiness; therefore examination of other modifications is needed.

The goal of this project was to investigate SFE extraction aids in relation to lipid extraction efficiency by measuring the lipid removal effectiveness. Extraction aids such as diatomaceous earth, Teflon® beads and physical mixing to mimic fluidized bed were used. Lipid as well as protein composition of the treated powders were assessed.

METHODS AND MATERIALS

Chemicals and Reagent

The following lipid Standards were purchased from Sigma Chemical Co.: sphingomyelin (**SM**), phosphatidylcholine (**PC**), and phosphatidylethanolamine (**PE**). Silica gel TLC plates (60 F_{254}) were by EMD Chemicals (Darmstadt, Germany), and the developing tanks by Kontes Glass Company (New Jersey). All solvents and other reagents were of analytical grade, purchased from Fisher Scientific (Tustin, CA).

Processing

Buttermilk production. Fresh regular manufacturing cream was provided by Fosters Farm (Modesto, CA). Butter was made using a continuous pilot scale butter churn (Egli, Switzerland). Recovered buttermilk was filtered and spray dried using a Niro Filterlab Spray Dryer (Hudson, WI) following the manufacturer's instructions. For reconstitution of the dried buttermilk, tap water was added to give a 10% total solids solution, and let sit overnight at 4°C to fully hydrate.

Microfiltration. The microfiltration system is 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, France). A membrane with a pore size of 0.45 μm was used. All runs were carried at low temperature (8-10°C) at a transmembrane pressure of 80 to 95 kPa. Microfiltration processes (**VCMF**) concentrated the buttermilk to a volumetric concentration factor of 2x. Final retentates were spray dried using a Niro Filterlab Spray Dryer (Hudson, WI).

Supercritical Fluid Extraction. The SFE system and components were acquired from Thar Designs, Inc. (Pittsburgh, PA) and are previously described by Astaire et al. (2003). Briefly, the in-house unit includes the following: 500 ml vessel, model P-50 high-pressure pump, automated back pressure regulator model BPR-A-200B, and PolyScience brand water bath and pump unit (model 9505). Circulated deionized water at 5°C was used for cooling different zones in the SFE apparatus. 50-lb 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). The running conditions were as follows: pressure, 350 bar; temperature, 50˚C; flow rate, 20g/min; total vessel flushes, 3.40 ± 0.51; total run time, 94.00 ± 0.14 min; and total CO_2 used, 1700.0 ± 226 grams. Following treatment, the SFE extraction vessel was rinsed with 50 mL – 100 mL of chloroform:methanol (2:1 v/v) into a pre-weighed, 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, v:v).

SFE Extraction Efficiency

A 2x VCMF BMP Retentate (% fat = 17.39% ± 0.06) was used. Four powder systems were used as follows: 1) without added Celite™ 566 or Teflon® beads and rings (TB), 2) with added Celite 566 (50:50 w/w) (World Minerals, Inc., Lompoc, CA), 3) with added Teflon® (Polytetrafluoroethylene) beads and rings (24.2g), 3 mm in diameter (Fisher Scientific #09-191-30A and 09-191-25A, respectively), and 4) without additions, fluidized bed simulation. To simulate the extraction of SFE coupled with a fluidized bed, the SFE vessel was opened during the run following one

vessel flush (500g CO_2), the powder was stirred and the extraction continued. The sample was stirred three times in a complete run. Each powder was given a total of 12 stirrings in a 4x run. SFE treatments at the specified parameters were performed on each powder, until the % fat in the powder remained unchanged. Following SFE treatments, the TB were removed by sifting the powder; Celite was not removed from the powders. The SFE extractions for all powder systems were carried out in triplicate. The total fat was extracted from the BMP samples in duplicate by Mojonnier Method, and analyzed by TLC following the procedures detailed below. The lipid extraction efficiency was compared between the four powder systems. Samples were labeled as follows: powder with no additions, OC; with added Celite 566, WC; with added Teflon beads and rings, TB; and stimulated fluidized bed, FB. Lipid fractions removed from the SFE extraction vessel were labeled as above with suffix –EXT.

Protein Determination. Percent protein was determined by testing for total nitrogen content by the Kjeldahl method (AOAC Official Method 955.04). Samples were heated on a Digestion System 20, model 1015 digester. Samples were distilled with the Kjeldahl System, 1026 Distilling Unit, and FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific, Tustin, CA) were used as reagent. All samples were analyzed in duplicate. Titrations were done using 0.1 N HCl, and the percent protein was calculated using the milk protein conversion factor, 6.38. All reagents were of Kjeldahl analysis analytical grade**.**

Lipid Quantification. The Mojonnier ether extraction method as described by Marshall (1992) was followed. All samples were extracted in duplicate.

Lipid Profiling by TLC. Silica gel glass plates were made by Merck (Darmstadt, Germany), and the developing tanks by Kontes Glass Company (New Jersey). The polar lipids were separated using chloroform-methanol-water (65:25:4, v:v); nonpolar lipids were separated using petroleum ether-ethyl ether-acetic acid (85:15:2, v:v). Lipids were prepared in chloroform-methanol (2:1, v:v) to 10 mg/ml; lipid standards were diluted to 1 mg/ml. 100 μg 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 (Astaire, 2003).

Statistical Analysis

Graphics, statistics and experimental design were performed with Minitab 14.0 software (Minitab Inc., PA). All comparisons were done by ANOVA with Tuckey's pairwise comparison using Minitab 14.0 software (Minitab Inc., PA). Results were considered statistically different at $p<0.05$.

RESULTS AND DISCUSSION

The objective of this study was to optimize an SFE process in relation to examining the effects of powder additions on lipid extraction efficiency. The analysis was based on protein and lipid compositional changes of the treated powders. Table 4.1 shows the protein and lipid composition as well as the percent of lipids removed by the SFE process after a total of four treatments. In relation to protein content, significant differences were shown between powders with different additives ($p = 0.003$). Results show the powder treated with added biosilicates is greater than the powders treated with added TB and the powder with no additives. Similarly, lipid content shows differences ($p = 0.000$) between the experimental powders with the

biosilicate powder being lower than the TB or no addition powder. The average % total fat removal for SFE treatments with added adsorbent is shown in Figure 4.1. For all powders the first SFE treatment greatly reduces lipid content by at least 50%, independently of powder modifications. SFE processing with added biosilicate materials (WC) produces a greater efficiency of total fat removal (77%) than without the biosilicates. An additional SFE treatment reduced the amount of total fat in the powders by an average of 85% and 63% with and without added biosilicates, respectively. From this, it is observed that a fourth SFE treatment on the WC powder has no effect on further fat removal. This is consistent with a previous study (Astaire, 2003). However, without any aids for extraction, four treatments are required to extract the same amount of lipid as that compared with the obtained in just one treatment when biosilicates are present. The addition of TB showed a 78% removal after three treatments. Although lipid removal in the powder with beads was less than the powder with biosilicates, the TB offers an attractive alternative since it is a removable aid. Using a bed fluidizer in conjunction with the SFE may create a constant flowing space between the powder particles, enabling greater lipid extraction. Mixing of the powder in intervals during the treatment served to mimic a fluidized bed and demonstrated that it may be ideal. According to the data presented in Figure 4.1, we show that two treatments with mixing were equivalent to three treatments with TB. We think that constant powder mixing within the SFE sample vessel would increase lipid extraction comparable to that of adding biosilicates by increasing the surface area within the powder and therefore enabling greater solvent penetration.

Another explanation into greater efficiency using biosilicate material is component interaction between the buttermilk powder matrix and the biosilicates, perhaps lipid or protein material. It has been previously shown that biosilicate material absorbed specific protein and phospholipids from the milk fat globule membrane during buttermilk filtration (Fryksdale, 2000). However, studies are limited to the interactions of dry biosilicate matter with milk powder. An overview of silica by Bergna (1994) describes natural biosilicates and the different groups that occur at their surfaces. Those that can occur are 1) strained and stable siloxane groups, 2) silanol groups: isolated hydroxyl groups, 3) hydrogen-bonded silanol groups: hydroxyl groups on adjacent surface silicon atoms, 4) silanediols: two hydroxyls associated with one silicon atom, 5) silanetriols: three hydroxyls associated with one silicon atom, and 6) physically absorbed H_2O hydrogen to all types of surface hydroxyl groups. Materials can attract and be anchored to the surface of biosilicate material by three types of binding forces: hydrogen bonding, hydrophobic interactions, and ionic attractions. Much of the observed whey protein binding can be explained by hydrophobic interactions, when the molecule is attracted to the biosilicate surface by hydrophobic forces. A study by Krisdhasima et al. (1993) compared the differences between milk proteins in relation to their adsorption and elution characteristics from hydrophilic and hydrophobic surfaces. α-lactalbumin, β-lactoglobulin and BSA all had a higher affinity for hydrophobic surfaces while β-casein had a higher affinity for hydrophilic surfaces. And among the globular proteins, α-lactalbumin, due to its smaller size, had a much greater affinity for hydrophobic surfaces than the other proteins.

In conclusion, the buttermilk powder products developed by combining microfiltration and SFE process are rich in polar lipids and protein. Addition of a physical aid, such as removable Teflon® beads or fluidized bed mixing ensures adequate non-polar lipid removal for polar lipid enrichment.

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Table 4.1. Compositional data for all BMP treated with SFE and additives for a total of four treatments and the untreated BMP.

Buttermilk Powder	% Protein	% Lipid	% Lipid Reduced
No Treatment	37.66 ± 1.02^{a}	17.39 ± 0.08^{a}	N/A
No Additives	34.45 ± 0.66^{a}	3.98 ± 0.26^{b}	65.49 ± 11.80
Biosilicate Addition	42.62 ± 0.39^{b}	2.32 ± 0.01^{c}	83.92 ± 4.97
Teflon Bead Addition	39.05 ± 0.03^{a}	4.00 ± 0.39^{b}	69.77 ± 10.70
Physical Mixing	38.90 ± 0.41^{ab}	3.00 ± 0.49^{bc}	66.51 ± 9.32

^{abc}Values in same column with different superscript differ significantly($p < 0.05$).

± indicates standard deviation.

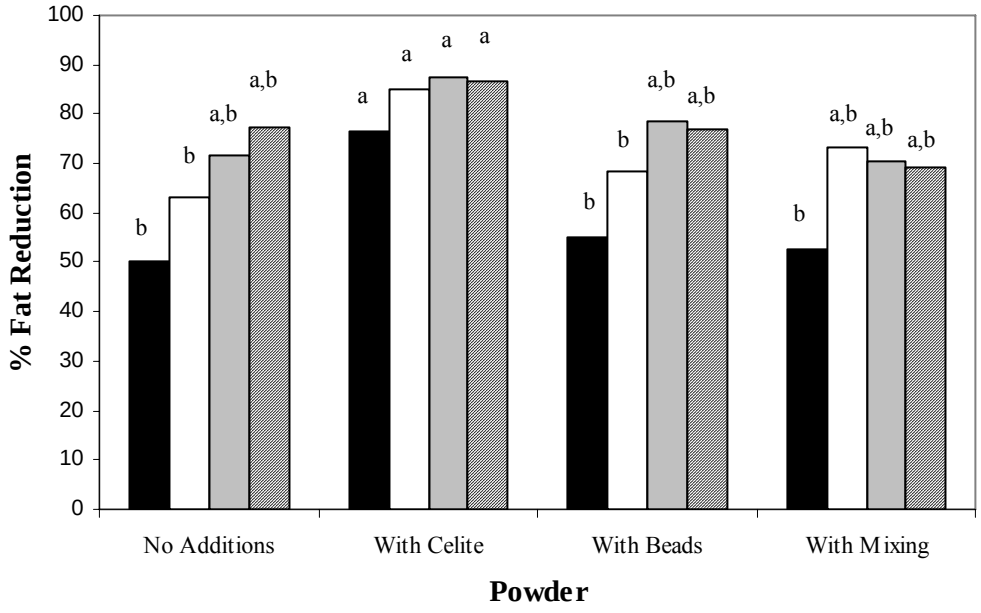

Figure 4.1. Average % fat removal for SFE treatments on powder with added biosilicates, no additions, added TB beads and rings, and fluidized bed simulation. Results marked with a different letter denote significant difference ($P < 0.05$). Each bar represents the treatment repetition (treatment #1, black bar; #2, white bar; #3, gray bar; #4, diagonal hatch) of extractions at 350 Bar, 50°C using 1683.36 ± 226.38 grams of CO_2.

Enrichment of Polar Lipids in Buttermilk and Whey Cream Buttermilk using Microfiltration and Supercritical Fluid Extraction

Amy J. Spence[†], Rafael Jimenez-Flores[‡*], Michael Qian[†], Lisbeth 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

[*]Rafael Jimenez-Flores

Tel: (805) 756-6103

Fax: (805) 756-2998

rjimenez@calpoly.edu

Abbreviations: SFE – supercritical fluid extraction; PL – phospholipid; SL – sphingolipid; MFGM – milk fat globule membrane; DF – diafiltered; VC – volumetric concentration; MF – microfiltration; RC – regular cream buttermilk; WC – whey cream buttermilk; PE – phosphatidylethanolamine; PI – phosphatidylinositol; PS – phosphatidylserine; PC – phosphatidylcholine; SM – sphingomyelin

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 such as buttermilk, whey, whey cream. However, PL are present in low concentration. To enrich PL in buttermilk powders, regular buttermilk and whey buttermilk (by-product of whey cream after making butter) were microfiltrated and then treated with SFE after drying. The total fat, namely non-polar lipids, in the powders was reduced by 38 – 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 in comparison to the others. However, most unsaturated species were not as abundant.

Keywords: Supercritical Fluid Extraction; Phospholipids; Buttermilk Lipids; Sphingomyelin

INTRODUCTION

Milk fat consists as 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 it 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 (Berra, 2002, Dacaranhe and Terao, 2001, Molkentin, 2000). During the process of churning cream into butter, the liquid fat sticks together and the surrounding MFGM is left as deflated membranous sacks in the buttermilk. MFGM contain 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, 1999). The major PL fractions consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS) (Barenholz and Thompson) (Renner et al., 1989). SL, in particular, are known to have essential roles in cell-to-cell interactions, differentiation, proliferation, immune recognition, and transmembrane signaling (Huwiler et al., 2000, Kim et al., 1991, Lightle et al., 2000, Okazaki et al., 1989). 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, 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, Cinque et al., 2003, Vesper et al., 1999). Sphingolipids consist of a long-chained sphingoid backbone and all together, may represent the most structurally diverse, as well as complex, group of lipids in nature (Berra, 2002, Vesper et al., 1999). Along with PCs, SMs form one of the major classes of PLs and comprise more than 50% of membrane PLs (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 budding uses as anticancer agents or for regulating disorders in which apoptosis plays a crucial role (Nava et al., 2000, Perry et al., 1996). Many of their roles in 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 its unique composition and concentration of these lipids, MFGM in buttermilk could be used as a source of PLs

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 in order 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 content was increased in the retentate when using a 0.1 μm membrane. In addition, improved separation between lipids and proteins was shown using fresh rather than reconstituted buttermilk.

Whey cream is primarily used to standardize milk fat prior to cheese making, the whey cream (WC) can also 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 - 6. Morin et al. (2006) compared the volumetric concentration (VCMF) and diafiltration (DFMF) of both regular and whey cream buttermilk in terms of separation efficacy and permeation flux. A two-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 can be effectively removed from buttermilk, whereas bioactive lipids of the MFGM could be

concentrated in buttermilk. To our knowledge, using these techniques in conjunction with each other to develop a novel buttermilk ingredient has not been explored any further. Additionally, there are no current uses for buttermilk obtained from whey cream; 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 and whey cream. 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. SM structure was described to show the unique composition of the final products.

METHODS AND MATERIALS

Chemicals and Reagent

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

Buttermilk Production.

For each trial, either 200 L fresh cream or whey cream was processed. Fresh regular (RC) manufacturing cream was provided by Fosters Farm (Modesto, CA) and whey cream (WC) was graciously donated by Hilmar Cheese Co. (Hilmar, CA). 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 three times and whey cream

buttermilk was repeated twice with different lots of both creams. The buttermilks for each process were then treated through the membrane filtration process, and the retentates obtained in every case were then spray dried in our pilot plant drier Niro Filterlab Spray Drier (Hudson, WI). The resulting powders were then subjected to SFE treatment (see below).

Microfiltration and Diafiltration. The microfiltration and diafiltration were performed according to the procedures described previously (Astaire, 2003, Morin et al., 2006). Briefly, the microfiltration unit consists of two tubular membranes containing Tami Sunflower Design ceramic membranes (Tami Industries, France) fitted in parallel on the module. Total surface area was 0.7 m^2. A membrane with a pore size of 0.45 μm was used and fitted in US filters stainless steel housings (Warrendale, PA). All runs were carried at low temperature (8-10°C) at a transmembrane pressure of 80 – 95 kPa. The microfiltration process termed VCMF consisted of concentrating the buttermilk by microfiltration to a volumetric concentration factor (VCF) of 2x. In the diafiltration of DFMF process, chilled tap water was continuously added to the retentate in order replace the extracted permeate until a dilution factor (DF) of 2x was reached (i.e. twice the amount of the starting volume). Final retentates from both processes were spray dried using a Niro Filterlab Spray Dryer (Hudson, WI).

Supercritical Fluid Extraction. The SFE system and components were acquired from Thar Designs, Inc. (Pittsburgh, PA) and operating conditions were previously described by Astaire et al. (Astaire, 2003). Briefly, the in-house unit includes the following: 500 ml vessel, model P-50 high-pressure pump, automated

back pressure regulator model BPR-A-200B, and PolyScience 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 were from Airgas West (San Luis Obispo, CA). The system conditions were controlled manually by Windows 2000-based software (Hewlett-Packard). BMP (300 g) was mixed 1:1 with Teflon® (Polytetrafluoroethylene) beads and rings (24.2g), 3 mm in diameter (Fisher Scientific, Tustin, CA), 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, 20g/min; total vessel flushes 3.13 ± 0.23; total run time, 86.00 ± 0.06 min; and total CO_2 used, 1560.0 ± 116 grams. 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, v:v).. To ensure complete removal of fat, the vessel was rinsed with 50 mL – 100 mL of chloroform:methanol (2:1 v/v) and discarded.

Buttermilk Powder Lipids

Lipid Profile Analysis. The lipids were extracted from the treated BMP in duplicate by Mojonnier Method, and lipid profiles were analyzed by TLC. The polar lipids were separated using chloroform-methanol-water (65:25:4, v:v); nonpolar lipids were separated using petroleum ether-ethyl ether-acetic acid (85:15:2, v:v). Lipids were prepared in chloroform-methanol (2:1, v:v) to 10 mg/ml; lipid standards were diluted to 1 mg/ml. 100 µg 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. x 150 mm (Alltech, Deerfield, IL). Lipids were collected and analyzed with an evaporative light-scattering detector, ELSD (SEDEX 55, SEDERE, France) and EZ-Logic software (Bio-Rad Laboratories). The injector valve (AVR 7-3) was fitted with a 25µL injection loop. 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 v/v/v of chloroform:methanol:triethylamine buffer (pH 3, 1 M formic acid) at t = 0 to 28:60:12 v/v/v 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 re-suspended in 3 mL chloroform. Polar lipids were separated by solid phase extraction using 500 mg silica columns (Strata SI-1 SPE silica, 55 µm, Phenomenex, Torrance, CA). Columns were conditioned with 6 mL of chloroform at 3-4 mL/min flow, samples were loaded at 1-2

mL/min, columns were washed with 6 mL of chloroform at 3-4 mL/min, and the polar lipids were eluted in 6 mL methanol. Following SPE, samples were hydrolyzed in methanolic KOH (0.1M, 40°C for 3 hours) to saponify glycerolipids. Hydrolyzed samples were re-extracted by a procedure described by Merrill and Wang (Merrill and Wang, 1986). A solution of 1:2 chloroform:methanol (1.5 mL) was added and mixed thoroughly; 1 mL each of chloroform and water were added and centrifuged (1000 x g for 2 min); the upper phase was discarded and the chloroform phase was washed twice with water and dried under N_2.

SM molecular species was analyzed using a LC/MS/MS system at the Linus Pauling Institute (Oregon State University). Liquid chromatography was performed with a Shimadzu system: two LC-10Advp pumps, a DGU-14A degasser, a SIL-HT autosampler, and a CTO-10Avp column oven (Columbia, MD). The column used was a Discovery C18 column, 50 x 2 mm i.d., 5 μm (Supelco, St. Louis, MO). The flow rate was 0.3 mL/min, the column temperature was 30°C, and the injection volume was 5 μL. 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 v/v) + 10mM ammonium acetate and solvent B was methanol-chloroform-formic acid (60:40:0.2 v/v) + 10mM 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 mass

spectrometry system was performed by scanning for m/z 184 at a flow rate of 10 μL/min. Data handling was performed using the Analyst 1.4.1 software (Applied Biosystems/MDS SCIEX). Prior to LC/MS/MS analysis, samples were diluted to 100 μg/ml in solvent A. Long-chain bases (LCB) and fatty amides (FA) 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 (TN), nitrogen soluble at pH 4.6 (Reid et al.) and non-protein nitrogen (NPN) from the treated BMP retentates were measured by Kjeldahl method (AOAC, 1995). Samples were heated on a Digestion System 20, model 1015 digester. Samples were distilled with the Kjeldahl System, 1026 Distilling Unit, and FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific, Tustin, CA) were used as reagent. Titrations were done using 0.1 N HCl, and the percent protein was calculated using the milk protein conversion factor, 6.38. Total protein measurements were taken in duplicate. The NPN, expressed in protein equivalent, was calculated as NPN x 6.38. The soluble protein at pH 4.6 was calculated as (SN – NPN) x 6.38. The insoluble protein at pH 4.6 was calculated as (TN – SN) x 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 ± 1°C. Percent moisture was calculated by subtraction. Percent ash was determined by incineration at 550°C

Statistical Analysis

Statistical analyses were performed with Minitab 14.0 software (Minitab Inc., PA). All comparisons were done by ANOVA with Tuckey's pairwise comparison

using Minitab 14.0 software (Minitab Inc., PA). Results were considered statistically different at $p<0.05$.

RESULTS AND DISCUSSION

Buttermilk Powder Lipids

The average total solids, total protein (% DM), total lipid (% DM), total phospholipid (% DM), ratio of phospholipid (mg) to protein (g), and final concentration factor (CF) of PL using SFE treatments is shown in Table 5.1. The total fat observed in the initial DFMF whey cream buttermilk has a high variability in comparison to the other buttermilks specifically due to commercial origin, since we did not select the kind of process parameters. Total lipids and total phospholipids between the DFMF whey BMP and the other powders differ significantly ($P = 0.002$ and 0.000, respectively). After three extractions, SFE processing reduced the total fat in the powders by 36 to 55% with a high variability between the samples for the VCMF filtrated powders, both whey cream and regular cream (as seen by the % weight SD 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 show less variability in relation to percentage of lipid reduction for both whey and regular cream (SD 5 and 3, respectively). However, there is no difference in percentage of lipid reduction between the powders ($P = 0.091$).

The variability 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, 1999). High variations in whey cream prior to production of buttermilk have previously been reported (Morin et

al., 2006, Morin et al., 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). Higher transmission was observed with DFMF, resulting from more stable fluxes and less fouling. And whey buttermilk had lower lipid transmission attributed to aggregation and presence of casein proteins in regular buttermilk. The accumulation of these factors leaves the final product with high variability.

Concentration factor of the PL was calculated in order to illustrate the overall effectiveness of the SFE treatment. CF was calculated on a dry basis by comparison of the PL concentration in the final BMP after SFE as compared to the initial BMP. Results show that the calculated CFs 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 regular and whey cream 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 (Astaire, 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 PLs (mg) to protein (mg) ratio also increased significantly ($P = 0.000$). The % PL is important, but more relevant is the ratio of PL to protein. This directly relates the PL content to actual

solids contained in the buttermilk powder. From Table 5.1, a 3x increase in the ratio is observed following SFE treatment for all buttermilk samples.

We have also described the specific fatty amide (FA) combinations as found on a sphingosine backbone for intact sphingomyelin in our BMP samples. Molecular species of intact SM from the mojonnier extracted lipids was separated by using a precursor scan at m/z 184, representative of phosphocholine, $[PO_4(CH_2)_2N(CH_3)_3 + H]^+$ and analysis of FA combinations as measured by a sphingosine LCB backbone (18:1) thereof. At least 28 different FA combinations were detected covering a range of molecular masses from 647.5 to 841.7 Da (Table 5.3). The combinations found are similar to those described in previous studies (Byrdwell and Perry, 2007, Karlsson et al., 1998). By using a number of techniques in combination with each other Karlsson et al. (1998) was able to describe intact SM from bovine milk by both the long chain base and fatty amide combinations. The most common LCBs were 16:1, 17:1, 18:1 and 19:1; moreover, the most common FAs 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 (Byrdwell and Perry, 2007; Karlsson, 1998).

In our study, the six most common saturated FA species (> 5% of total) for SM detected were 16:0, 20:0, 21:0, 22:0, 23:0, and 24:0. Unsaturated FA species were not as abundant; however FA 24:1 represented greater than 1% of total species for all

BMP samples. The FA 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 FA combinations with 18 carbons or fewer are observed for this sample. For this sample, FA 16:0 was rather high (16.91%) in comparison to the other powders (8.29%, 6.81%, and 9.04%), possibly explained by variability in the starting material and processing methods. There were two molecular species detected that had significant differences between the DFMF and VCMF BMP samples, 23:1 and 24:1. For both of these FAs, the VCMF WC powder had a greater amount than the other powders. DFMF WC powder was elevated in comparison to the other two, 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 FAs is a result of the VCMF process. Other interesting observations include similarities between FA for the different filtration modes. FAs 22:0, 23:0, 24:0, and 25:0 have slightly higher amounts in both powders for DFMF than VCMF. Perhaps the higher fouling and flux rates observed with VCMF attributes to a lower retention of the higher chained unsaturated SM species.

Compositional Study

Results for protein content in BMP retentates is shown in Table 5.4. For all samples, the NPN and SP fractions increased and the insoluble protein fraction decreased following SFE treatment. NPN 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 in comparison to 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 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 in comparison to 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.

Results for % solids, moisture, and ash are shown in Table 5.5. For both VCMF and DFMF whey cream samples, the total solids increased following SFE whereas the total solids for both VCMF and DFMF regular cream samples decreased; however, no significant difference was observed for total solids or moisture (P =0.167). The ash content increased slightly following treatment for all samples;

however, there was no significant difference between the samples (P = 0.106). It is expected for % protein and % ash of the powder to increase and the % fat to decrease following SFE treatment. According to Table 5.1, this was found to be the case in all samples except VCMF RC cream where the % protein amount decreased slightly (0.20%) following treatment.

The mean phospholipid distribution (as % of PL) in the buttermilk samples is shown in Table 5.2. 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 in comparison to DFMF, possibly attributed to the filtration method. The distribution of PE and PI differ among the powders. PE content for the regular cream samples shows that the VCMF value is slightly higher than DFMF and the opposite is true for the whey cream samples. Similarly PI content is higher for DFMF and lower for VCMF regular cream and opposite effects are shown for the whey cream buttermilk.

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.

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Table 5.1. The average total solids, total protein (% DM), total lipid (% DM), total phospholipid (% DM), ratio of phospholipid (mg) to protein (mg), and final concentration factor (CF) of PL in supercritical fluid extraction treated regular and whey buttermilk powder.

Sample		Total Solids	Total Protein (%DM)	Total Lipid (%DM)	Total PL (%DM)	PL (mg) / Protein (mg)	CF of PL	% Lipid Reduced
BMP Before SFE	DFMF RC	96.1 ± 1.2	45.6 ± 1.9^{a}	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	38.7 ± 1.3^{a}	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	22.6 ± 4.1^{b}	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	17.2 ± 1.4^{b}	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	51.1 ± 2.9^{a}	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	38.5 ± 4.5^{a}	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	95.6 ± 2.9	26.1 ± 2.1^{b}	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	96.9 ± 2.4	20.8 ± 0.4^{b}	9.8 ± 0.6^{a}	3.0 ± 0.2^{b}	0.15 ± 0.01^{b}	4.2 ± 0.8^{b}	55.3 ± 10.7

^{ab}Values in same line for each product with different superscript differs significantly ($P < 0.05$)

± indicates standard deviation.

BMP – buttermilk powder; SFE – supercritical fluid extraction; DM – dry matter; CF-concentration factor; PL – phospholipid; DFMF – diafiltration; VCMF – volumetric concentration; RC – regular cream buttermilk; WC – whey cream buttermilk

Table 5.2. The average % lipid reduction and average phospholipid content (% of PL) in SFE-treated regular and whey buttermilk powder.

		Regular Buttermilk Powder		Whey Buttermilk Powder	
		VCMF	DFMF	VCMF	DFMF
(% of PL)	PE	18.6	16.9	10.4	22.0
	PI	0.7	4.4	10.0	2.4
	PS	6.3	6.0	5.1	4.8
	PC	31.3	23.1	35.7	23.0
	SM	43.1	49.6	38.8	47.8

PL – phospholipid; DFMF – diafiltration; VCMF – volumetric concentration; PE – phosphatidylethanolamine; PI – phosphatidylinositol; PS – phosphatidylserine; PC – phosphatidylcholine; SM - sphingomyelin

Table 5.3. The fatty amide composition (% of total) of SM found in SFE-modified treatments for regular and whey buttermilk powder (% of total).

	DFRC	VCRC	DFWC	VCWC
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	ND	ND

[ab]Values in same row with different superscript differ significantly ($p < 0.05$).

ND = Not detected

DF – diafiltration; VC – volumetric concentration; RC – regular cream buttermilk;

WC – whey cream buttermilk

Table 5.4. Content of the nitrogen fraction of buttermilk retentate powders before and after SFE treatment: non-protein nitrogen (as % of DM), soluble protein at pH 4.6 (as % of DM), and insoluble protein at pH 4.6 (as % of DM).

Sample		Non Protein Nitrogen	Soluble Protein (pH 4.6)	Insoluble Protein (pH 4.6)
BMP Before SFE	DFMF RC	3.0 ± 0.3[a]	8.9 ± 1.7[a]	88.1 ± 2.1[a]
	VCMF RC	4.7 ± 0.4[b]	10.9 ± 0.4[a]	84.3 ± 0.1[a]
	DFMF WC	12.3 ± 0.8[c]	24.9 ± 1.1[b]	62.7 ± 1.9[b]
	VCMF WC	20.3 ± 0.0[d]	25.4 ± 0.0[b]	54.3 ± 0.0[c]
BMP After SFE	DFMF RC	3.7 ± 0.5[a]	9.9 ± 2.1[a]	86.4 ± 2.4[a]
	VCMF RC	5.8 ± 1.1[b]	12.2 ± 2.3[a]	82.0 ± 3.4[a]
	DFMF WC	12.8 ± 1.8[c]	27.4 ± 4.4[b]	59.9 ± 6.1[b]
	VCMF WC	21.7 ± 1.5[d]	33.8 ± 1.0[c]	44.5 ± 0.5[c]

[abcd]Values in same column with different superscript differs significantly ($P < 0.05$)

± indicates standard deviation

DFMF – diafiltration; VCMF – volumetric concentration; RC – regular cream buttermilk; WC – whey cream buttermilk

Table 5.5. Composition of regular and whey cream BMP retentates before and after SFE treatment.

Sample		Total Solids (%)	Moisture (%)	Ash (% DM)
BMP Before SFE	DFMF RC	96.1 ± 1.2	3.9 ± 1.2	6.1 ± 0.2
	VCMF RC	97.3 ± 0.8	2.7 ± 0.8	6.2 ± 0.2
	DFMF WC	95.3 ± 2.9	4.8 ± 2.9	5.5 ± 1.2
	VCMF WC	95.4 ± 0.8	4.6 ± 0.8	6.0 ± 0.7
BMP After SFE	DFMF RC	94.8 ± 0.3	5.2 ± 0.3	7.3 ± 0.2
	VCMF RC	95.8 ± 1.4	4.2 ± 1.4	7.1 ± 0.3
	DFMF WC	96.9 ± 2.4	3.1 ± 2.4	7.2 ± 0.3
	VCMF WC	95.6 ± 2.9	4.4 ± 2.9	7.4 ± 0.2

± indicates standard deviation

DFMF – diafiltration; VCMF – volumetric concentration; RC – regular cream buttermilk; WC – whey cream buttermilk

A Thermal Stability Study of Whey Cream Buttermilk Powder Treated with Membrane Filtration and Supercritical Fluid Extraction

A. J. Spence[1], L. Goddik[1*], R. Jimenez-Flores[2], M. Qian[1],

[*]Lisbeth Goddik

100 Wiegand Hall, Corvallis, Oregon 97331-6602

Tel: (541) 737-8322

Fax: (541) 737-1877

lisbeth.goddik@oregonstate.edu

[1]Department of Food Science and Technology, Oregon State University, Corvallis, 97330

[2]Dairy Products and Technology Center, California Polytechnic State University, San Luis Obispo, 93407

Thermal Study of Whey Cream Buttermilk….

JFS: Food Chemistry

ABSTRACT

Buttermilk is rich in milk fat globule membrane material, which contains complex lipids implicated in an assortment of biological processes. Whey cream buttermilk contains low amounts of caseins, giving it a distinctive composition. Membrane filtration coupled with supercritical fluid extraction (SFE) has been shown to provide a method of concentrating these nutritionally valuable lipids into a novel ingredient. The objective of this study was to observe the thermal stability of whey cream buttermilk powder prior to and following SFE treatment in order to learn about its potential application into food products. Whey cream buttermilk was ultrafiltrated prior to drying and subsequently processed using supercritical fluid extraction. Salt as well as pH levels were adjusted in the reconstituted powders and protein agglomeration upon heating (80 °C) was assessed. SFE treatment significantly affected protein aggregation in the whey cream buttermilk powders ($p = 0.009$), observed by a decrease in optical density and mean particle size ($p = 0.000$) following a short heat treatment (5 min). This observation was pH-dependent. Addition of divalent salts was shown to aid in thermal stability, but only at an acidic pH and for a short period of heating. In comparison to whey protein isolate, the SFE process enhances thermal stability by reducing protein aggregation, indicating the processed whey cream buttermilk powder to be a unique product with an interesting thermal profile and composition.

Keywords: Whey Cream, Buttermilk Powder, Supercritical Fluid Extraction, Thermal Stability, Phospholipids

INTRODUCTION

Whey cream is obtained by separation of the fat from cheese whey with centrifugation. In producing cheese, the cheese maker's objective is to maximize casein recovery in the cheese; therefore, whey cream is practically devoid of caseins. Though primarily used to standardize milk fat prior to cheese making, the whey cream can also be churned to make whey butter and, as a by-product, whey cream buttermilk. Buttermilk is the aqueous phase left over from churning butter into cream and contains material derived from milk fat globule membranes (MFGM). Buttermilk contains a higher percentage of phospholipids in comparison to separated milk, 0.13% and 0.015% respectively, which gives buttermilk unique properties as a functional dairy ingredient (Walstra et al., 1999). Buttermilk has an ability to increase the heat stability of recombined milks (Singh and Tokley, 1990), mainly due to phospholipid-protein interactions (McCrae, 1999). Additionally, phospholipids have been shown to possess biological activities (Huwiler et al., 2000, Kim et al., 1991, Lightle et al., 2000, Okazaki et al., 1989).

Whey cream buttermilk composition is distinct, offering an ingredient with promising functionality. Sodini et al. (2006) compared the compositional and functional properties of whey buttermilk, cultured buttermilk, and sweet cream buttermilk. Their results showed that whey cream buttermilk is a potentially novel ingredient having higher emulsification properties and lower foaming ability, along with stable protein solubility, viscosity, and emulsifying capacity over a pH range of 4 - 6. Moreover, concentrating these distinctive functional components using filtration coupled with supercritical fluid extraction (SFE) may lead to a product rich in whey

protein and bioactive lipids (Morin et al., 2006). SFE is a method that uses CO_2 in the supercritical state and can selectively extract nonpolar lipid components of a complex mixture. A study by Astaire et al. (2003) successfully increased the polar MFGM lipids in buttermilk powder by the removal of nonpolar lipids, specifically triacylglycerides.

An important functional property of whey proteins is the ability, under appropriate conditions, to form heat-induced gel structures (Kuhn and Foegeding, 1991). Although the inevitable heat treatments used during processing can affect the stability and gelling properties of the proteins. A study by deWit (1990) found that within the temperature range of 70°C and 90°C, whey proteins undergo unfolding due to irreversible denaturation and that the thermal stability is affected by environmental conditions, such as pH and ionic strength. In-depth studies on the heat-induced aggregation behavior of buttermilk, as well as individual whey proteins, have been completed (Kuhn and Foegeding, 1991, Rombaut and Dewettinck, 2007). However, little is known about the thermal stability of these proteins in a whey cream buttermilk system, rich in phospholipids and devoid of caseins.

The objective of this study was to document the thermal stability of whey cream buttermilk powder prior to and following SFE treatment in order to learn about its potential applications in food products. The whey cream buttermilk was ultrafiltrated prior to drying and subsequently processed using SFE. The SFE-treated buttermilk powder (BMP) was compared to a whey protein isolate (WPI) powder. Salt as well as pH levels were adjusted in the reconstituted powders and protein agglomeration upon heating was assessed.

METHODS AND MATERIALS

Processing

Buttermilk Production. Three batches of approximately 59.0 kg of whey cream, donated by Hilmar Cheese Co. (Hilmar, CA), were churned in a pilot plant process cheese cooker, Model CC-45 (Blentech Corp, Rohnert Park, CA). Butter fines were removed from the buttermilk by filtering into milk cans through cheese cloth.

Ultrafiltration with Diafiltration. Three batches of buttermilk (total of 176.9 kg) were passed through a R12 pilot plant Niro filtration unit with diafiltration (Niro Inc., Hudson, WI) using a membrane of 10,000 MW. Briefly, the UF system contains 2 spiral pressure vessels and 1 ceramic pressure vessels. The feed streams to the recirculation loop which consists of pressure vessels, recirculation pump, heat exchanger, loop inlet, control valve, gauges, and piping. All runs were carried out at 25°C. In the diafiltration process, chilled tap water replaced the extracted permeate until reaching a diafiltration factor (DF) of 5x (884.5 kg of total permeate removed). Permeate flux (1 h^{-1} m^{-2}) was measured at fixed intervals during the experiments. Final buttermilk retentates were spray-dried using a Niro Filterlab Spray Dryer.

Supercritical Fluid Extraction. The SFE system and components were acquired from Thar Designs, Inc. (Pittsburgh, PA) and was previously described by Astaire et al. (2003). Briefly, the in-house unit includes the following: 500 mL vessel, model P-50 high-pressure pump, automated back pressure regulator model BPR-A-200B, and PolyScience brand water bath, and pump unit (model 9505). Circulated deionized water at 5°C was used for cooling different zones in the SFE apparatus. The 22.7-kg 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). Approximately 100 g of BMP was placed in inert Rapid-flow milk filter tubes (Filter Fabrics, Inc., Goshen, IN) and subjected to SFE 3x at the parameters indicated below. For each run, the running conditions were as follows: flow rate: 20 g/min; total vessel flushes: 3.014 ± 0.024; total run time: 82 ± 6 min; and total CO_2 used: 1507.22 ± 11.33 g. Following treatment, the fat in the SFE extraction vessel was drained and diluted to 10 mg/mL in chloroform-methanol (2:1, v:v) (Fisher Scientific, Tustin, CA).

BMP Composition

Total Lipid. The total fat from the SFE-treated BMP was extracted and composition was calculated by the Mojonnier Method as described by Marshall (1992). Total lipid measurements were taken in duplicate. Extracted lipids were diluted in chloroform-methanol (2:1, v:v) to 10 mg/mL for further analysis.

Nitrogen and Protein Determination. The amount of total nitrogen (TN), from the SFE-treated BMP retentates, was measured by the Kjeldahl method (AOAC, 1995). Samples were heated on a Digestion System 20, Model 1015 Digester and distilled with the Kjeldahl System, 1026 Distilling Unit (Foss, Eden Prarie, MN). FisherTab LCT-40 Kjeldahl Tablets (Fisher Scientific, Tustin, CA) were used as reagent. Titrations were done using 0.1 N HCl, and the percent protein was calculated using the milk protein conversion factor, TN x 6.38 (Sodini et al., 2006). All reagents were of Kjeldahl analysis analytical grade (Sigma Chemical Company, St. Louis, MO). Total protein measurements were made in duplicate.

Total Solids and Ash. Total solids were determined using the direct oven drying method for milk using a forced air oven at 100 ± 1°C as described by Marshall (1992). Percent ash was determined by incineration at 550°C as described by Marshall (1992). Total solids and ash measurements were taken in duplicate.

Chemicals and Reagent. All solvents and other reagents were of analytical grade, purchased from Fisher Scientific (Tustin, CA).

Application Study

Experimental Design. The product application study of SFE-treated BMP focused on heat stability at different levels of salt, concentration, and pH. A general full factorial design was employed for these experiments: calcium, magnesium, and barium were added at 2 concentrations (low, 20 mM; and high, 100 mM) under two pH conditions (3.0 and 6.8) and were heated to 80°C for a short (5 min) or long (30 min) period of time. A commercial whey protein isolate, BarFlex® WPI, 90 – 94% protein, 6% minerals, 1% lactose, <1% fat (Glanbia, Monroe, WI), was SFE treated according to the parameters above and subjected to the experimental design. Control samples of the powders at the two pH levels were not mixed with salt but heated in the same manner. SFE-treated buttermilk and WPI powders were reconstituted to 10% total solids and allowed to rehydrate for at least 12 h. Solutions were adjusted by pH using 1.0 M HCl and desired salt concentration using $CaCl_2$, $MgCl_2$, or $BaCl_2$, and were placed into an 80°C water bath. Immediately following the indicated treatment time, the solutions were placed in an ice water bath to cool.

Optical Density. Optical density measurements of the heated samples were taken with a Spectra Max Plus spectrometer (Molecular Devices, Sunnyvale, CA).

Prior to heating, each sample was diluted 1/10 with deionized water. Immediately following the heat treatment and cooling as described above, 200 μL of each solution was placed in the wells of a Whatman 96 well clear microplate (Fisher Scientific, Tustin, CA). The wavelength was set at 630 nm and absorbance was recorded.

Particle Size Distribution. Particle size distributions were determined using a laser diffraction particle size analyzer in the Polarization Intensity Differential Scattering (PIDS) optical mode of a Beckman Coulter PSA, model LS 230 (Miami, FL). Following the sample preparation and heat treatment described above, each sample was added into the analysis chamber containing deionized water, until the required obscuration (about 50%) was achieved. The run conditions were as follows: pump speed, 10%; laser power, 84%, 57.8 mA; run length, 60 s; and temperature, ambient (23 to 25˚C). Three wavelengths of light (450 nm, 600 nm, and 900 nm) at two polarizations were used for the PIDS system. Measurements were taken in duplicate.

Statistical Analysis. Statistical analyses were performed with Minitab 14.0 software (Minitab Inc., State College, PA). Data were analyzed by analysis of variance using the general linear model procedure and all comparisons by Tukey's pairwise comparison. Results were considered statistically different at $p<0.05$.

RESULTS AND DISCUSSION

Composition

Whey cream buttermilk powder composition, on a dry matter basis, is shown in Table 6.1 for the triplicate runs. There is a significant difference between the

powders before and after SFE treatment for total solids, lipids, proteins, and ash ($P < 0.05$). No significant differences were observed between each set of triplicate samples. Both protein and ash contents increased following SFE while lipid content decreased. The percent of lipids that was extracted by the SFE treatment ranged from 53 – 64% with differences between the triplicate analyses ($P = 0.005$). From previous studies and lipid profiling by thin-layer chromatography (results not shown) we know that this extraction contains nonpolar and neutral lipids, while polar lipids remain in the BMP (Arul et al., 1994, Astaire, 2003, Hierro et al., 1995).

Protein profiling (results not shown) and previous studies have shown that whey cream buttermilk is devoid of caseins (Morin et al., 2006, Sodini et al., 2006). It is observed to have a high percentage of nonprotein nitrogen and nitrogen soluble at pH 4.6 in comparison to cultured whey or sweet cream buttermilk (Sodini et al., 2006). Although caseins are not present, there is still a high portion of insoluble protein at pH 4.6, explained by the presence of denatured whey protein during processing; it also contains a nonnegligible fraction of MFGM protein (Sodini et al., 2006). Our treated BMPs were compared to a WPI powder because the protein fraction of whey buttermilk is composed primarily of whey protein, and there are already a number of studies to understand whey protein behavior and modifications to their structures upon heating (deWit, 1990, Kuhn and Foegeding, 1991, Kulmyrzaev et al., 2000, McSweeney, 2004). In addition, lipid content in WPI is low (<1%) and the SFE process should not alter composition. This allowed the observation of differences created in the whey cream BMP by decreasing nonpolar lipid concentration.

Optical Density and Particle Size Analysis

Whey proteins are susceptible to thermal denaturation, changes in globular protein structure occur at or above 50 and 80°C for α-lactalbumin and β-lactoglobulin, respectively (Hong and Creamer, 2002). Unfolding of the protein structure increases intra- and intermolecular interactions, which leads to protein aggregations and subsequent insolubility (Walstra et al., 1999). Other factors, such as pH, ionic strenth, and protein concentration during processing and preservation can affect the thiol/disulfide exchange and hydrophobic interactions that lead to aggregations (Iametti et al., 1996, Mleko, 1999). Our study attempted to show the changes in heat stability after altering pH and salt content by studying protein aggregates, determined by OD and PSA. The formation of larger aggregates reflects more light and the turbidity of protein solutions increases (Mleko, 1999). Likewise, PSA measurements can explain protein aggregation by determining mean particle size in solution (Ju and Kilara, 1998).

Optical density measurements ($\lambda = 630$) following the 2 heat treatments for the whey cream BMP are shown in Table 6.2. Significant effects were seen as a result of SFE treatment ($P = 0.009$) and pH ($P = 0.003$). The interaction between SFE and pH also showed a significant effect on OD ($P = 0.001$). At pH 3.0, OD decreased in value after the SFE treatment for all samples. The control solution, with no added salt, also had an observed decrease in value following SFE treatment. At pH 6.8, OD decreased following SFE treatment, with the exception of a few samples. After a 5-min heat treatment, an increase in OD was observed for 20 mM calcium, 20 mM magnesium, and 100 mM calcium. After a 30 min heat treatment, an increase in OD was observed for 20 mM and 100 mM Barium, 20 mM magnesium, and 100 mM calcium.

According to OD results, whey cream BMP had greater heat stability following SFE treatment; large aggregates are less likely to be formed. The difference in heat stability was greater at pH 3.0 than at 6.8.

Changes in heat stability, when observing mean particle size (μm), showed that a number of variables had a significant effect (Table 6.3). Not only did SFE ($P = 0.000$), pH ($P = 0.000$), and the interaction between the two ($P = 0.000$) show differences, but type of salt ($P = 0.019$) and time of treatment ($P = 0.000$) as well. Salt concentration had an effect on particle size, but only as an interaction with pH ($P = 0.005$). At an acidic pH, the SFE-treated powders had a decreased particle size following a 5-min heat treatment; however, after 30 min, particle size increased. The control powders, as well as 100 mM magnesium, followed the same trend at 5 min but not at 30 min. At pH 6.8, particle size increased following both a short and long heat treatment for all powders including the control. Although the powders showed differences, the change in mean particle size was small (i.e., 14.02 to 15.68 μm). The largest particle size difference was observed for the solution at pH 6.8 with 100 mM calcium heated for 5 min (9.96 to 19.91 μm).

For heat stability comparisons, measurements for WPI are shown in Table 6.4. Results show that pH had a significant effect on the thermal stability of the proteins ($P = 0.000$). Although it was observed that OD decreased following SFE treatment for the samples at pH 6.8, SFE treatment or the interaction between SFE and pH were not significant effects ($P = 0.066$ and 0.057, respectively). Our data show that WPI aggregation was not significantly affected by the presence, concentration, or type of divalent cation added. These results have been shown previously (Corredig and

Dalgleish, 1996, Kuhn and Foegeding, 1991, Onwulata et al., 2003). OD results showed a drastic variation from pH 3.0 to 6.8 (namely, 0.2553 to 2.0432, respectively). The WPI samples showed a greater difference in mean particle size between the treatment variables (Table 6.5). It was observed that pH, type of salt added, and heat treatment time all had an effect (P = 0.000, 0.002, and 0.000, respectively). However, whether or not the powder was SFE-treated did not have an effect (P = 0.799). In general, at pH 3.0, heat treatment time was observed to have the greatest effect. Mean particle size was increased for all samples, including the control, from 5 min to 30 min; type or concentration of salt added did not appear to show a general trend. Similar to OD results, at the lower pH, particle size was small, even after a short heat treatment (0.1135 μm) and increased more than 10-fold after a 30-min heating step (1.708 μm). At pH 6.8, it was observed that heat serves to further increase particle size (32.22 to 109.4 μm). The OD and PSA support previous research on the effect of pH on whey protein aggregation (Xiong, 1992). Preheating whey protein solutions exposes the sulfhydryl groups and disulfide bonds of β-lactoglobulin, leading to the formation of soluble aggregates; however, pH can alter aggregate formation. At a low pH, β-lactoglobulin has increased thermostability, explained by the strong action of the disulfide bonds in its tertiary structure. The reactivity of the thiol group increases above pH 6, by deprotonation of the groups and as a consequence increasing the probability of disulfide bond formation (deWit and Klarenbeek, 1984).

In comparison to WPI and SFE-treated WPI, we have shown that SFE-treated whey cream BMP behaves differently upon thermal treatment; protein aggregation of

whey cream BMP is not as drastic under intense heat. It is apparent that, without taking salt into consideration, the SFE process enhances thermal stability by reducing aggregation of the solutions. This may be explained by phospholipid-protein interactions within the system. A study by McCrae (1999) showed that the addition of lecithin to fresh whole milk increased heat stability by interaction with membrane proteins, preventing coagulation before sterilization. Because the SFE treatment serves to reduce the nonpolar lipids from the powder and enrich the phospholipid fraction, a type of "shielding" from protein-protein aggregation may be taking place in the whey cream BMP samples, at both acidic and neutral pH. Previous investigations into the interaction of whey proteins in the presence of sugars have shown that sugars can increase the thermal denaturation temperature of globular proteins and WPI, mainly by increasing the surface free energy between oil and water (Kulmyrzaev et al., 2000, Taiwo et al., 1996). When the surface free energy increases, protein unfolding is opposed and thermal stability is enhanced (Kulmyrzaev et al., 2000). Having both lipophilic and hydrophilic properties, phospholipids associate at the oil-water interface and, like sugars, can increase the surface free energy and, therefore, increase the thermal denaturation temperature to retard aggregation.

Other studies have shown that altering salt concentration is an effective way to adjust whey protein aggregations. In relation to WPI gelation and modification of rheological properties, including the gel structure, firmness, and water-holding capacity, Kuhn and Foegeding (1991) showed that divalent salts, at lower concentrations than monovalent salts, increase the hardness of WPI gels by promoting aggregation. By observing mean particle size of the whey cream BMP samples, at pH

6.8, the addition of salts does not appear to promote protein aggregation, but rather hinders it when comparing with the control. However, at a lower pH, the addition of divalent salts may help somewhat in thermal stability but only for a short period (5 min). We have also shown that not all divalent salts behave in the same manner with respect to heat stability.

CONCLUSIONS

Whey cream BMP has a unique composition, which provides interesting functional characteristics. We believe that we have developed a product by membrane filtration and SFE treatment that contains not only an increased amount of high-value whey proteins and bioactive MFGM lipids, but has unique thermal stability profiles as well. Potential product application is not limited by heat treatment, acidic conditions, or added divalent salts. Because it has little to no casein proteins present, its heat stability would exceed that of buttermilk and other milk products which contain casein proteins. Future studies of SFE-treated whey cream BMP may indicate this is a novel ingredient, giving rise to new products with added health benefits.

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Table 6.1. Composition of whey cream buttermilk powder (WBMP) prior to and following SFE treatment (WBMP-SFE); total solids, total ash (% Dry Matter), total protein (% DM), total lipid (% DM) and % lipid extracted.

	Total Ash (% DM)	Total Protein (%DM)	Total Lipid (% DM)	% Lipid Extracted
WBMP 1	2.29 ± 0.06^{a}	44.45 ± 0.16^{a}	49.80 ± 0.54^{a}	
WBMP 2	2.16 ± 0.01^{a}	51.11 ± 0.04^{a}	49.43 ± 0.20^{a}	
WBMP 3	2.44 ± 0.06^{a}	43.23 ± 0.93^{a}	42.54 ± 0.19^{a}	
WBMP-SFE 1	3.33 ± 0.40^{b}	65.96 ± 0.27^{b}	23.16 ± 0.30^{b}	53.49 ± 1.12^{a}
WBMP-SFE 2	3.08 ± 0.15^{b}	66.01 ± 0.35^{b}	19.30 ± 0.25^{b}	60.95 ± 1.45^{b}
WBMP-SFE 3	3.21 ± 0.15^{b}	71.95 ± 0.24^{b}	15.17 ± 0.36^{b}	64.33 ± 0.68^{b}

± indicates standard deviation

^{a,b}values in same column with different superscripts differ significantly ($p<0.05$).

Table 6.2. Mean optical density measurements at pH 3.0 and 6.8 for the whey cream buttermilk powder samples following the 80°C heat treatment for 5 min and 30 min.

pH 3.0		Salt						
		Barium		Calcium		Magnesium		Control
		20mM	100mM	20mM	100mM	20mM	100mM	
Before SFE	5min	0.7132	0.7165	0.8198	0.7414	0.7515	0.7795	0.7372
	30min	0.8076	0.6744	0.7357	0.7145	0.7307	0.6901	1.0214
After SFE	5min	0.5944	0.5333	0.3947	0.4724	0.6434	0.6246	0.4380
	30min	0.5334	0.6154	0.6702	0.5979	0.4319	0.5962	0.5790
pH 6.8								
Before SFE	5min	0.7607	0.6982	0.7741	0.7018	0.7377	0.6456	0.9140
	30min	0.7986	0.6965	0.8194	0.7063	0.7041	0.6599	0.9866
After SFE	5min	0.5839	0.6391	0.9641	0.7625	0.8301	0.6240	0.5278
	30min	0.8033	0.7648	0.7311	0.8008	0.9873	0.5995	0.7558

Table 6.3. Mean particle size (μm) for the whey cream buttermilk powder samples at pH 3.0 and 6.8 following the 80°C heat treatment for 5 min and 30 min.

pH 3.0		Salt						
		Barium		Calcium		Magnesium		Control
		20mM	100mM	20mM	100mM	20mM	100mM	
Before SFE	5min	16.81	18.95	22.90	17.77	14.86	16.01	20.41
	30min	16.24	19.93	19.49	15.33	17.5	18.70	18.22
After SFE	5min	15.32	11.98	18.52	14.37	14.38	12.23	18.69
	30min	20.35	20.20	24.14	20.19	20.90	18.26	17.07
pH 6.8								
Before SFE	5min	14.02	9.16	15.42	9.96	13.60	12.83	15.34
	30min	14.24	15.00	13.62	17.17	15.69	17.69	20.89
After SFE	5min	15.68	17.02	17.39	19.91	15.54	19.80	21.22
	30min	19.06	19.74	16.61	19.60	18.32	19.21	21.64

Table 6.4. Mean optical density measurements for the whey protein isolate samples at pH 3.0 and 6.8 following the 80°C heat treatment for 5 min and 30 min.

pH 3.0		Salt						
		Barium		Calcium		Magnesium		Control
		20mM	100mM	20mM	100mM	20mM	100mM	
Before SFE	5min	0.2553	0.2829	0.2510	0.2830	0.2663	0.2850	0.2745
	30min	0.2633	0.2618	0.2701	0.2819	0.2566	0.5567	0.2642
After SFE	5min	0.2723	0.2758	0.2680	0.2865	0.2649	0.3039	0.2772
	30min	0.2650	0.3074	0.2728	0.3821	0.2602	0.4412	0.2727
pH 6.8								
Before SFE	5min	2.0432	2.0754	2.0605	2.0509	2.0549	2.0186	1.9779
	30min	2.0546	1.0182	1.9233	2.0602	2.0136	1.0255	1.7974
After SFE	5min	1.4431	1.2929	1.8257	1.3012	1.4003	1.1422	1.5665
	30min	1.5429	1.4553	1.5844	1.6070	1.5178	1.3102	1.2210

Table 6.5. Mean particle size (μm) for the whey protein isolate samples at pH 3.0 and 6.8 following the 80°C heat treatment for 5 min and 30 min.

pH 3.0		Salt						
		Barium		Calcium		Magnesium		Control
		20mM	100mM	20mM	100mM	20mM	100mM	
Before SFE	5min	0.1135	0.1355	0.1255	0.1500	0.1195	1.175	0.1195
	30min	1.708	2.304	2.007	1.391	1.411	4.955	1.246
After SFE	5min	0.1773	0.2842	0.2508	0.2965	0.2508	0.1201	0.1150
	30min	7.491	8.325	6.031	12.19	6.448	2.275	8.452
pH 6.8								
Before SFE	5min	32.22	92.20	113.0	68.90	26.90	52.29	9.855
	30min	109.4	567.5	746.8	323.4	416.3	212.6	18.65
After SFE	5min	49.72	111.9	152.1	105.9	147.0	55.49	8.452
	30min	153.5	589.6	746.8	88.79	392.2	123.0	25.82

General Conclusion

Bioactive sphingolipids along with other phospholipids are present in the milk fat globule membrane, a major component in buttermilk. These interesting lipids are known to have essential roles in numerous cell functions, including antioxidative, anticarcinogenic, and antiatherogenic properties. We have explored and analyzed the possibility of utilizing a process that couples filtration methods with supercritical fluid extraction (SFE) as a method of enriching these nutritionally valuable lipids into a novel ingredient.

We have optimized the SFE temperature and pressure parameters to ensure adequate lipid removal of nonpolar lipids from the powder without major disruption of other components. Experimental values showed non-linearity of the process parameters in contrast to theoretical calculations; it was also determined that pressure values have a greater effect on extraction efficiency. The optimized parameters limit browning and lactosylation of the powder and powder functionality, determined by protein solubility, was not lost.

Lipid extraction efficiency was improved by the addition of a physical supplement to the powder during the SFE process. Biosilicate material improves lipid removal greatly however leaves the product inconsumable. Although the addition of a physical aid, such as small, removable Teflon® beads resolves this problem, lipid removal effectiveness is hindered. Using a bed fluidizer in conjunction with the SFE creates a constant flowing space between the powder particles, enabling greater lipid extraction. Mixing of the powder in intervals during the treatment served to mimic a

fluidized bed and demonstrated that it may be an ideal alternative for an edible product.

We have compared the SFE treatment on two different sources of buttermilk powder passed through two different filtration modes prior to drying and have shown that SFE coupled with microfiltration processing helps to enrich the milk fat globule membrane components in buttermilk powder created from both whey cream and regular cream. Phospholipid concentrations were shown to increase considerably with a SFE treatment following both a volumetric concentration and a diafiltration process. Chemical composition of the buttermilk powders showed that other constituents (i.e. protein, ash) were not altered to a great degree by the SFE process but rather by the preceding filtration process. We have also characterized the specific molecular fatty acid combinations of sphingomyelin for all of the buttermilk powders, showing the unique composition of SFE treated buttermilk powder.

Lastly, the thermal stability of SFE treated whey cream buttermilk powder was observed in order to study its potential application into food products. Results showed that the SFE process enhances thermal stability by reducing protein aggregation, and its unique thermal profiles indicate that potential product application is not limited by heat treatment, acidic conditions or added divalent salts. Therefore, membrane processed and SFE treated whey cream buttermilk powder is a product with interesting functionality and composition.

Future studies of SFE treated buttermilk powder and whey cream buttermilk powder may indicate this is a novel ingredient - giving rise to new products with unique functionality and components. Although further knowledge into the metabolic

effects and mechanisms of these products is needed, we believe combining these processes has revealed a product with enriched components that are indicative to having an increase benefit to ones health.

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