MICROFILTRATION AND SUPERCRITICAL FLUID EXTRACTION OF BUTTERMILK TO CONCENTRATE BIOLOGICAL LIPID MESSENGERS

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ABSTRACT

Microfiltration and Supercritical Fluid Extraction Fractionate

Buttermilk to Concentrate Biological Lipid Messengers

by

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Buttermilk contains the milk fat globule membrane (MFGM), a material that possesses several lipids that function as intracellular signaling molecules. Certain sphingolipids contained therein influence cellular apoptotic pathways, and affect numerous other imperative functions. Their anti-cancer effects make them good candidates as potential health-enhancing therapeutics, or as part of an anti-cancer regimen. Concentration of these lipids using conventional methods utilizes toxic solvents, rendering the product hazardous to organisms or cells. We developed a method to increase the purity of the MFGM phospholipids by fractionating reconstituted buttermilk using a two-step processing schematic. Firstly, a crossflow microfiltration process using a ceramic tubular membrane with a 0.8 micron pore size concentrated the total lipid material in the buttermilk. Secondly, a supercritical fluid extraction (SFE) process using supercritical carbon dioxide (SC-CO_2) removed exclusively nonpolar lipid material from the microfiltered buttermilk; the final product retained all of the polar MFGM lipids in increased concentrations. Through reaching pressure and temperature conditions above a substance's critical points, a supercritical state is achieved. Here, the substance possesses a gas-like viscosity, and behaves as a strong solvent easily penetrating complex matrices,

and solublizing selective compounds within. Optimum conditions of the extraction process were 375 bar, 55-60^{o}C, and a flow rate of 20 g/min for 3 successive runs of 75 min. Quantitative lipid profiling through both processing steps was done by TLC using the following solvent systems: petroleum ether-ethyl ether-acetic acid (85:15:2, v:v) to analyze nonpolar lipids, and chloroform-methanol-water (65:25:4, v:v) to analyze polar lipids. Standards were used to verify lipids. Particle size analysis revealed shifts in the distribution of the particle sizes. By using microfiltration and SFE to fractionate the lipids in reconstituted buttermilk, we produced an edible fraction containing an increased concentration of the polar lipids of the MFGM without utilizing toxic solvents.

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Chapter 1.0.

Introduction and Background

Sphingolipids are of interest not only because they help define the structural properties of membranes and lipoproteins, but they also function as intracellular signaling molecules in a huge variety of biological functions, including the regulation of cell growth, development, adhesion, cross membrane trafficking, roles in aging and age-related diseases, stress responses, and apoptosis (Hidari et al., 1996; Kim et al., 1991 Cutler and Mattson, 2001). Their roles in apoptotic pathways cause speculation that they may be beneficial dietary supplements or anticancer agents (Parodi, 1997). The sphingolipids sphingomyelin (SM), sphingosine (S), sphingosine 1-phosphate, ceramide, and ceramide 1-phosphate, which are metabolites of one another, are of particular interest for their roles as lipid messengers (Bajjalieh and Batchelor, 1999; Chatterjee and Ghosh, 1991; Huwiler et al., 2000).

Unlike the glycerol-containing lipids, all sphingolipids are composed of a sphingosine backbone. SM represents a phosphorus-containing subclass of sphingolipids, and together with phosphatidylcholines (PCs) comprises more than half of all phospholipids (PLs) (Cutler and Mattson, 2001; Garret and Grisham, 1995). SM is commonly found in the cell membrane, brain and neural tissue, retina, and within some genera of microbes (Batrakov et al., 2000; Huwiler et al., 2000; Ilangumaran, 1997). However, buttermilk contains the MFGM, a material that contains comparatively large amounts of several phospholipids involved in intracellular signaling. The MFGM contains proteins (25-60%), glycoproteins, carbohydrates, triacylglycerides (TAGs), and PLs (including SM, PC, and phosphatidylethanolamine (PE)) (Deeth, 2000;

Goudedranche et al., 2000; Lambertsen and Christiansen, 1997; Mather, 2000; Walstra et al., 1999; Ye et al., 2002). Buttermilk in particular contains a high percentage of PLs compared to regular milk, 0.13% versus 0.035% respectively (Walstra et al., 1990). Furthermore, SM represents about one third of its total PLs (Parodi, 1997).

Using buttermilk as a source for these bioactive lipids is a sound alternative considering its unique properties as a functional ingredient, its low cost, and availability (Fryksdale, 2000; Walstra et al., 1990). Compared to other abundant sources of these lipids, buttermilk is both easily accessible, and available in large quantities from butter-producing dairies as a waste product. When cream is mechanically churned during butter production, the fat globules rupture, releasing the fat as butter, and the membranous MFGM sac is left in the aqueous buttermilk. In the U.S., the average monthly production of butter is about 110.2 million pounds; the monthly production of buttermilk is 4.4-5.5 million pounds (USDA, 2001). Although there are numerous conventional methods to isolate these types of lipids (Christie, 1982), due to their reliance on toxic solvents their application would render the products unsafe for consumption, or for use in live cell research. Since our goals include producing a product that is nondamaging to organisms and cells, our main challenge in achieving this isolation is avoiding the use of toxic solvents.

Microfiltration and SFE allow for the isolation and purification of the MFGM lipids from buttermilk without using conventional solvents. Microfiltration is an indispensable tool for the fractionation of various dairy components. It has been used to concentrate and purify dilute solutions of macromolecules, such as proteins, and lipids. Particularly in the dairy industry, the concentration of lipids, caseins, and whey proteins

has responded well to microfiltration systems (Cheryan, 1998; Samuelesson, 1997). Designing a microfiltration system to fractionate buttermilk offers a chemical-free method to increase the concentration of the MFGM material in the retentate fraction. The success of this process depends on numerous factors, an important one being using a membrane pore size that would allow the permeation of various proteins, lactose, and minerals, and the retention of the MFGM. Because this system separates primarily based on particle size, other components would be retained, such as triacylglycerides (TAGs), and other nonpolar lipids, complexes of proteins and fats, and other protein complexes.

SFE also offers an environmentally benign alternative to other lipid extraction procedures. This process uses the unique properties exhibited by substances at the supercritical phase to easily penetrate a wide range of samples, solublize specific components therein. Carbon dioxide is a common solvent (including extractions of nonpolar lipids) due primarily to its low critical parameters (31.1°C, 73.8 bar), low cost, non-toxicity, chemical inertness, and non-flammability (Hauthal, 2001; Rozzi and Singh, 2000; Turner et al., 2001). By using SC-CO_2 to remove nonpolar lipids from the buttermilk retentate fraction, it would become more concentrated in the MFGM lipids, and be safe for consumption or live cell research. Because buttermilk is a complex system, the complete isolation of MFGM using microfiltration and SFE is an unrealistic goal. Furthermore, complexes between proteins, other lipids, and the MFGM material may in fact lend unique characterists to the product not offered by other sources.

It is poorly understood whether dietary sphingomyelin and the absorption of its metabolites influences cell signaling pathways. Though there is no nutritional requirement for sphingolipids, emerging from research is a vast array of evidence

strongly suggesting this relationship. Dietary manipulations designed to reduce or increase specific sphingolipid signaling cascades may lead to an increased control we can claim over our health.

Chapter 2.0

Literature Review

2.1. Sphingolipids and Cell Signaling

Sphingolipids are a broad and diverse class of molecules. They are of interest not only because they help define the structural properties of membranes and lipoproteins, but they also participate in a huge variety of biological functions including cell growth and development, adhesion, cross membrane trafficking, aging, and signaling processes including stress responses, apoptosis (cell death) (Cutler and Mattson, 2001; Dreyfus et al., 1997; Hidari et al., 1996; Ilangumaran, 1997; Kim et al., 1999). Their roles in cellular apoptotic pathways give scientists reason to speculate that they have potential uses as anticancer agents, and possibly may be a beneficial dietary supplement for cancer preventative purposes (Parodi, 1997). Many of their roles in activities such as these are not thoroughly understood or described, and still a broad area of research, in particular regarding their roles in as intracellular signaling molecules. The sphingolipids sphingomyelin, sphingosine, sphingosine 1-phosphate, ceramide, and ceramide 1-phosphate are of particular interest for their signaling activities, and are the lipids of focus in this review. The diverse functions of these lipids greatly depend on their structures, which can vary in polar head group components, fatty acid components, and in their associations with other molecules, such as glycoproteins (Sullards, 2000).

All sphingolipids are composed of a sphingosine backbone; the molecular structure of which is shown in Figure 2.1.1. This component distinguishes sphingolipids from other glycerol-containing lipids, such as triacylglycerides consumed in our diets.

An amide linkage between a fatty acid and sphingosine results in the formation of ceramide; the naturally-occurring structure of which is shown in Figure 2.1.2 (Garret and Grisham, 1995). Sphingomyelins are a phosphorus-containing subclass of sphingolipids; together with phosphatidylcholines comprise more than half of all phospholipids (Cutler and Mattson, 2001). They are formed by the esterification of a phosphocholine head group to the 1-hydroxy group of ceramide; this action is carried out by the enzyme sphingomyelin synthase. There are two stereoisomers of sphingomyelin, the naturally occurring form is *D-erythro*-(2S3R), and is shown in Figure 2.1.3. It has been separated from the *L-threo*-(2S3R) form of sphingomyelin through normal-phase HPLC, which results from the semi-synthetic preparations of sphingomyelin (Ramstedt and Slotte, 2000).

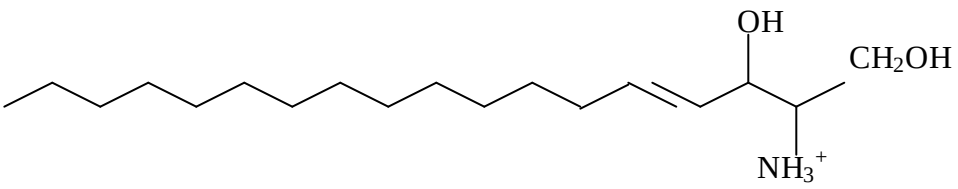


Figure 2.1.1. The molecular structure of sphingosine, which forms the backbone of all sphingolipids (Garret and Grisham, 1995).

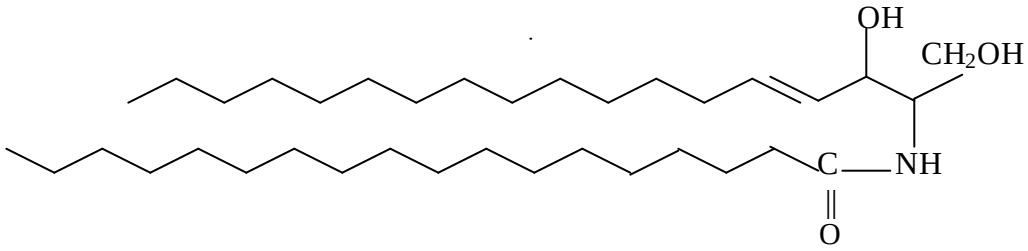


Figure 2.1.2. The molecular structure of ceramide in its naturally occurring form, *D-erythro*-(2S,3R) (Kim et al., 1991). The R chain of the fatty acid linked to the carboxamide group can vary in length. Note the sphingosine component.

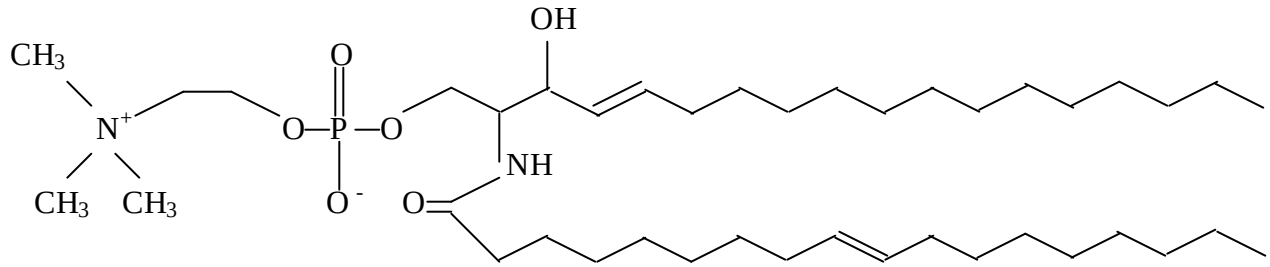


Figure 2.1.3. The molecular structure of the naturally-occurring form of sphingomyelin, *D-erythro-N*-18:1-(2S3R) (Ramstedt and Slotte, 2000). The phosphocholine headgroup is esterified to the ceramide component.

In animals sphingolipids are found in cell membranes, in particularly large concentrations in brain and neural tissues, and retina (Cutler and Mattson, 2001; Dreyfus et al., 1997; Horne and Holt-Larkin, 1997). In the cell membrane, glycosylphosphatidylinositol (GPI)-anchored glycoproteins are embedded glycoproteins that are physically connected to GPI molecules in the cell membrane. These glycoproteins are found in areas of the membrane that are rich in sphingolipids, and also are known to play important roles in interactions between adjacent cells, and other extracellular substrates. The association between the GPI-acyl chains and the sphingolipids themselves is what gives these glycoproteins their physical characteristics, thus influencing how the cell interacts with neighboring cell-surface molecules and other substrates. This was investigated in a mutant cell line derived from mouse melanoma cells. It was found that the complete removal of sphingolipids from the plasma membrane caused defects in mechanisms of cell-substratum adhesion. The presence of either glycosphingolipids (glycosylated sphingolipids) or sphingomyelin was sufficient for normal cell adhesion to occur (Ilangumaran, 1997). Although the presence of glycosphingolipids on the plasma membrane is not essential to the survival of cells

growing in culture, they take part in cell type-specific adhesion processes. It is known that they serve as specific binding sites for extracellular molecules, such as bacteria, toxins, and viruses, and are thought to interact with other cellular receptors and enzyme domains as well (Huwiler et al., 2000). Besides sphingomyelin having a T_m of 37°C (near body temperature), it has a large difference between the long, highly saturated hydrocarbon chains; these properties allow for integration within the phospholipid bilayer of the cell membrane (Cutler and Mattson, 2001).

Generally, sphingolipids are only typical of the eykaryotic cell and organisms, not found within the prokaryotic cell; however, some bacterial taxa contain these lipids in relatively high concentrations, and can even synthesize sphingolipids. These unique characteristics have served as the basis for grouping them into two individual genera, *Sphingobacterium*, and *Sphingomonas.* In some strict anaerobe species, sphingolipids have been shown to compose up to 70% of the total extractable lipid. It was also found that of the total extractable lipid in the Gram-negative bacterium *Flectobacillus major*, 90% was found to be polar in nature (Batrakov et al., 2000).

Only within the past 30 years has research implicated that sphingomyelins and other membrane phospholipids served prominent roles in intracellular signal transduction. In response to a variety of stimuli, the cleavage of one or more phospholipids release biologically active cellular messengers. For example, sphingomyelin is enzymatically hydrolyzed by the sphingomyelinase enzymes releasing ceramide, and phosphocoline. This is the first step leading to a cascade of reactions ultimately affecting numerous cell-signaling pathways, including those regulating tissue development, cell growth and differerentiation, and oncogenesis (Hidari et al., 1996; Kim et al., 1991). One such

pathway has been identified as the "Sphingomyelin Cycle," and was demonstrated by Okazaki et al. (1989) in HL-60 human myelocytic leukemia cells. In this study it was found that vitamin D_3 specifically activated the enzymatic hydrolysis of sphingomyelin to ceramide and phosphorylcoline by sphingomyelinase. Ceramide, which is known to be involved in numerous cell-signaling pathways, is phosphorylated by ceramide kinase, producing the lipid ceramide 1-Phosphate; this lipid is also hypothesized to act as a unique intracellular signal. When ceramide is enzymatically hydrolyzed by ceramidase, sphingosine results. The phosphorylation of sphingosine by sphingosine kinase produces sphingosine 1-Phosphate. Both sphingosine and sphingosine 1-Phosphate are involved in intracellular signaling pathways (Chatterjee and Ghosh, 1991; Bajjalieh and Batchelor, 1999; Huwiler et al., 2000). After induced activation, this cycle peaked and returned to normal activity levels within four hours, during which time the effects of the cytokines tumor necrosis factor-alpha (TNF-alpha) and gamma-interferon (gamma-INF), were mediated (Kim et al., 1991; Okazaki et. al, 1989). Figure 2.1.4 demonstrates this sphingolipid cascade, producing metabolites that lead to numerous cell-signaling pathways.

Some final results of intracellular signaling induced by the metabolites involved in this particular cascade include the activation and regulation of specific protein phosphatases; protein kinases, including the inhibition of protein kinase C; and the activation of protein kinase A and tyrosine kinase; transcription factors; cellular growth and development; and apoptosis (Bajjalieh and Batchelor, 1999; Chatterjee and Ghosh, 1991, Gavrilova and Petkova, 1995).

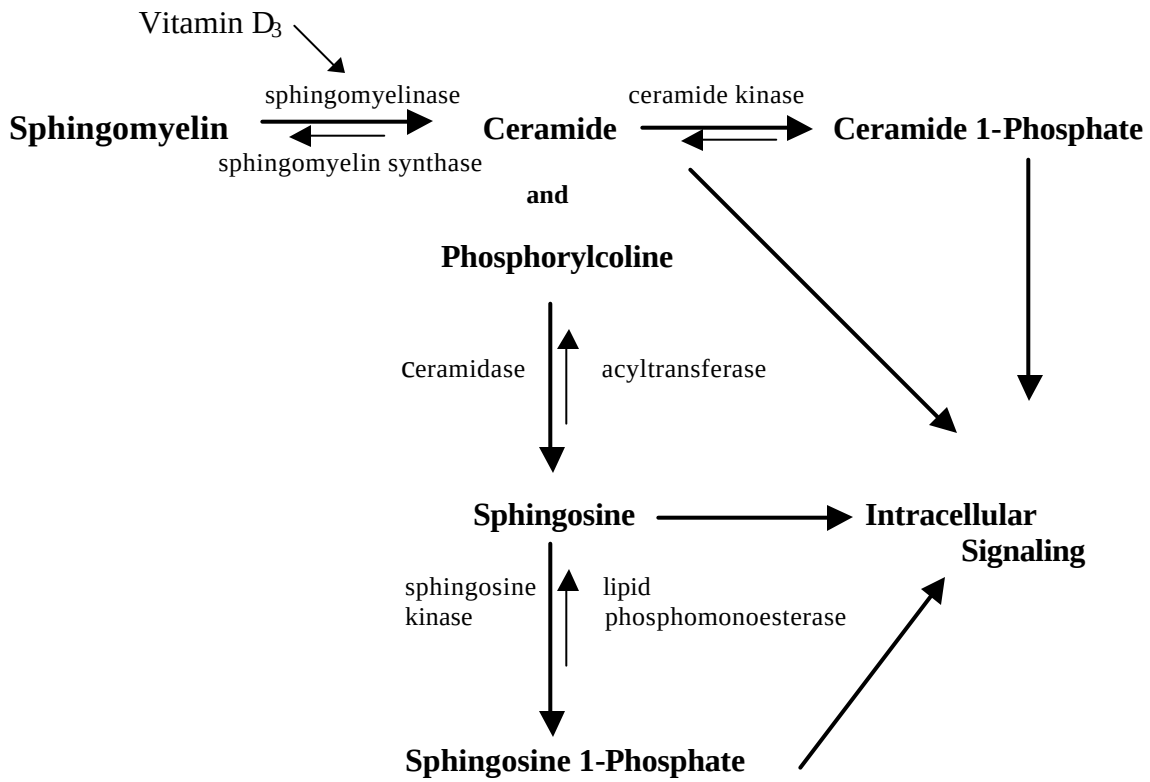


Figure 2.1.4. The sphingolipid signaling cascade involving the generation of many metabolites, which are involved in regulating cell function through participation in intracellular signaling.

There are at least five different types of sphingomyelinases identified so far; among these, acid sphingomyelinase and neutral sphingomyelinase have been investigated more thoroughly for their roles in cellular metabolism. Membrane-bound neutral sphingomyelinase is the enzyme acting in the sphingomyelin cycle discussed above, responsible for the formation of the lipid messengers ceramide, sphingosine, and their phosphorylated forms (Chatterjee and Ghosh, 1991; Bajjalieh and Batchelor, 1999). Signals that activate sphingomyelinases range from growth factors and cytokines, to neurotransmitters, hormones, and reactive oxygen species. Depending on the

concentrations, sphingomyelin and ceramide can stimulate cell proliferation and survival, or induce cell dysfunction and death (Cutler and Mattson, 2001).

Apoptosis is a form of programmed cell death that takes place when an external or internal stimuli causes the cell to enter a pathway resulting in its own demise. Apoptosis is thought to play a vital role in numerous biological and pathological conditions, including cell and tissue development, cancer, ischemia and infarction, immune disorders, removal of unwanted cells, and neurodegenerative diseases. Defective apoptosis can contribute to serious disorders, such as cancer pathogenesis and autoimmune disease. As cell biology advanced, it became understood that apoptosis is a biochemically regulated process, with many cellular components involved in regulating the process; sphingolipid metabolites are thought to be key regulators of apoptosis (Perry et al., 1996).

Further research on the signaling pathways stimulated by the metabolites in the sphingomyelin cycle revealed that sphingomyelin signaling is associated with cellular stress response. In response to stimuli such as physical trauma, infection, atherosclerosis, ischemia, radiation, heat shock, and serum withdrawal, an increase in ceramide production is observed in both cells that survive, or perish. While low levels of ceramide accumulation can provide resistance to the cell, higher levels can induce death (Cutler and Mattson, 2001). As a natural defense to these sorts of stress, specific protein kinases are activated to aid in survival. TNF-alpha (a well known inducer of cellular apoptosis, as well as an inducer of neutral sphingomyelinase activity) triggered the generation of ceramide from sphingomyelin through sphingomyelinase. This specific growth arrest pathway is mediated by the retinoblastoma (Rb) gene product. Alternatively, ceramide

can act through a protein phosphatase, or the stress activated protein kinases to induce cell death (Gavrilova and Petkova, 1995; Perry et al., 1996; Sawai and Hannun, 2000).

In efforts to directly tie the activation of neutral sphingomyelinase activity to TNF-alpha initiated apoptosis a study was done which utilized an antibody against neutral sphingomyelinase in HL-60 cells. The presence of this antibody completely inhibited TNF-alpha induced neutral sphingomyelinase activity, yet did not completely inhibit TNF-alpha induced apoptosis. From these results, it was interpreted that the inhibition of neutral sphingomyelinase activity by the antibody affected specific pathways involved in TNF-alpha induced cellular apoptosis, but the final fatality of the cell involves other contributing factors. Some other contributing factors are speculated to be other isoforms of sphingomyelinase, cytosolic neutral sphingomyelinase, or perhaps acid sphingomyelinase (Chatterjee, 1999). It is not fully understood whether ceramide is the cause of other recognized cell responses to stress, but knowing the significance it holds as a cell-signaling molecule gives ample reason to suspect it helps the cell to regulate stress responses. More research in this area is further investigating these relationships.

The induction of cellular apoptosis by sphingomyelin derivatives was studied in human prostate cancer cells as well. It was suggested from this study that the relative levels of sphingolipid metabolites, ceramide, sphingosine, and sphingosine 1-Phosphate, might play a role in determining the radiosensitivity of prostate cancer cells to ionizing gamma-irradiation. The human prostate cancer cells used in this study were resistant to ionizing gamma-irradiation. It was found that increased amounts of the above-mentioned sphingolipid metabolites increased the susceptibility of these previously resistant cells to

radiation treatment by inducing apoptosis. This may be of particular therapeutic value to the treatment regimes of cancer patients. Cancer cells frequently become resistant to radiation or chemotherapy, and do not exhibit normal pathways leading to cell apoptosis (Nava et al., 2000). These findings were further supported by similar research by Modrak et al. (2002), who found that with increased intracellular sphingomyelin levels there was an increased sensitivity to chemotherapeutics in colonic tumor xenografts. If the presence of these lipid messengers can increase the susceptibility of cancer cells to treatment, then treatments that otherwise may be relatively ineffective or detrimental to other aspects of health may prove to be increasingly beneficial in battling cancer.

It has been proposed that membrane-bound neutral sphingomyelinase may contribute to the rupture of plaques in advanced atherosclerosis. The aortic smooth muscle seen with atherosclerosis compared with a normal vascular wall experiences proliferation at a higher rate due to increased amounts of growth factors. Over time, this results in plaque formation, which can rupture and cause death. It has been found that neutral sphingomyelinase activity is elevated in the atherosclerotic plaque, causing scientists to speculate that it may be a contributing factor in plaque rupture through the instigation or apoptosis, as witnessed in other systems (Chattergee, 1999). As scientists progressively discover more of apoptosis induction by neutral sphingomyelinase, the connections between these signaling pathways and their applications to human health will undoubtedly provide new venues towards battling premature plaque rupture in advanced atherosclerosis.

It has also been suggested that sphingomyelin and its metabolites are also associated with controlling rates of development and aging, and life span. This was

investigated in liver cells. It is known that as the liver naturally ages, its ability to metabolize drugs and secrete important proteins in response to stimuli is decreased. In a study using Fisher 344 rats of 24 months and five months of age, levels of ceramide and sphingosine were shown to be elevated in the livers of the older rats. Enzyme analysis showed that in the older rats activities of both acid and neutral sphingomyelinases were elevated, while sphingomyelin synthase activity was lowered. These observations showed that the aging of liver cells was accompanied by an increase in sphingomyelin turnover, and a concurrent decrease in its synthesis (Lightle et al., 2000). It has also been hypothesized that as aging progresses, sphingolipids accumulate to unstable levels due to long-chain sphingolipids (particularly ceramides with 18 Carbons and higher) that cannot autonomously transport themselves across cell membranes due to their physicality. This accumulation is implicated to be involved in the regulation of development, aging rate, and the onset of age-related diseases (Cutler and Mattson, 2001).

The proper functioning of sphingolipid metabolism is of great importance to human medical health, as defects can lead to serious diseases known as sphingolipidoses. Sphingolipidoses are diseases caused by defects in specific enzymes involved in the hydrolysis of sphingolipids; two examples of ten different types of this disease are Niemann-Pick disease, and Farber disease. Neimann-Pick disease is a recessively inherited genetic defect that results in deficient amounts of the enzyme human acid sphingomyelinase. Human acid sphingomelinase is a lysosomal enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Without this enzyme, sphingomyelin accumulates to very high levels in lysosomes, which causes serious problems (Lansmann et al., 1999). Those born with this disease usually die early in life,

and rarely live to their teen years. The abundance of sphingomyelin causes enlargement of the liver and spleen, inability to control motor functions, a loss in vision and hearing, the inability to mobilize cholesterol, and other serious neurological disorders (Fujiwaki, et al., 1999).

Farber disease is another type of recessively inherited sphingolipidosis, but the enzymatic defect results in deficiencies of lysosomal acid ceramidase, which hydrolyzes ceramide to sphingosine and free fatty acids. Without the presence of ceramidase, ceramide accumulates to abnormal levels, as it is unable to be hydrolyzed to its components, sphingosine and fatty acids. In this disease, also called lipogranulomatosis, there is painful arthritis, subcutaneous nodules, a hoarse voice and a progressive course, usually with early death, although a few patients survive to their teens. There is usually progressive psychomotor deterioration as well (Fujiwaki, et al., 1999).

Proper diagnosis of sphingolipidoses has been challenging as it is dependent upon the identification of clinical features, which may vary from patient to patient. Techniques used to assess these characteristics have been inspection of light and electron microscopy showing lipid accumulation in bone marrow cells, or rectal mucosa; detecting abnormalities in lipid analysis of urine, and tissues; and detecting defective enzymatic activity in cultured cells. In addition to this, analysis of sphingolipids has been done using HPLC, and TLC. A new method of diagnosis of sphingolipidoses has been developed using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (DE MALDI-TOF-MS). This type of mass spectrophotometry allows for the precise determination of high molecular weight macromolecules; in this situation sphingolipids and other related compounds are being

identified. DE MALDI-TOF-MS is advantageous in that only a small amount of tissue is needed to produce a rapid diagnosis of these rare diseases; however, its use has only been employed on autopsied liver, spleen, and brain tissue. Further studies on more available tissues or cultured cells will hopefully prove this method a reliable diagnostic tool on living patients (Fujiwaki et al, 1999).

It is still poorly understood whether dietary sphingomyelin and the absorption of its metabolites can influences the cellular metabolic pathways described above. Though there is no nutritional requirement for sphingolipids, studies with experimental animals have shown that ingestion of sphingolipids can inhibit colon cancer (Colombo et al., 2002). Dillehay et al. investigated the effects a sphingomyelin-enhanced diet may have on the induced propagation of colon cancer in rats. These rats were fed a 0.025 % sphingomyelin-enriched diet after the initiation of colon cancer with the carcinogen 1,2-dimethylhyrdrazine. The incidence of colon tumors in the sphingomyelin diet-enhanced rats was 20% versus 47% in the controls. It was also shown that rats fed sphingomyelin-rich diets showed increased circulating serum sphingomyelin levels relative to the doses given (Parodi, 1997). Similar findings have been further substantiated in recent research (Colombo et al., 2002; Modrak et al., 2002). It has been hypothesized that dietary sphingolipids act to suppress colon cancer by bypassing a sphingolipid signaling defect in cancer cells, enabling normal cell propogation (Colombo et al., 2002). Similarly, it was suggested by Modrak et al. (2002) that dietary sphingomyelin can be a good, nontoxic chemotherapeutic for the treatment of specific lines of colon cancers by reversing defective apoptotic signals in those cancer cells.

Having shown that increased sphingomyelin signaling and ceramide levels are associated with cellular responses to severe stress conditions, aging and age related diseases, and atherosclerosis, it suggests that reducing sphingomyelin signaling may be of benefit. Alternatively, increasing sphingomyelin signaling activities and related sphingolipid metabolites may aid in the suppression of certain cancers. Dietary manipulations designed to reduce or increase sphingomyelin and ceramide production may lead to an increased control we can claim over our health.

2.2. Lipid Analysis by Thin Layer Chromatography (TLC)

For the analysis and identification of both simple and complex lipid classes, chromatographic methods are by far the most widely used (Christie, 1982). TLC is a variation of column chromatography that separates mixtures of compounds. It utilizes a strip of glass, plastic, or aluminum as a backing to a plate, and a thin layer of silica gel (sometimes mixed with other substances) as the adsorbent. Depending on the type of adsorbent on the plate, different qualities of separation can be achieved (Fessenden et al., 1993).

As an analytical tool, TLC offers numerous advantages over other forms of chromatography. It is simple, quick, inexpensive, and requires only small amounts of sample. It is commonly used as a qualitative analytical tool, by checking the purity of a compound, or determining the number of components in a mixture (Fessenden et al., 1993); however, if performed correctly, it can also be used quantitatively. For example, it can be used to follow the course of a reaction by identifying the disappearance of a starting material, and the formation of a product. By controlling the quantity of lipid analyzed, relative amounts of components present can be determined, and when equal amounts of lipid analyzed, compared between samples with the same component. It can also be used to identify the species present in an unknown sample by co-analyzing a known compound, or standard.

Another way to identify the species present is by calculating the retention factor (R_f) for a particular compound; the R_f value is the distance traveled by the compound divided by the distance traveled by the solvent. For a particular compound the R_f value is

constant only if all variables during the analysis are held constant, including the following: temperature, solvent mixtures, atmosphere in tank, adsorbent, thickness of adsorbent, amount of compound on plate, and the distance the solvent travels. Clearly, preventing any variation in these numerous factors between individual analyses can present a challenge. Furthermore, the R_f value may not necessarily identify the compound correctly; multiple TLC trials utilizing different solvent systems producing the same R_f values for the spots identified increases the chance that the R_f value obtained can identify the species. For these reasons, analyzing a standard on the same plate as the unknown sample is the best way to determine the constituents of a mixture without further analyses such as mass spectroscopy (Fessenden et al., 1993; Christie, 1982).

In order to visualize the compounds analyzed by TLC, the plate must be subjected to one of a variety of treatments. There are numerous chemicals that can be applied to the plate to unveil its inhabitants by reacting with the compounds present, forming colors. Specific chemical reagents react with specific compounds, so a particular spray may only reveal one type of compound, leaving other species present unnoted. For example, ferric chloride sprays identify phenols, 2,4-dinitrophenylhydrazine identifies aldehydes and ketones, ninhydrin identifies amino acids, the "Zinzadze" and "Dittmer-Lester" reagents identify phosphorus-containing lipids, and benzidine (a strong carcinogen) identifies sphingolipids. For the identification of a much more broad array of compounds, charring the plate (that can withstand heat) can unveil organic substances, as can exposure to iodine fumes. (Christie, 1982; Fessenden et al., 1993; van Echten-Deckert, 2000). Iodine fumes are selectively adsorbed onto the plate wherever there is a collection of organic

compounds, causing the compound to turn yellowish-brown (or purplish) in color; the spots shortly fade when taken away from the fumes (Fessenden et al., 1993).

A variety of solvent systems can be used for TLC, the most appropriate ones for a particular analysis depend on what compounds are present in the sample. The more nonpolar a compound is, the further it will travel up the plate during analysis; alternatively, highly polar compounds do not migrate as far. A common solvent system for the analysis of nonpolar lipids is petroleum ether, ethyl ether, and acetic acid (85:15:2, v:v). Using this solvent system, the analysis of simple lipids on a silica plate results in spots occurring 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) (Christie, 1982).

For the analysis of complex lipids a variety of solvent systems exist as well; furthermore, there is no single TLC system that can be used to separate all known complex lipids in one dimension. A selection of a particular solvent system depends on what lipids are desired to be visualized from an analysis. Many more complex lipid components can be separated on a single TLC plate by using two-dimensional systems; this may resolve the components that appear as one compound in the first dimension. A further check of the purity of a given sample can be verified using this method. When needed, it offers considerable advantages, but the double development time makes it more time consuming (Christie, 1982; van Echten-Deckert, 2000, Smith et al., 1996).

For the analysis of sphingolipids and phospholipids, chloroform-methanol-aqueous mixtures of different polarities are most widely used. A solvent system

consisting of chloroform, methanol, and water (65:25:4, v:v) have been used successfully for the resolution of complex lipids commonly found in membrane systems of uni, and multicellular organisms; the major classes of membrane lipids are phospholipids, sphingolipids, and cholesterol (Gomes-Quintana et al., 1998; Hafenbradl et al., 1996; Sul and Erwin, 1997; van Echten-Deckert, 2000; and Smith et al., 1996). For example, a complex mixture containing sphingomyelin, cholesterol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cardiolipin, and other nonpolar lipids would separate in the above-mentioned solvent system in the following order: nonpolar lipids at the solvent head, followed by cholesterol, cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, then sphingomyelin closest to the source spotted (Christie, 1982). All of these compounds can be identified by the exposure to iodine vapors, as described above.

2.3. Buttermilk

Other than cell membranes, brain, and neural tissue, another source that is rich in naturally occurring sphingolipids is buttermilk; this is due to the presence of the milk fat globule membrane (MFGM), a phospholipid bilayer system that surrounds milk fat globules. It lends structural integrity, protection, and allows compatibity with the aqueous environment (Goudedranche et al., 2000; Ye et al., 2002). In milk and milk products, the fat present is primarily found in the form of spherical globules, which can vary in size from 0.1μm to 15μm (average size of 8μm); their abundance per ml is between 10^{10}-10^{11}. The MFGM is closely involved in the natural processes in milk, such as creaming and agglutination, and the properties it lends to milk and milk products can be greatly affected by treatments, such as heating, cooling, and homogenization. The MFGM contributes to the stability, emulsion properties, and other factors influencing the overall acceptability of milk products (Corredig and Dalgleish, 1997; Ye et al., 2002).

Buttermilk is the main by-product of the butter-making process. During the churning of butter, the milk fat globules are mechanically disrupted, causing the composing fat to be released from the MFGM, becoming butter. The surrounding MFGM is left as a deflated membranous sack free in the aqueous buttermilk fluid; Figure 2.3.1 shows a scanning electron micrograph of buttermilk.

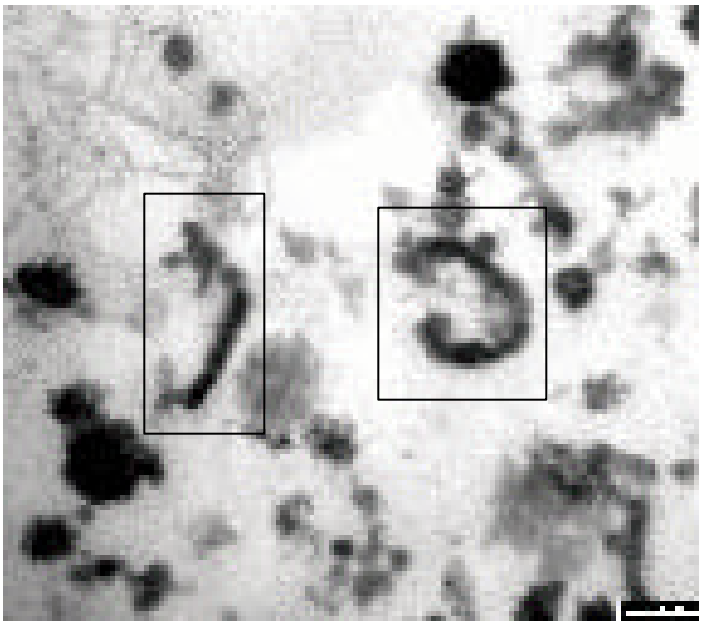


Figure 2.3.1. Scanning electron micrograph of buttermilk showing the MFGM. The deflated membranous sacks are easily distinguished from the proteins and fat in the surrounding fluid as the crescent shaped objects (photo compliments of Beth Fryksdale, Cal Poly State University, San Luis Obispo).

Buttermilk is commonly discarded, and is not generally desirable for consumption on the market (Walstra et al., 1999). However, it is a highly functional food ingredient, used in a variety of foods such as bread and other baked products, ice cream, yogurt, chocolate, toffee, dry mixes, soups, and sauces. When used as an ingredient, it can impart beneficial characterists, such as adding flavor, firmness, moisture retention, and improving the whipping and emulsion properties; it also can prolong shelf life by inhibiting staling (Fryksdale, 2001). It is also frequently added as a source for extra protein (Corredig and Dalgleish, 1998). Unfortunately, buttermilk is highly susceptible to spoilage due to rancidity, so it is frequently dried and sold as a low quality food ingredient, often to companies looking for a cheap alternative to milk solids (Fryksdale,

2001; Walstra et al., 1999). Buttermilk's diverse functionality was extensively reviewed by Fryksdale (2001).

The composition of the MFGM is quite complex, and though extensively reviewed, not thoroughly understood (Eigel et al., 1984; Mather, 2000). It contains numerous proteins (25-60%), polypeptides (over 40), glycoproteins (at least 8), carbohydrates, triacylglycerides, and various phospholipids (including sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine) that count for about 33% of the MFGM (Deeth, 2000; Goudedranche et al., 2000; Hui, 1998; Lambertsen and Christiansen, 1997; Mather, 2000; Walstra et al., 1999; Ye et al., 2002).

Buttermilk in particular contains a high percentage of phospholipids as compared to regular dairy milk, 0.13% versus 0.035% respectively (Walstra et al., 1990). Furthermore, sphingomyelin represents about one third of total buttermilk phospholipids (Parodi, 1997).

The phospholipids of the MFGM have also been shown to contribute significantly in the oxidation process of milk, which results in an off flavor, and other defects in milk products. Phosphatidylcholine and phosphatidylethanolamine, two of the above-mentioned major phospholipids of the MFGM, contain 40-60% unsaturated fatty acids, about one third of which are polyunsaturated; this makes them highly susceptible to oxidation. Likewise, isolated MFGM is easily subject to oxidation (Deeth, 2000).

The amounts of the specific protein components can vary with the stage of lactation (Ye et al., 2002). Many of the proteins found in the MFGM are unique to that source as they are secreted from the mammary glands. Their functions are not all clearly defined, and many are not easily purified. When separated by SDS-PAGE, the MFGM is

resolved into 7 to 8 major bands of protein. In order from heaviest molecular weight to lightest, the major proteins of the MFGM are as follows: Mucin 1 (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). All proteins but MUC1 and PAS III (both glycoproteins) stain with Coomassie blue stain; the presence of these proteins can be detected staining with PAS reagent or a modified silver stain reagent (Mather, 2000).

The biological functions of MUC1 are undefined despite the detailed physical and chemical characterizations that have been done. With an approximate molecular weight of 254.3 kD, MUC1 is heavily glycosylated (up to 50% its weight), and can vary in its degree of glycosylation. It is expressed on the apical plasma membrane of epithelial cells of many tissues besides the MFGM. It is presumed to protect exposed surfaces from physical damage and pathogens; it may play immunoprotective roles as well (Mather, 2000).

XDH/XO is a molybdenum-containing redox enzyme with an approximate molecular weight of 155 kD. An extensively studied protein, XDH/XO oxidizes a variety of compounds by reducing water, and transferring the free reducing counterparts to redox centers within the protein. It possesses further functions that are still undefined (Mather, 2000).

PASIII is a glycoprotein with a molecular weight of approximately 95kD that is visualized by staining with PAS reagent after separation by SDS-PAGE. The PASIII glycoprotein is poorly characterized (Mather, 2000).

CD36 is a glycoprotein consisting of about 24% carbohydrates; though it satins best with PAS reagent, it is detectable when stained with Coomassie blue. It's molecular weight is approximately 77kD, and comprises only 5% or less of the total MFGM protein. It is presumed to possess numerous functions, including serving as a receptor for collagen and thrombospondin, in platelet activation and aggregation, intracellular adhesion, and thrombospondin mediated angiogenesis. It may also act as a scavenger molecule, helping the body to rid itself of dead cell and fragments. Its purpose on the MFGM has yet to be thoroughly determined (Mather, 2000).

The most abundant protein in the MFGM, BTN comprises 34-43% of the total protein in Holstein milk, and approximately 20% in Jersey milk. It's molecular weight is approximately 66kD, and due to its high affinity for butter fat is firmly membrane-bound. It's function is not clearly defined (Mather, 2000).

PAS 6/7 proteins are major components of the MFGM of many species that vary widely in their size due to posttranslational modifications of a single protein. This causes the proteins to range in molecular weight from 43-59kD. Their function in the MFGM is unknown, through in other locations they are known to function as acetyl transferases (Mather, 2000).

ADPH is easily overlooked in SDS-PAGE due to its relative insolubility in sample buffers, and it's molecular weight of approximately 52kD is very close to the dominant protein of PAS 6/7. ADPH is associated with lipid droplets in many cultured cell lines; in tissues its distribution is more limited (Mather, 2000).

FABP in the MFGM is an isoform of the mammary-derived growth inhibitor (MGDI) protein found in heart tissue; heart MGDI was shown to stimulate cellular

differentiation of mammary cells as well as inhibiting cell division. It has not been clearly demonstrated that the MFGM FABP possesses the same functions. It's molecular weight is approximately 13kD (Mather, 2000).

In addition to these characteristics of the major MFGM proteins, they appear to have complex interactions with each other, other proteins, lipids, and other molecules through molecular interactions such as hydrophobic and hydrophilic interactions, and the formation of disulfide bonds (Kim and Jiménez-Flores, 1995; Lefèvre and Subirade, 2000; Ye et al., 2002). It has recently been shown that XDH/XO and BTN form a high molecular weight aggregate through the formation of intermolecular disulfide bonds after a heat treatment at 60^{o}C for 10 minutes (Ye et al., 2002), while previous studies have shown that with higher temperature heat treatments MFGM proteins form similar high molecular weight complexes with milk proteins. Kim and Jiménez-Flores (1995) reported that with heat treatment of 87^{o}C, ß-lactoglobulin (BLG) and other milk serum proteins interact, forming high molecular weight complexes with the MFGM proteins PAS6/7. The mechanism of formation was undefined, but did not appear to be solely due to the formation of disulfide bonds (Kim and Jiménez-Flores, 1995).

Because of the high phospholipid content in MFGM, it is accepted that isolated MFGM and buttermilk have good emulsifying properties, an important characteristic of a functional ingredient. Because of the close association of the MFGM lipids and proteins, the effects of proteolysis of the MFGM proteins were investigated with respect to the emulsion properties. It was found that after treatment with trypsin and chymotrypsin, the emulsion properties of MFGM isolates improved. MFGM isolated from industrial buttermilk exhibited a greater improvement than did MFGM from cream (Corredig and

Dalgleish, 1998). This implies that some aspect of the MFGM structure is changed during buttermilk processing, perhaps affecting the associations between the proteins and lipids of the MFGM.

Buttermilk is also a natural source for numerous other bovine milk lipids, many possessing bioactive properties as well. Conjugated linoleic acids, which are gaining acceptability as being antiatherogenic and reducing adipose fat, are primarily consumed in meat products, and secondarily as bovine milk lipids. Alternatively, saturated fats and trans fatty acids, which are generally accepted as atherogenic, are present in bovine milk lipids also. Saturated, monounsaturated, and polyunsaturated fatty acids account for only 0.1-0.4% of the total milk lipids, their bioactivities include regulating in the proper functioning of lipid metabolism; monounsaturated and polyunsaturated fats have both been associated with promoting healthy cholesterol levels. Also present in milk lipids are steroids, which are necessary for the resorption of fats, and ether lipids, which are thought to influence biochemical and biophysical processes (Molkentin, 2000).

2.4. Microfiltration

The use of filtration in the food industry is extremely diverse, extending to the processing of dairy products, water treatment, wastewater and sewage treatment, textiles, pharmaceuticals, latex emulsions, sugars, oils, grains, animal products, juices, alcoholic beverages, and other applications. Though all applications have distinct, unique goals for using filtration, they all achieve them using the same basic principles of the filtration process. Generally, filtration processes select for the concentration of specific components in a mixture by passage through a membrane, which acts as a selective barrier. By doing so, either the permeating or retaining phase becomes enriched in one or more components of the mixture. The degree of selectivity depends not only on the type of membrane being used (pore size and reactivity), but also on the other conditions of the process, including temperature, pressure, velocity, flow schematic, and other conditions (Boyd, 1999; Cheryan, 1998; Samuelsson et al., 1997; Short, 1998; Tanny et al., 1982).

Based on the selectivity of the membrane used for the process, filtration processes can be grouped into four general categories; in order from least to most selective, the are microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Figure 2.4.1 depicts these classifications along with their selectivity ranges, and what type of components may be separated through their application. These classifications were developed to give a better description of the membrane selectivity, and are most appropriately used as a guide to understand the processes, not as strict definitions of their limitations. Microfiltration processes are most commonly used in the dairy industry for fat separation,

bacterial removal, and casein protein concentrations (Cheryan, 1998); and will be the focus of this review.

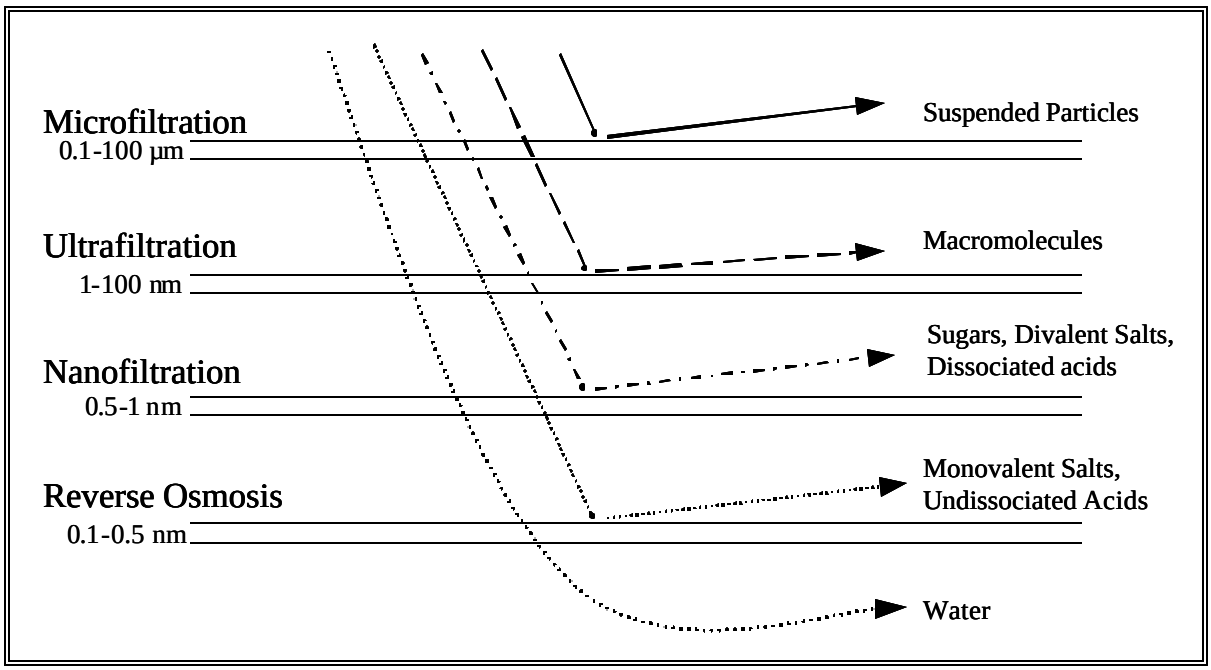


Figure 2.4.1. The varying selectivity of pressure-driven membrane filtration processes is shown by how components of a complex mixture are separated. The size ranges for a given process are approximate; the sizes of the components separated may vary in the actual process (Cheryan, 1998).

For microfiltration and ultrafiltration processes, though numerous membrane materials have been developed and tested, relatively few have demonstrated success commercially. Of these, polyvinylidene fluoride (PVDF), polyacrylonitrile, polysulfone, polypropylene, and ceramic materials continue to be the most widely used. Ceramic membranes are of particular advantage in that they are extremely long-lived (with proper care), and can withstand extreme temperatures. They are usually in a tubular form, either as one, or a series of tubes that run in channels down the length of the cylinder. During the microfiltration process the batch being treated is fed into the channels within the

cylinder, and permeable substances exit the channels through the pores leaving the system (Cheryan, 1998). Figure 2.4.2 shows a representation of this type of membrane configuration.

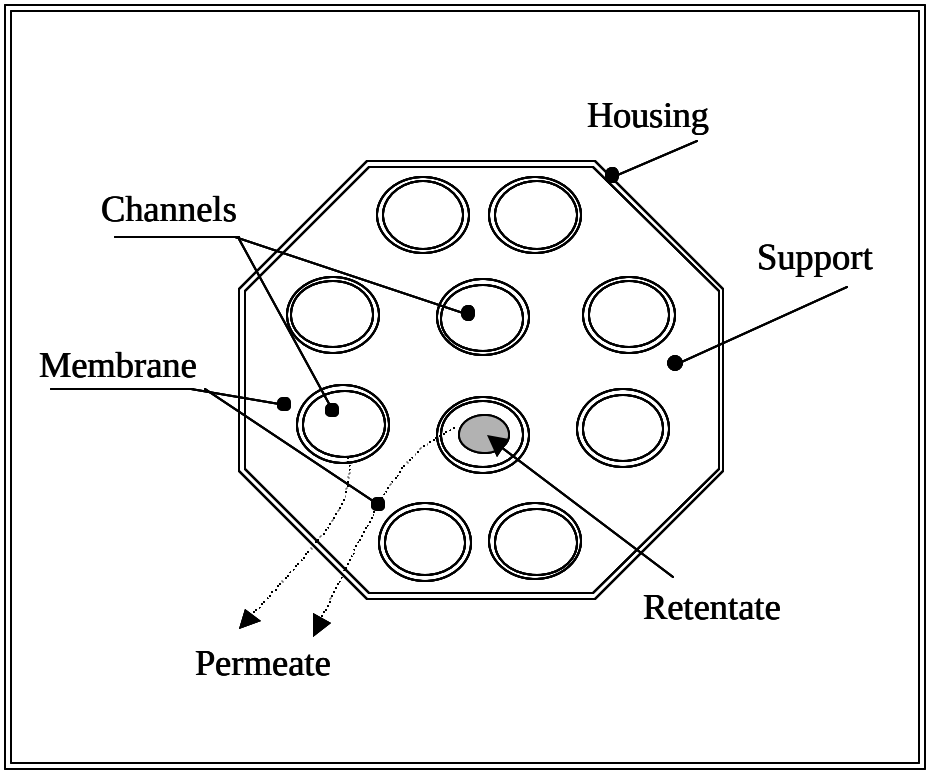


Figure 2.4.2. Configuration, and flow path for a cylindrical ceramic membrane shown as a cross section. As the substance flows through the channels permeable substances exit the system through the pores, while the retained substances continue through the channels.

One major problem that arises with microfiltration processes is fouling of the membrane. Fouling occurs when components adsorb to the membrane surface or within the pores forming a filter cake, preventing normal separation to occur. The effects of fouling are detrimental as selectivity and efficiency are lost, and it is timely to clean the system to restore proper functioning. Unfortunately, all systems will eventually show a decline in performance due to a gradual build up of foulants, but certain design features

can be adopted to reduce this tendency. Using a crossflow method of microfiltration is one way to reduce fouling. This design uses a pump as the driving force to pass the feed stream tangentially to the membrane (as opposed to perpendicularly). A general schematic of a crossflow microfiltration system is given in Figure 2.4.3. The concentration of the retained species in the loop increases as permeate is removed from the system (Akhtar et al., 1995; Short, 1988; Tanny et al., 1982).

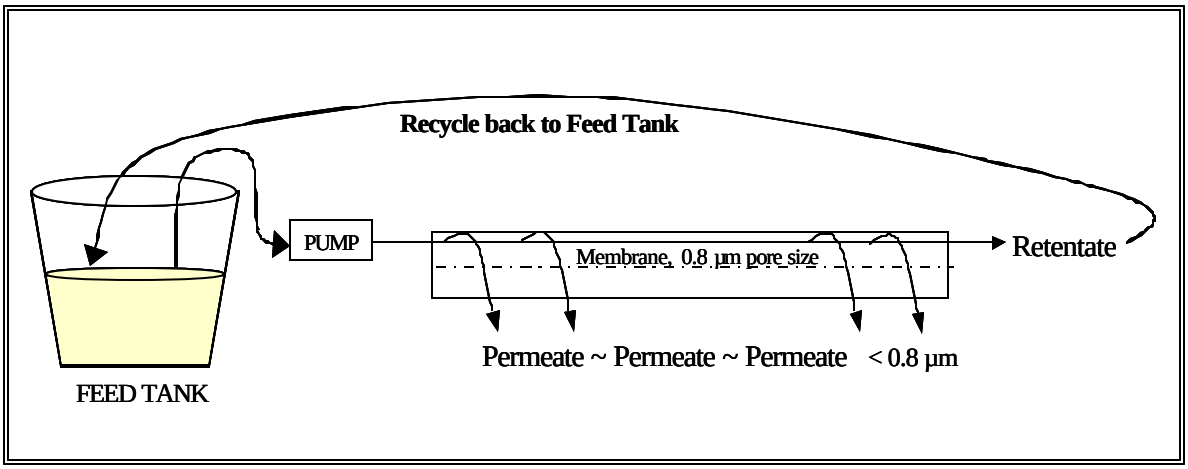


Figure 2.4.3. General schematic of a pressure-driven crossflow microfiltration system, using a 0.8 µm membrane as an example. The feed is pumped over the surface of the membrane, and the components separated through the membrane yielding the permeate and retentate fractions.

Another way to reduce fouling is by the application of coatings to the membrane surface. A phospholipids bilayer applied to both PVDF and cellulose acetate membranes was found to reduce the fouling of proteins to the membrane Because of the natural tendency of cellular plasma membranes to resist protein fouling, a similar bilayer structure was applied to the above-mentioned membranes. An increase in performance and a decrease in protein fouling was seen in these systems (Akhtar, 1995). However, the

charge characteristics can affect what is permeated and retained in a system, and if a coating alters the nature of the membrane from what is desired, selectivity can be drastically affected (Cheryan, 1998)

Traditionally, crossflow microfiltration has been used to concentrate and purify dilute solutions of macromolecules, such as proteins, and lipids. Particularly in the dairy industry, the concentration of lipids, caseins, and whey proteins has responded well to microfiltration systems (Samuelesson, 1997). Altering the functionality of a product by altering its composition; such as concentrating the microbial loads in fermented products, or minimizing whey proteins in cheese; is a powerful application of microfiltration to the dairy industry. Particularly at a time where the nutritional benefits of numerous dairy components make them desirable ingredients, microfiltration is an indispensable tool for the fractionation of various dairy components.

2.5. Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction is a relatively new method that uses the unique physical properties of a substance at the superceitical state to extract components of a complex mixture. The supercritical state is reached when temperature and pressure conditions above a substance's critical temperature and pressure points are reached. In this state the substance possesses a gas-like viscosity, and can easily penetrate a wide range of sample matrices, enabling it to have strong solvent capabilities. Supercritical fluid extraction processes offer an environmentally benign alternative to other extraction methods, which commonly employ hazardous solvents that would render the product unsafe for consumption or contact. It has been referred to as the "greener" solvent for its usefulness in replacing traditional solvents in certain chemistry-related applications (Hauthal, 2001). Selecting appropriate supercritical solvents as replacement solvents has been greatly accommodated by the recent development of solvent databases; these databases provide valuable information such as the solubility parameters and critical points for various compounds (Hauthal, 2001). Carbon dioxide is a common solvent for many SFE applications, including the extraction of nonpolar lipid material. This is due primarily to its low critical temperature and pressure parameters (31.1°C, 73.8 bar), low cost, non-toxicity, chemical inertness, and non-flamability. It can be used with or without the addition of a polar co-solvent (such as alcohols or other organic chemicals) to enhance extraction efficiency, and widen the range of what is solvated in the supercritical fluid (Boselli and Caboni, 2000; King, 1995; Montanari et al., 1999; Rónyai et al., 1998; Rozzi and Singh, 2000; Turner et al., 2001). Without modification by polar cosolvents, carbon dioxide can only extract nonpolar materials.

A wide range of supercritical fluids has been used for commercial, developmental, pharmaceutical, and scientific processes; the critical properties of some

commonly used SCFs are listed in Table 2.5.1. Many are more commonly used as a cosolvent with carbon dioxide, such as ethanol, which is polar enough to extract a wider range of lipids than is carbon dioxide alone.

Fluid	Critical Temperature (K)	Critical Pressure (bar)
Carbon dioxide	304.1	73.8
Ethanol	514.15	61.8
Ethane	305.4	48.8
Ethylene	282.4	50.4
Propane	369.8	42.5
Propylene	364.9	46.0
Ammonia	405.5	113.5
Water	647.3	221.2
Cyclohexane	553.5	40.7
Toluene	591.8	41.0

Table 2.5.1. The critical temperatures and pressures of some commonly used solvents in supercritical fluid extraction processes (Rozzi and Singh, 2000). Of these, carbon dioxide, ethanol, and water are generally recognized as safe (GRAS).

When a supercritical fluid is introduced to a sample, specific compounds are solublized in it; the efficiency of the extraction depends on numerous conditions, including polarities of the solvent and solute, pressure and temperature conditions, and the phase equilibria (Hauthal, 2001; King, 1995; Rozzi and Singh, 2000; Turner et al., 2001). The process can be though of as occurring in four steps: penetration of the supercritical fluid, release of solutes within the sample matrix, diffusion of solutes out of the sample matrix, and final removal of the solutes from the sample. Once solvated, the solutes can be removed from the supercritical solvent when it is brought back to ambient conditions. The supercritical solvent is returned to a gas (if that is its normal state at ambient conditions) and either released as waste, or recycled for further use (King, 1995; Turner et al., 2001).

The effects pressure and temperature have on a pure substance are described by its phase diagram; the phase diagram for carbon dioxide is given in Figure 2.5.1. Here, four main phases exist, the solid, liquid, gas, and supercritical; they are all governed by the environmental temperature and pressure conditions. The triple point is when the combination of temperature and pressure is such that the substance can exist as a gas, liquid, and solid, simultaneously. It is also the lowest pressure at which liquid CO_2 can exist; the triple point for CO_2 is at 5.07 bar at -56.6°C. Below this point, the pressure is low enough that if you heat the material up it will sublimate directly from a solid to a gas. The critical point is along the boiling point curve where the line between the liquid and gaseous phases disappears; the critical point for CO_2 is at 73.8 bar, at 31.06^{o}C (Ebbing, 1996). At temperatures higher than the critical point the only phase that exists is the supercritical phase. Contrary to terminology, supercritical fluids are neither liquids nor gasses; they are more accurately described as very dense gasses with unique solvating characteristics.

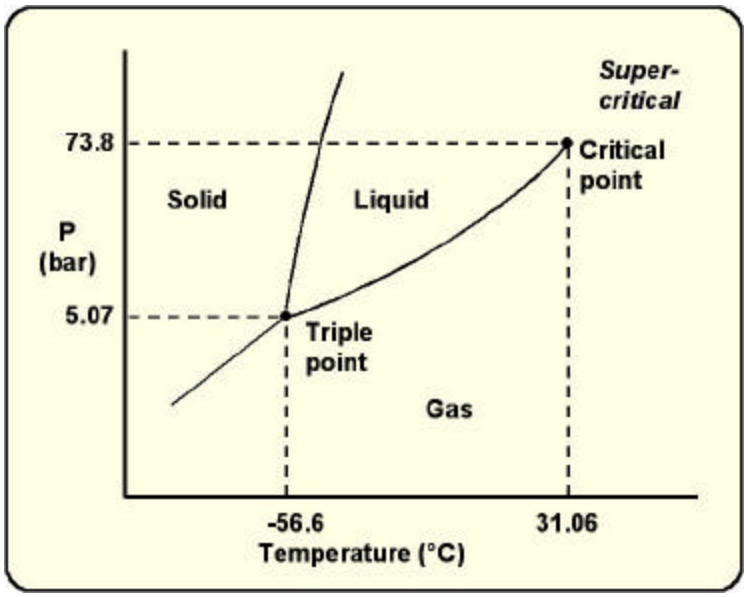


Figure 2.5.1. Phase diagram for carbon dioxide, showing the triple point and critical point; above the critical point it exists as a supercritical fluid. Image adopted from IEA Greenhouse Gas Research and Development Programme website (2002).

At a constant temperature, increasing the pressure of the supercritical fluid results in increasing its density, thereby increasing its solvent strength. The Hildebrand Solubility Parameter, the formula of which is given in Figure 2.5.2, can describe the solvating capability of some compressed gasses. This equation provides a rough measure of the ability of a solvent to dissolve in a solute; a property that is related to the density of both the gas and the liquid. From this equation, with increased pressure, the solubility parameter increases, with large changes occurring as the critical pressure is reached (Rozzi and Singh, 2000; Turner et al., 2001). This equation can be used successfully when applied to some solutes in supercritical fluids; however, it does not extend to esters, ketones, alcohols, and other polar liquids (Rozzi and Singh, 2000).

$$d = 1.25P_c^{½} [\ ^{?}/?_{liq}]$$

Figure 2.5.2. The Hildebrand Solubility Parameter, describing the solvating capability of a compressed gas, where d is the solubility parameter, P_c is the critical pressure, ? is the gas density, and d_{liq} is the density of the liquid (Rozzi and Singh, 2000).

If the temperature of a supercritical fluid is increased with pressure held constant, the solvent strength can be either increased or decreased, depending on the pressure. Above the point where the vapor pressure is increased (above the critical points) an increased temperature can increase the extraction efficiency. Below this point, the efficiency can be decreased with increased temperature; this is due to the decreased fluid density (Turner et al., 2001). The appropriate conditions for a particular extraction type should be customized to what is to be extracted from the mixture, then suitable pressure and temperature conditions can be determined for that process. Experimentally optimizing the procedure by varying the pressure, temperature, and flow rate conditions can determine the most efficient extraction conditions for the system being used (de Frankca et al., 1999; Montanari et al., 1999. Spricigo et al., 1999)

Numerous SFE methods have been developed to extract lipid and lipid soluble materials from a complex mixture, including (but not limited to) the extraction of lipid-soluble vitamins (A, D, E, and K), various seed, nut, bean, and wood oils, essential oils, meat fats, phospholipids, pesticides, cholesterol, and pharmaceutical components (Berg et al., 1997; Boselli et al., 2000; de Franca et al., 1999; Gideo et al., 1996; Gonzáles-Vila et al., 2000; Hopper and King, 1991; King et al., 2001; Montanari et al., 1999; Ramos et al., 2000; Rónyai et al., 1998; Sovová et al., 2001; Spricigo et al., 1999; Turner and Mathiasson, 2000; Turner et al., 2001; Wigfield et al., 1996). Many of these methods

preferred the use of SFE over conventional methods because the products were free from organic solvent residues, and there was a minimal risk of thermal oxidation; such was the case for Turner et al., (2001), who extracted fat-soluble vitamins. These compounds, which are commonly used as natural additives to foods and pharmaceuticals, are sensitive to light, oxygen, heat, and pH changes. By using SFE they were able to effectively purify the selected compounds without rendering their product unfit for further applications, as conventional methods might. Interestingly, when milk powder was used as a source for vitamins A and E, it was found that the presence of lactose interfered with the extraction process. Pretreating the powder to remove the lactose, and using ethanol as a cosolvent enabled the vitamins to be extracted with over 96% efficiency (Turner and Mathiasson, 2000).

The efficiency of SFE for the extraction of lipids has been compared favorably to conventional methods. Berg et al., (1997) compared a SFE method using ethanol-modified carbon dioxide with the Bligh and Dyer method (chloroform-methanol extraction), and the Schmid, Bondzynski, and Ratzlaff method (acid hydrolysis extraction). With optimization of their method, they found no significant difference between the methods for the extraction of total fat and lipid classes from meat. Furthermore, the total time for extraction was considerable shorter than that required for the Bligh and Dyer method (Berg et al., 1997). Similar findings were presented by Taylor et al., (1997). It is indicated from their work that SFE (using ethanol-modified carbon dioxide) is a reasonable alternative analytical technique to AOCS/AOAC official methods for determining total fat content of oilseed and ground beef (Berg et al., 1997).

SFE may prove to be increasingly beneficial to the food industry by its ability to remove pesticides from food products. Ogranochlorine, a common pesticide used in eggs, is typically screened for using a Soxhlet method with acetone and hexane, a time and solvent-consuming method. A SFE method was developed that used carbon dioxide to remove the pesticide with comparable efficiency to the Soxhlet method, and an additional solvent alternative method to the Soxhlet (developed by Scheneck et al., 1994). (Wigflield et al., 1996).

Supercritical carbon dioxide was also used by Hopper and King (1991) to extract over 30 different types of pesticides from a variety of foods, including carrots, lettuce, peanut butter, hamburger, and fortified butterfat. Using pelletized diatomaceous earth as an extraction enhancer, they were able to recover pesticides with efficiencies in excess of 85% for all pesticide types, with levels ranging from 0.005 to 2 ppm (Hopper and King, 1991). Similarly, Polychlorinated biphenyls (PCBs), which are a known health risk, have been removed from powdered full-fat milk along with cholesterol, and a variety of glycerides. The goal of the study was to characterize the distribution of the PCBs in the milk fat globule, not to necessarily purify it as an extract. It was found to be closely associated with the cholesterol, and the short and medium chain triacylglycerides as they all extracted together under the same range of conditions (136-233 bar, at 50^{o} C) (Ramos et al., 2000).

In addition to SFE using supercritical carbon dioxide as a method to extract components from a complex mixture, it can also offer many of the same benefits derived by other high pressure and carbon dioxide processing practices. High pressure processing is commonly used as a non-thermal processing technique to kill vegetative

microorganisms, endospores, and to inactivate undesirable enzymes. It is a desirable method because it does not cause loss of color, nutrients, or flavor of the food. Atmospheric carbon dioxide is also used as an antimicrobial treatment in the food industry; it helps preserve food by displacing oxygen, reducing the pH, and penetrating cells rapidly. Likewise, supercritical carbon dioxide has been shown to successfully kill a wide range of microbes, vegetative cells, and spores, as well as inactivating deleterious enzymes in certain food products (Corwin and Shellhammer, 2002; Enomoto et al., 1997; Smelt and Rijke, 1997; Spilimbergo et al., 2002; Stewart et al., 2000). This makes SFE using carbon dioxide as a solvent an attractive option to those working in the food industry.

Using high-pressure conditions at room temperature, vegetative cells can easily be killed at an operating pressure of 2000-3000 bar, while spores can endure the treatment and continue to form vegetative cells (Spilimbergo et al., 2002). Treatments employing multiple pressure and heat treatments were developed to firstly provoke spore germination, and then secondarily kill the vegetative cells (Lundgren, 1966). Adding the use of SC-CO_2 to pressure-based sterilization methods greatly reduced the conditions necessary to obtain equal results; a 15 minute treatment with SC-CO_2 at 150 bar at room temperature gave the same microbial reduction as at 3000 bar at the same temperature (Smelt and Rijke, 1997). While this alone did not inactivate spores adequately, it was later found that coupling the process with a heat treatment allowed for the deactivation of spores. Spilimbergo et al. (2002) demonstrated that complete deactivation of *Bacillus subtilis* and *Pseudomonas aeruginosa* spores occurred with SC-CO_2 cycled (30 cycles per

hour) at 80 bar at 75^{o}C; partial deactivation of the spores occurred at 35^{o}C, with all other conditions being the same (Spilimbergo et al., 2002).

In addition to all the benefits SFE offers as an alternative to solvent extractions, by the nature of the process it also gives the benefits of high pressure, and carbon dioxide processing. Not only can products for human use (food, cosmetics, pharmaceuticals) be processed without traditional solvents, an assurance can be made regarding microbial quality of the product, and shelf life may be extended due to the processing.

Chapter 3.0
Materials and Methods
3.1. Microfiltration and Ultrafiltration

Equipment

The microfiltration unit was an in-house manufactured stainless steel shell and tube module containing a GEA Niro brand ceramic membrane (Hudson, WI) with a pore size of 0.8µm. The process was driven by an APV Crepaco Inc. (Lake Mills, WI) positive displacement pump. A 10,000 molecular weight cut off membrane was used for the ultrafiltration process using the same unit as for microfiltration.

Microfiltration Processing and Diafiltration

A two factorial design was employed for these experiments with the following variables selected: temperature (4^oC and 50^oC), and origin of the buttermilk (reconstituted from powder, or fresh). These run types are referred to as cold reconstituted, cold fresh, hot reconstituted and hot fresh.

Spray dried buttermilk powder (product code 26048, Land O'Lakes, Inc., MN, U.S.A.) was reconstituted in tap water to give 20 L of a 10% total solids solution, and let sit overnight at 4^oC to fully hydrate. Appendix A gives the product specifications obtained from the Land O'Lakes Food Ingredients Division.

For all microfiltration processing, the same conditions were applied to the system. The total pressure of the system was maintained at approximately 200 psi; the inlet was approximately 150 psi, and the retentate outlet was approximately 50 psi; the transmembrane pressure was maintained at all times at less than 5 psi. The pump was operated at 48-54 Hz. The flux rates were recorded for all processes.

Diafiltration was commenced with tap water replacing the lost volume of the retentate when the volume was reduced by 50%; this was continued until five times (5X) the starting volume had been added to the system (100 L total).

Sampling

Throughout the microfiltration and diafiltration process samples were drawn of both the retentate (R) and permeate (P) according to the stage of the diafiltration process. The first samples were taken when the retentate reached 50% its starting concentration (before diafiltration); these samples were labeled as R50 and P50. Throughout the diafiltration process samples were taken at 5 intervals when water had been added to the retentate at 1, 2, 3, 4, and 5 times the starting volume. The samples were labeled in terms of their source, and their relationship to the initial volume of water: R1X, R2X, R3X, R4X, R5X, and P1X, P2X, P3X, P4X, P5X.

For the triplicate cold reconstituted runs, samples were drawn and analyzed in duplicate at the R/P50, 1X, 2X, 3X, 4X, and 5X points during the process.

For the triplicate cold fresh buttermilk processing runs, samples were drawn at all points in the run, and analyzed in duplicate at the R/P1X, 3X, and 5X points.

For the duplicate hot reconstituted buttermilk runs, samples were drawn at all points discussed in the processing, and analyzed in duplicate at the R/P1X, 3X, and 5X points. The retentate samples were pooled from both runs and analyzed in duplicate because inadequate sample volumes were drawn; the permeate samples were all analyzed independently in duplicate.

For the hot fresh buttermilk processing runs, samples were drawn at all points in the run, and analyzed in duplicate at the 1X, 3X, and 5X points.

Cleaning the System

The microfiltration system should be cleaned after use, or when fouling occurs. The following steps give the cleaning procedure:

1. Rinse all solids out with hot water.
2. Add ~100ml of caustic cleaner (Ultrasil 25 (Klenzade #51094)) to 1/3 of the sanitation tank full of hot water
3. Run at 50 Hz for 45 min after verifying that the pH is 10-12
4. Position return pipe out of tank
5. Add water/rinse until pH 7 (run pump at 35Hz)
6. Replace return pipe into tank.
7. Add ~100ml of acid cleaner (Ultrasil 78 (Ecolab #52618)) to 1/3 of the sanitation tank full of hot water
8. Run at 50 Hz for 45 min after verifying that the pH is 2-4.
9. Position return pipe out of tank
10. Add water/rinse until pH 7 (run pump at 35Hz)
11. Leave water in the system.

Ultrafiltration Processing

Following the microfiltration process, ultrafiltration was performed using the 10,000 molecular weight cut off membrane to remove water from the retentate to increase

the total solids content to 6-10%. The same conditions were maintained during ultrafiltration as for the microfiltration processes.

3.2. Spray Drying of Retentate Powder

Retentate that has been concentrated to approximately 10% total solids was spray dried in the pilot plant on a Niro Filterlab Spray Dryer (Hudson, WI) following the manufactures instructions. The equipment has a water evaporating capacity of 100 pounds per hour. The spray nozzles had a core of size 16, and the orifice was size 70; the pressure was 500 psi.

3.3. Total Solids (% TS)

Direct Oven Drying Method for Determination of Total Solids Content of Milk was used for all determination of total solids. Weighing approximately 5 g of the product, drying the product in a forced air oven for 4 hours at 100+1 degrees Celsius, and weighing the dried residue determined total solids. All weights were taken using an analytical balance, and read to the nearest 0.0001 g. Total solids content is the weight of dried product residue expressed as a percentage of original product weight. A blank was analyzed initially to ensure accuracy of the analysis, and then not employed for further anlayses. Crucibles were handled using tongs, and cooled prior to weighing in a dessicator. The following formula gives the calculation of total solids.

$$\text{Percent TS} = \frac{[(\text{weight of dish + sample}) - (\text{wt of dish})] - (\text{mean blank})}{[(\text{wt of dish + sample}) - (\text{wt of dish})]} \times (100)$$

All samples were analyzed in duplicate, and the average taken as the final value for that sample. The maximum recommended difference between duplicates is 0.05% total solids.

3.4. Percent Ash

Ash content was determined directly following determination of total solids. The resulting crucible from total solids analysis was covered containing solids, and placed in the incinerator oven at 550^{o}C for 24 hours. Upon completion, crucibles were removed with tongs, cooled in a dessicator, and the weight of the crucible and ash was recorded on an analytical balance to the nearest 0.0001 g. The percent ash was determined using the same formula as for total solids calculation. All samples were analyzed in duplicate, and the average taken as the final value for that sample.

3.5. Protein Analysis

The 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) (catalog # K304-1000) were used as reagent. For aqueous samples, approximately 2.0 grams were analyzed, and for powder samples approximately 0.80 grams. Titrations were done using 0.1N HCl, and the percent protein was calculated using the milk protein conversion factor, 6.38. All reagents were of Kjeldahl analysis analytical grade. Samples mixed in a 1:1 ratio with biosilicate were converted to express the protein concentration of the powder alone.

3.6. Protein Profiling by SDS-PAGE

Based on the percent protein determined by Kjeldahl analysis, samples were diluted in de-ionized water to yield a protein concentration of 1 mg/ml. Samples were then added to reducing-denaturing sample buffer in a 1:1 dilution, vortexed, boiled for 10 minutes, and centrifuged for 2 minutes at 12,000 rcf; 10µl (0.5 µg) was then loaded to the

gels. The gels were run at 90 V for approximately 10 minutes (or until the sample reached the resolving gel), then increased to 120 V for the remainder of the run. BlueRange® Prestained Protein Molecular Weight Marker Mix (Pierce, Rockford, IL) (Lot # KL1120) was used as the molecular weight standard. Gels were stained in Coomassie Brilliant Blue (Sigma Chemical Company, St. Louis, MO, U.S.A.) (Catalog # B-8647) for 2-12 hours, then destained, and visualized. All relevant buffer and reagent recipes for SDS-PAGE are given in Appendix B.

For silver stains, the *Silver Stain Plus Kit* (Bio-Rad, Hercules, CA, U.S.A.) (Catalog Number 161-0449) was followed according to manufacture's protocol.

3.7. Percent Lipid Determination and Extraction

For the determination and extraction of lipids from sample, the Mojonnier ether extraction method was followed as described in *Standard Methods for the Examination of Dairy Products, 16th* Edition (Marshall, 1992). In addition to providing an accurate quantification of polar and nonpolar lipids in a given sample, this method also extracts the total lipids present in a sample without destroying their molecular structures. All reagents were of analytical grade, and purchased from Fisher Scientific (Tustin, CA, U.S.A.).

The following modifications from the methods described in Marshall (1992) were made: the amount of a particular sample extracted was based on its total solids content, not volume of the liquid. Equal solids from each point in the microfiltration process were analyzed, and the fat extracted expressed as a concentration: g fat / g dry solid. Because the aqueous samples had increasingly low amount of solids throughout the processing, analyzing their fat contents based on total solids allowed the concentrations between

samples to be comparative. For powdered samples the methods described in Marshall (1992) were followed.

All samples were extracted in duplicate, and the average taken as the sample value; duplicates are to be within 0.03% of one another. Gross outliers were not included in the data set, and repeated if necessary. Samples mixed in a 1:1 ratio with biosilicate were converted to express the fat of the powder alone.

3.8. Lipid Profiling by Thin Layer Chromatography (TLC)

The following lipid Standards were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.): Sphingomyelin (SM), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Cardiolipin (CL), and Sphingosine (S). Silica gel glass plates for TLC analysis were purchased from Merck (Darmstadt, Germany). TLC developing tanks were purchased from Kontes Glass Company (New Jersey, U.S.A.). All solvents and other reagents were of analytical grade, purchased from Fisher Scientific (Tustin, CA, U.S.A.).

For the analysis of polar and nonpolar lipids, the following solvent systems were used respectively: chloroform-methanol-water (65:25:4, v:v), and petroleum ether-ethyl ether-acetic acid (85:15:2, v:v). These solvent systems were chosen based on their quality separations of the lipids present. After addition of the appropriate solvent system to its designated tank, the lid was placed on with weights to seal them firmly, and left to sit without agitation for a minimum of 30 minutes before analysis.

All lipids were prepared in chloroform-methanol (1:2, v:v). Lipid standards were diluted to a concentration of 1 mg/ml. Experimentally extracted lipid samples (from Mojonnier extraction described above) were diluted to a concentration of 10 mg/ml, and

the duplicates combined. Glass capillary tubes were used to apply the diluted lipids to vacuum-dried plates approximately 2 inches from the bottom of the plate, at least 1 cm apart from one another; 5 µg of lipid standard, and 100 µg of unknown lipid sample were spotted to the plates. After sample application, the plates were placed in a vacuum oven at room temperature for 10 minutes, then placed in the prepared tightly closed developing tank, and left to run to completion undisturbed. Plates were removed from the tank when the solvent head was approximately 2 cm from the end of the plate, and allowed to thoroughly dry in a vacuum oven at room temperature a minimum of 30 minutes. The lipids were visualized by exposure to iodine vapor, and the species identified by comparison to the corresponding standard. The plates were scanned using Vistascan® software on a Windows-controlled system (Hewlett-Packard).

3.9. Supercritical Fluid Extraction (SFE)

Equipment

The SFE machine and components used were made by Thar Designs, Inc. (Pittsburgh, PA, U.S.A.), with 500 ml vessel, model P-50 high-pressure pump (Serial # 61926-2), automated back pressure reglator model BPR-A-200B, and PolyScience® brand water bath and pump unit model 9505. 50 lb carbon dioxide tanks were filled and inspected by A & R Welding Supply (San Luis Obispo, CA, U.S.A.). The system was controlled by Windows 2000-based software (Hwelett-Packard).

Processing

Samples were prepared for SFE by mixing Celite® 566 biosilicate (Celite® Corporation, Lompoc, CA, U.S.A) in a 1:1 ratio with the powder material (buttermilk, or retentate samples processed at the D.P.T.C., Cal Poly State University, San Luis Obispo). This type of biosilicate is composed of flux calcined diatomaceous earth, and was chosen due to its large grain size, chemical inertness, non-toxicity, and availiability. It has been shown that extraction efficiency is increased by the addition of granular particles to the product (Berg et al., 1997). These particles enhance the extraction by preventing channeling of the solvent through the product during extraction, enabling for an even penetration of the sample.

The conditions of the extractions, temperatures, pressure, and flow rate, were controlled manually; Appendix C gives the stepwise protocol followed for a manual run, adapted from Thar Designs, Inc. (PA, U.S.A.). Extractions were carried out using 100 g of prepared sample product. Circulated de-ionized water at 5^{o} C was used for cooling different zones in the SFE apparatus. Carbon dioxide was used as the solvent. The temperature, pressure, and flow rate of CO_2 used for each run were varied during the optimization of the extractions. Table 3.9.1 gives the settings for the conditions of extractions on buttermilk powder (BMP); Table 3.9.2 gives the run conditions for the 5X and 3X Retentate (5X Ret, 3X Ret) extractions. At optimal conditions the pressure was set at 375 bar, and the temperatures for the back pressure regulator (BPR), heat exchanger (Heat XC), product vessel, and cyclone vessel were set to 50^{o}C, 77^{o}C, 77^{o}C, and 55^{o}C respectively; the flow rate used was 20 g/min. The actual conditions achieved during the extraction are given in parenthesis below the set conditions. Notably, the actual

temperature of the sample vessel is lower than the set temperature; the temperature profile is observed to be a slow ramp towards the set temperature during the run. The actual temperatures are given as a range, taken when the ramping stabilized after a sharp initial increase (the lower end), then where it leveled off for the remainder of the run (the higher end). The optimization was based on the actual conditions in response to a set value. The length of a given run within a series was chosen so that at the chosen flow rate, approximately three flushes of CO_2 would run through the 500 ml vessel.

The extracted fat was collected from the cyclone in a pre-weighed tin by rinsing it thoroughly with chloroform-methanol solution (1:2, v:v). The solvent was thoroughly evaporated on a hot plate at 100^{o} C, and then put under vacuum pressure of 25 Hg at 100^{o} C for 5 minutes to ensure complete evaporation of the solvent. The tin was cooled in a dessicator, weighed, and the fat present determined by weight difference of the tin. The fat was diluted to 10 mg/ml in chloroform-methanol solution (1:2, v:v), and stored in glass amber vials at -15^{o}C until analysis by TLC.

The efficiency of an extraction was determined based on the decrease in fat concentration in the starting material, and by its polar and nonpolar lipid profiles. The starting material, and post-extraction material (defatted material), were analyzed and extracted by the Mojonnier method, and analyzed by TLC following the methods described above.

In between runs using a different sample type for extraction, the product vessel and cyclone were cleaned thoroughly with warm soapy water to prevent cross contamination.

Run*	BMP 1	BMP 2	BMP 4/16	BMP 4/22
Back Pressure Regulator, P set (actual)	320 (319-324)	320 (319-324)	375 (373-378)	390 (388-395)
Back Pressure Regulator, T set (actual ramp)	60 (60)	55 (55)	55 (55)	50 (50)
Heat Exchanger, T set (actual)	80 (46-52)	65 (48-63)	75 (50-65)	83 (55-70)
Vessel T set (actual ramp)	80 (55-63)	65 (45-51)	73 (43-51)	77 (55-65)
Cyclone T set (actual ramp)	50 (45-50)	50 (45-50)	50 (48-50)	50 (48-50)
Flow Rate set (g/min) (actual)	50 (38-47)	20 (18-19)	20 (17-19)	15 (13-14)
Weight samp** (total in vessel)	150 (150)	100 (100)	102 (102)	50 (100)

* Runs are abbreviated as follows: BMP, Buttermilk Powder; followed by the run number.
** Denotes weight of actual sample only—not including celite® mixed in.

Table 3.9.1. Set conditions and actual conditions (in parenthesis) for SFE runs using buttermilk powder during optimization. Pressure is given in bar, temperature in ^{o}C, and weight in grams. Optimum conditions were determined based on extraction efficiency of the runs.

Run*	5X Ret 5/6	5X Ret 5/15	3X Ret Runs A,B, & C
Back Pressure Regulator, P set (actual)	375 (374-378)	375 (374-378)	375 (374-378)
Back Pressure Regulator, T set (actual ramp)	50 (48-50)	50 (48-50)	50 (49-50)
Heat Exchanger, T set (actual)	77 (72-80)	77 (50-82)	77 (50-85)
Vessel, T set (actual ramp)	77 (50-65)	77 (55-75)	77 (48-65)
Cyclone, T set (actual ramp)	50 (43-48)	50 (45-50)	50 (47-51)
Flow Rate, set (actual)	20 (18-19)	20 (18-19)	20 (18-19)
Weight sample** (total in vessel)	50 (100)	50 (100)	50 (100)

* Runs are abbreviated as follows: BMP, Buttermilk Powder; followed by the run number.
** Denotes weight of actual sample only, not including celite® component of mixture.

Table 3.9.2. Set conditions and actual conditions (in parenthesis) for SFE runs using spray dried retentate powders. Optimum conditions were used for extraction parameters.

3.10. Particle Size Distribution

Particle size was determined using a dynamic light scattering particle size analyzer by Beckman Coulter, model LS 230 (Miami, Florida). Buttermilk and retentate powder was dissolved to a 10% solution in phosphate buffered saline (PBS) (0.10 M),

and defatted powder materials were dissolved to a 5% solution; the carrier fluid was water. All analyses were made in duplicate, and averaged to give the reported value. Conditions for the analysis were as follows: Obscuration, 10%; PIDs Obscuration, 51%; Optical Model, Fraunhofer; pump speed, 10%; laser power, 84%, 57.8 mA; run length, 61 sec; temperature, 23.6ºC. Data was graphed by both volume percent and number percent versus particle diameter. The number percent graph describes the number of particles that are a given size, while the volume percent describes the sizes of the particles that occupy the majority of the volume.

3.11. Statistical Analysis

All statistical analyses were carried out using Minitab® software, version 12.0. Comparisons were made using Analysis of Variance, only after data was confirmed to be normal.

Chapter 4.0

Results

4.1. MICROFILTRATION – COMPOSITION ANALYSIS

COLD, RECONSTITUTED BUTTERMILK POWDER

Based on Analysis of Variance, there was no significant difference between the triplicate microfiltration runs, allowing the runs to be pooled for further analysis. The averaged compositional data with standard deviations from the analyses of the Permeate and Retentate samples through the runs are given in Table 4.1.1; Figure 4.1.1 describes these data graphically. Protein, Fat, and Ash data are expressed in percent of dry material; total solids is of the liquid sample. During the microfiltration runs using cold conditions and reconstituted buttermilk, the R3X point in the process showed the highest concentrations of both total protein, and total fat. When compared to buttermilk, the percent protein in the 3X Retentate increased from 35.46% to 83.02%, while the percent fat increased from 4.56% to 8.25 %; both significant increases (p=0.000). Compared to buttermilk, the concentration of ash in the 3X Retentate increased slightly, but not significantly from 7.49% to 7.97%.

	Avg % Prot	Std Dev % Prot	Avg % Fat	Std Dev % Fat	Avg % Ash	St Dev % Ash	Avg % TS	St Dev % TS
BM	35.46	1.49	4.56	0.45	7.49	0.52	10.05	0.50
P50	20.76	2.31	2.88	0.73	7.96	0.29	6.92	0.37
P1X	25.47	3.44	3.67	0.66	8.18	0.22	4.06	0.24
P2X	41.17	4.94	5.07	0.28	8.37	0.27	2.03	0.13
P3X	60.81	9.83	6.10	1.93	9.26	1.04	0.88	0.10
P4X	61.97	5.40	5.52	0.55	11.32	3.82	0.40	0.05
P5X	65.51	7.06	6.65	1.29	14.28	1.70	0.22	0.02
R50	43.66	1.87	5.34	0.74	7.30	0.39	9.58	3.43
R1X	53.54	1.79	6.20	0.11	7.50	0.36	4.09	0.22
R2X	62.80	10.43	6.35	0.82	7.64	0.34	2.12	0.19
R3X	83.02	7.63	8.25	2.16	7.97	0.43	1.27	0.12
R4X	79.68	2.53	6.72	1.31	8.02	0.43	0.89	0.12
R5X	82.08	5.93	7.76	1.35	7.96	0.56	0.76	0.09

Table 4.1.1. The average compositions with standard deviations for % Protein, % Fat, % Ash, and % Total Solids from the microfiltration runs using reconstituted buttermilk powder and cold conditions.

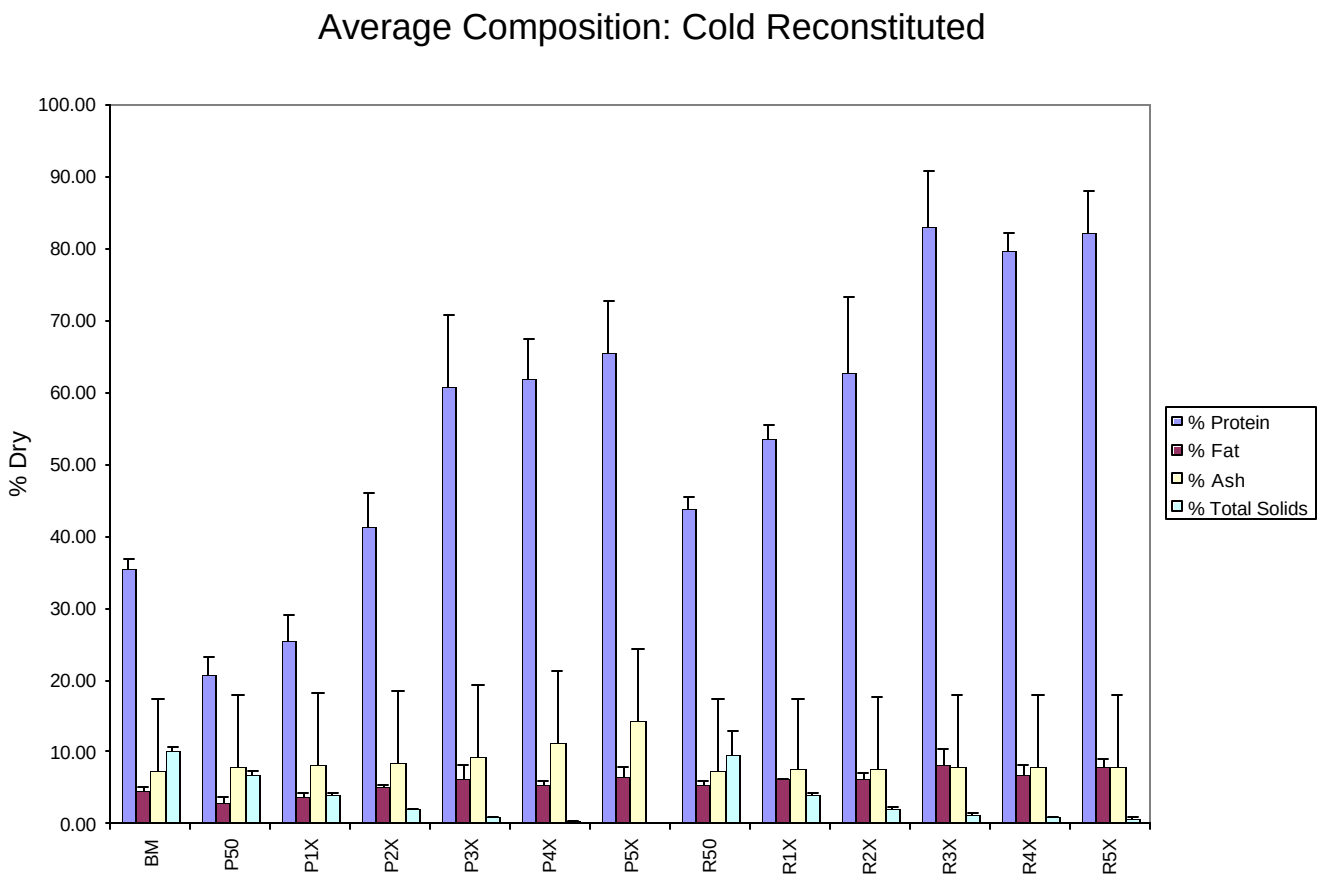


Figure 4.1.1. Average composition of percent fat, protein, ash, and total solids for the microfiltration runs using cold conditions, and reconstituted buttermilk powder.

The runs using hot conditions with reconstituted buttermilk, and hot and cold conditions using fresh buttermilk were analyzed only at the 1X, 3X, and 5X points (permeates and retentates) because it was not necessary to analyze every interval in the diafiltration process to spot the trends in the composition shifts. More importantly, it was desired to see main differences in the compositions between the various conditions of the processing.

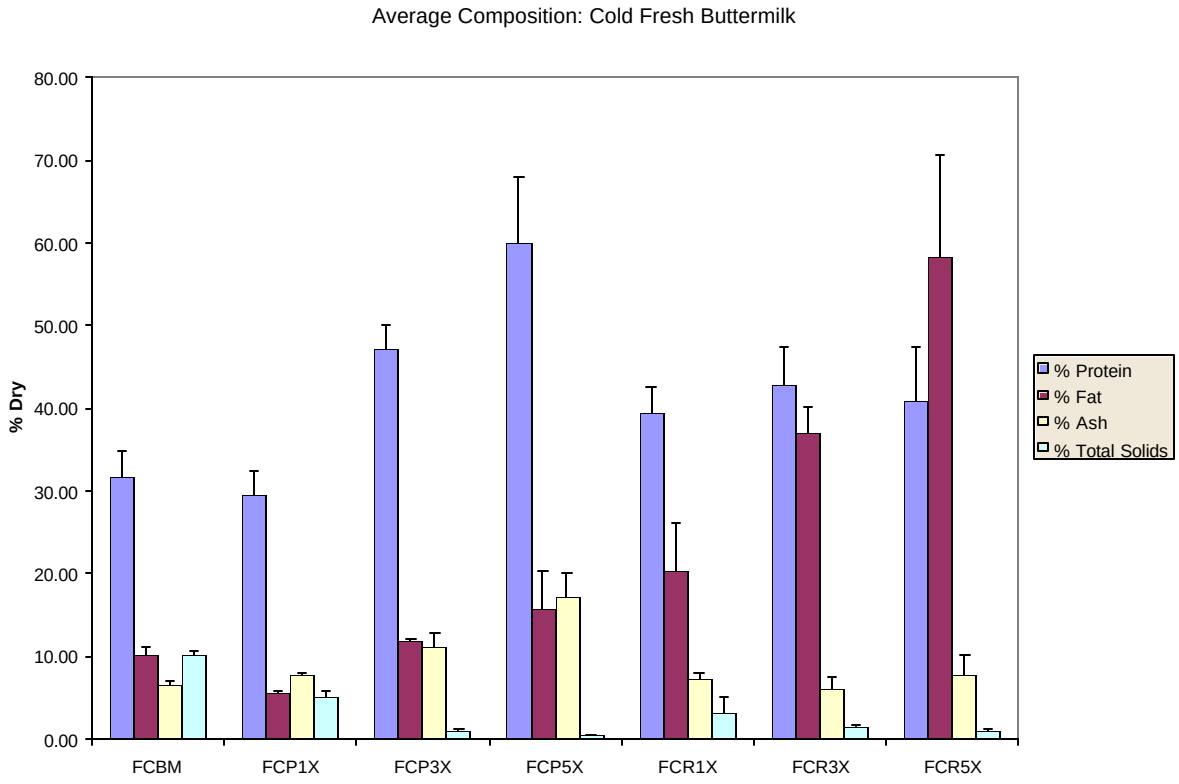
COLD, FRESH BUTTERMILK

Based on Analysis of Variance, there was no significant difference between the triplicate microfiltration runs, allowing the runs to be pooled for further analysis. The averaged data with standard deviations from the composition analyses of the Permeate and Retentate samples through the runs are given in Table 4.1.2; Figure 4.1.2 describes these data graphically. Protein, Fat, and Ash data are expressed in percent of dry material; Total Solids is of the liquid sample. The protein and fat concentrations were at their maximum at the R5X diafiltration point. From buttermilk, the increase in protein was from 31.60% to 40.76%; for fat the increase was from 10.15% to 58.28%. Compared to the cold reconstituted system, the retententions of protein and fat in the fresh system were significantly different (p=0.000).

	Avg % Prot	Std Dev % Prot	Avg % Fat	Std Dev % Fat	Avg % Ash	Std Dev % Ash	Avg % TS	Std Dev % TS
FC-BM	31.60	3.33	10.15	0.95	6.52	0.47	10.13	0.40
FC-P1X	29.39	3.02	5.55	0.27	7.75	0.28	5.04	0.77
FC-P3X	47.18	2.80	11.84	0.34	11.03	1.86	0.94	0.30
FC-P5X	60.04	7.98	15.68	4.71	17.11	2.96	0.34	0.09
FC-R1X	39.34	3.35	20.22	5.89	7.15	0.82	3.12	1.85
FC-R3X	42.84	4.61	37.12	3.14	6.02	1.53	1.36	0.29
FC-R5X	40.76	6.65	58.28	12.32	7.71	2.42	1.01	0.23

Table 4.1.2. The average compositions with standard deviations for % Protein, % Fat, % Ash, and % Total Solids from the microfiltration runs using cold conditions, and fresh buttermilk (denoted by the “F” and “C” prefix, respectively).

Figure 4.1.2. Average composition of percent fat, protein, ash, and total solids for the



microfiltration runs using cold conditions, and fresh buttermilk.

HOT, RECONSTITUTED BUTTERMILK POWDER

Based on Analysis of Variance, there was no significant difference between the duplicate microfiltration runs, allowing the runs to be pooled for further analysis. The averaged data with standard deviations from the composition analyses of the Permeate and Retentate samples through the runs are given in Table 4.1.3; Figure 4.1.3 describes these data graphically. Protein, Fat, and Ash data are expressed in percent of dry material; Total Solids is of the liquid sample. The protein concentration reached a maximum at the R5X point, increasing from 34.15% to 84.45% from buttermilk. The fat concentration reached a maximum at the R3X point, increasing from 5.14% to 9.00% from buttermilk.

	Avg % Prot	St Dev % Prot	Avg % Fat	St Dev % Fat	Avg % Ash	St Dev % Ash	Avg % TS	St Dev % TS
H-BM	34.05	0.54	5.14	0.54	7.54	0.20	9.36	0.04
H-P1X	29.80	0.39	4.56	0.27	8.10	0.10	3.87	0.29
H-P3X	37.54	0.51	5.78	0.14	8.90	0.32	2.25	0.00
H-P5X	65.65	10.45	15.17	1.93	18.24	6.09	0.21	0.01
H-R1X-pool	48.47	0.62	6.28	0.12	9.55	0.09	2.75	0.01
H-R3X-pool	57.98	1.55	9.00	No data	16.05	1.97	0.80	0.00
H-R5X-pool	84.43	4.03	0.75	No data	23.32	1.05	0.51	0.00

Table 4.1.3. The average compositions with standard deviations for % Protein, % Fat, % Ash, and % Total Solids from the microfiltration runs using hot conditions, and reconstituted buttermilk (denoted by the "H" prefix).

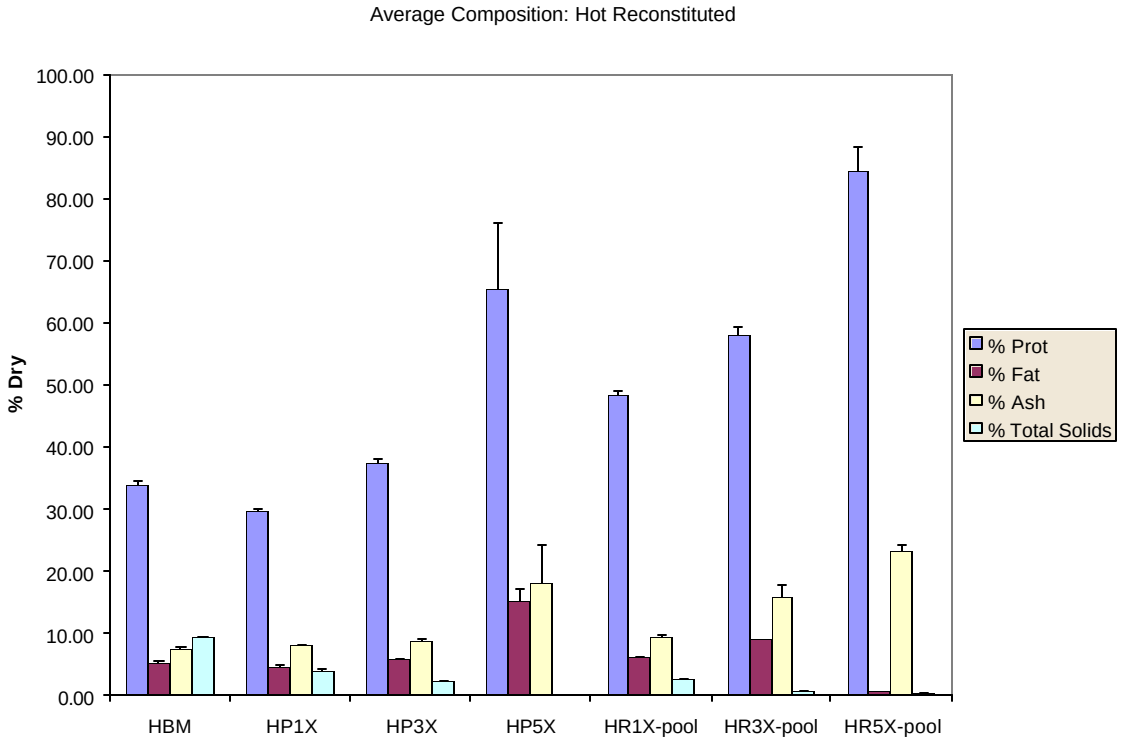


Figure 4.1.3. Average composition of percent fat, protein, ash, and total solids for the microfiltration runs using hot conditions, and reconstituted buttermilk powder.

HOT, FRESH BUTTERMILK

Based on Analysis of Variance, there was no significant difference between the triplicate microfiltration runs, allowing the runs to be pooled for further analysis. The averaged data with standard deviations from the composition analyses of the Permeate and Retentate samples through the runs are given in Table 4.1.4; Figure 4.1.4 describes these data graphically. Protein, Fat, and Ash data are expressed in percent of dry material; Total Solids is of the liquid sample. In the Retentates, the protein concentration reached a maximum at the R3X mark, increasing from 31.06 to 55.20 from buttermilk. The fat concentration was at a maximum in the original buttermilk; the system did not increase the concentration of the fat in any of the retentates.

	Avg % Prot	St Dev % Prot	Avg % Fat	St Dev % Fat	Avg % Ash	St Dev % Ash	Avg % TS	St Dev % TS
HF-BM	31.06	1.52	9.74	0.24	6.68	0.31	10.22	0.56
HF-P1X	30.96	0.30	3.39	0.17	7.56	0.21	6.61	0.18
HF-P3X	52.87	4.28	1.34	0.14	13.10	0.80	0.41	0.01
HF-P5X	82.44	10.39	0.74	0.11	35.88	3.34	0.12	0.01
HF-R1X	34.50	16.90	7.36	2.09	6.88	0.41	2.47	0.02
HF-R3X	55.20	2.65	3.24	1.71	8.73	2.10	0.72	0.02
HF-R5X	53.96	3.47	3.79	0.14	10.98	1.04	0.50	0.01

Table 4.1.4. The average compositions with standard deviations for % Protein, % Fat, % Ash, and % Total Solids from the microfiltration runs using hot conditions, and fresh buttermilk (denoted by the "H" and "F" prefix, respectively).

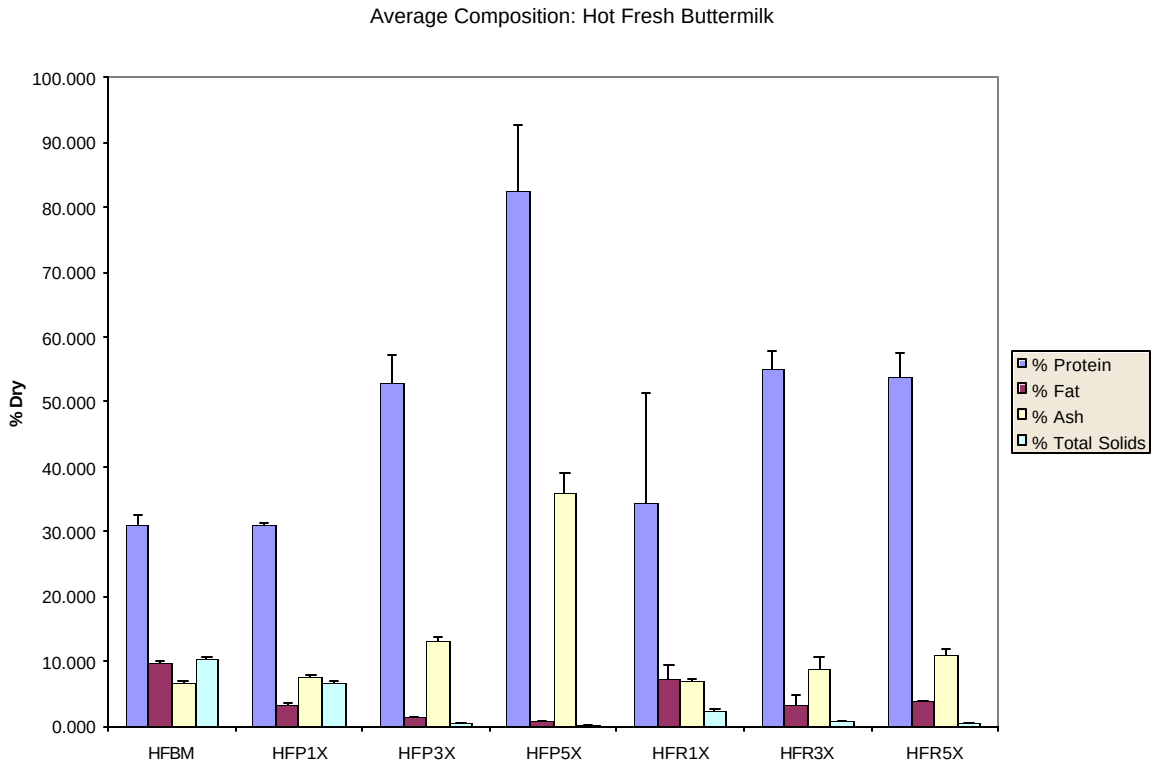


Figure 4.1.4. Average composition of percent fat, protein, ash, and total solids for the microfiltration runs using hot conditions, and fresh buttermilk.

FLUX RATES

Flux rates were observed for all runs, with a large difference seen between the processing runs using cold conditions and hot conditions. The average flux rate for the cold reconstituted and cold fresh runs were 0.24 L/min and 0.23 L/min, respectively. The average flux rate for the hot reconstituted and hot fresh runs were 0.61 L/min and 0.68 L/min respectively.

4.2. Microfiltration – Protein Profiling by SDS-PAGE

SDS-PAGE produced protein profiles of the samples collected during the microfiltration process for all four types of processing systems. Figures 4.2.1, 4.2.2, 4.2.3, and 4.2.4 show the profiles for the cold reconstituted, cold fresh, hot reconstituted,

and hot fresh microfiltration runs, respectively. The molecular weight markers have been identified, and the major protein components blocked into groups as existing as part of the MFGM, caseins, or whey proteins. As the amount of diafiltration increased, the concentration of caseins decreased in all samples; this is most clear in the hot fresh samples. While the caseins appear equal in concentration in the permeate and retentate samples after the 1X diafiltration mark, the MFGM proteins are consistently more concentrated in the Retentate samples throughout the diafiltration process. In the cold fresh samples, while the band at ~80K remains relatively stable between the Permeate and Retentate samples, the bands at ~48K and ~70K are clearly increasing with concentration in the Retentate samples with increased diafiltration. In the hot systems of both the reconstituted and fresh buttermilks, there is no distinct fractionation of the MFGM proteins between the samples, except for the P5X, which appears to contain less total protein altogether. There is a clear difference in the casein rejection between the hot and cold reconstituted runs.

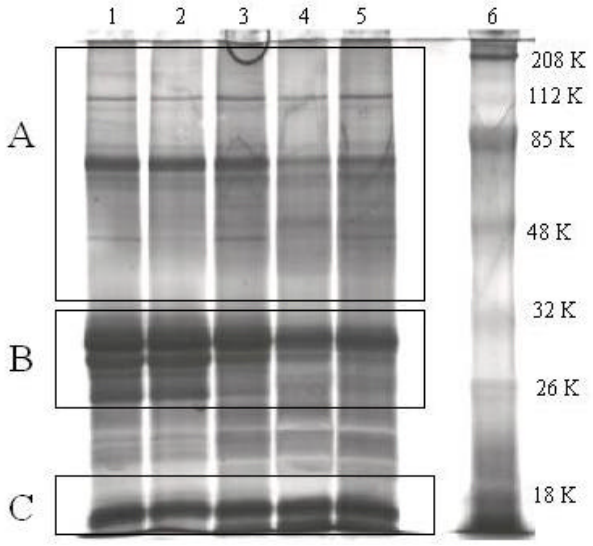


Figure 4.2.1. 12% SDS-PAGE gel of samples drawn during the reconstituted buttermilk microfiltration run using cold conditions. 0.5 µg protein per well, and stained with

coomassie brilliant blue, and silver stain reagent. Lane 1, BM; 2, P3X; 3, R3X; 4, P5X; 5, R5X; 6, MW marker. Block A, Proteins of the MFGM; B, Caseins (alpha, beta, and kappa, from top to bottom); C, whey proteins (not all visible).

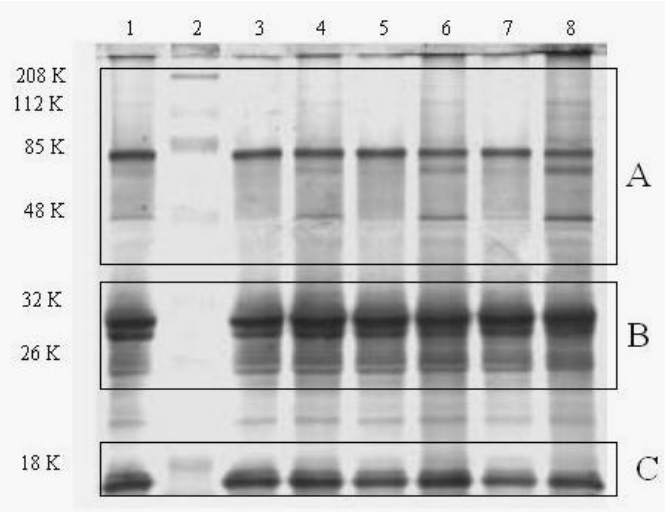


Figure 4.2.2. 12% SDS-PAGE gel of samples drawn during the fresh buttermilk microfiltration run using cold conditions. 0.5 µg protein per well, and stained with coomassie brilliant blue, and silver stain reagent. Lane 1, BM; 2, MW; 3, P1X; 4, R1X; 5, P3X; 6, R3X; 7, P5X; 8, R5X. Block A, Proteins of the MFGM; B, Caseins (alpha, beta, and kappa, from top to bottom); C, whey proteins (not all visible).

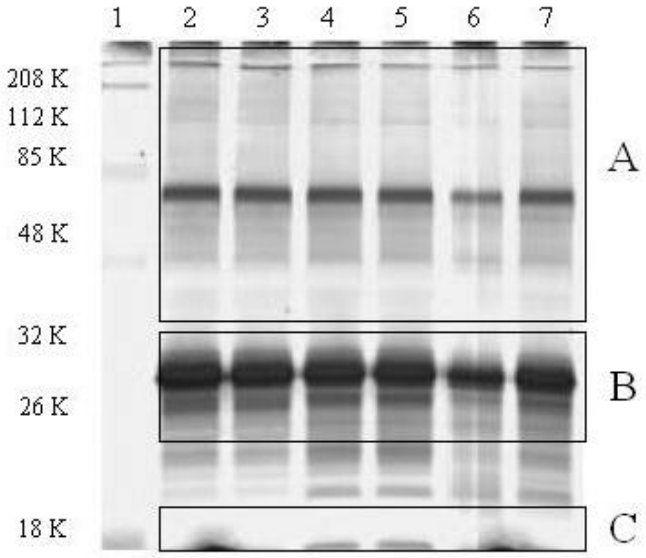


Figure 4.2.3. 12% SDS-PAGE gel of samples drawn during the reconstituted buttermilk microfiltration run using hot conditions. 0.5 µg protein per well, and stained with coomassie brilliant blue, and silver stain reagent. Lane 1, MW marker; 2, P1X; 3, R1X;

4, P3X; 5, R3X; 6, P5X; 7, R5X. Block A, Proteins of the MFGM; B, Caseins (alpha, beta, and kappa, from top to bottom); C, whey proteins (not all visible).

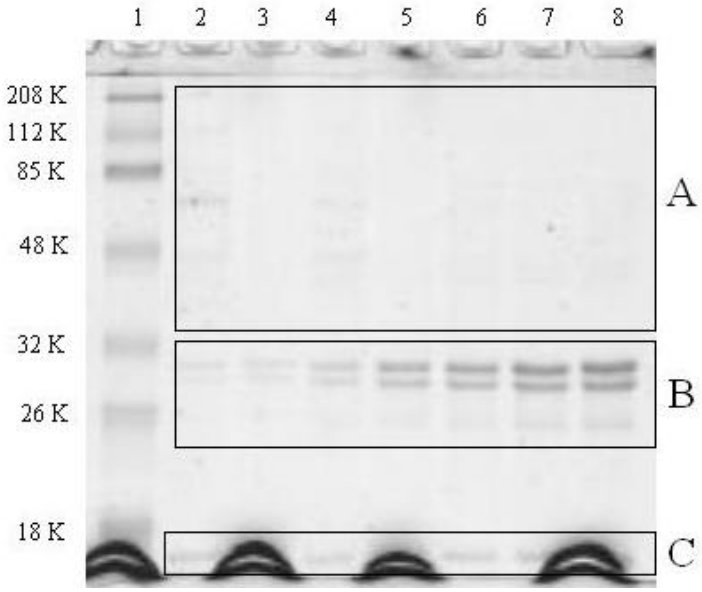


Figure 4.2.4. 12% SDS-PAGE gel of samples drawn during the fresh buttermilk microfiltration run using hot conditions. 0.5 µg protein per well, and stained with coomassie brilliant blue. Lane 1, MW marker; 2, R5X; 3, P5X; 4, R3X; 5, P3X; 6, R1X; 7, P1X; 8, BM. Block A, Proteins of the MFGM; B, Caseins (alpha, beta, and kappa, respectively); C, whey proteins (not all visible).

4.2. Microfiltration – Lipid Profiling byTLC

Cold Reconstituted Buttermilk Powder

The polar and nonpolar lipid profiles from a representative processing run using cold conditions, and reconstituted buttermilk are given in Figures 4.3.1 and 4.3.2, respectively. Though not all standards are shown on the plates, the spots have been identified, and labeled accordingly using the following abbreviations: sphingomyelin, SM; phosphatidylserine, PS; phosphatidylcholine, PC; phosphatidylehtanolamine, PE; nonpolar lipids (general), NP; monoacylglycerides, MAGs; diacylglycerides, DAGs; cholesterol, chol; free fatty acids, FFAs; triacylglycerides, TAGs; cholesterol esters, chol esters. Based on the intensity of the spots, there is no apparent concentration of the polar lipids until the 3X diafiltration point; the 3X, 4X, and 5X Retentates were more concentrated in the polar lipids than are the Permeates. Compared to

buttermilk, all Retentate samples are more concentrated in the polar lipids. The nonpolar lipid profiles do not show a clear difference between the Permeate and Retentate samples except for the P5X, which shows very little nonpolar lipid material.

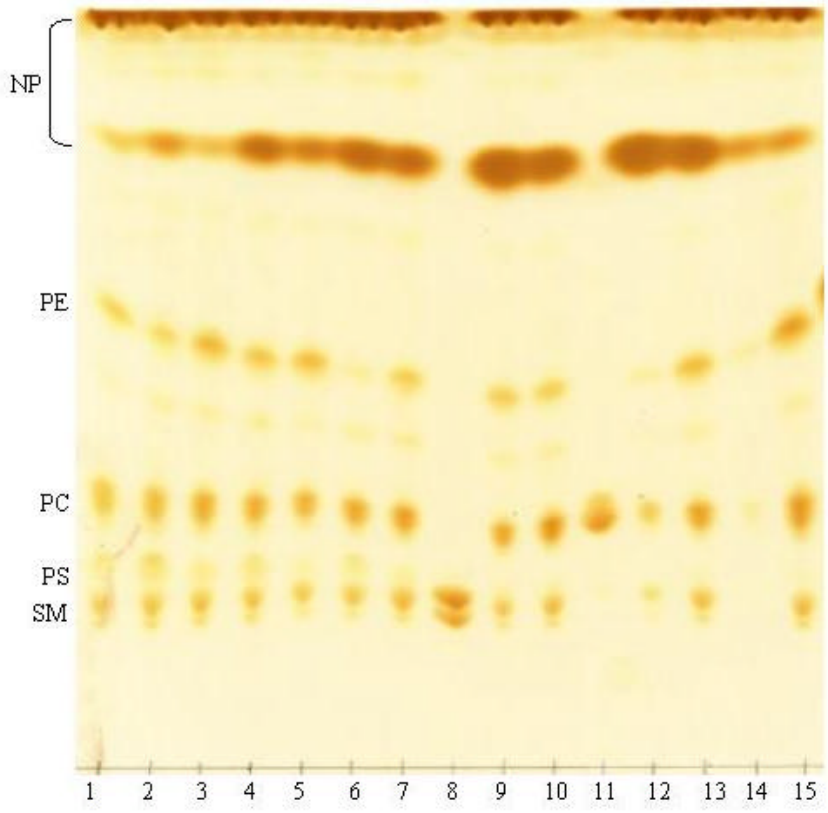


Figure 4.3.1. Polar lipid profiles of the permeate and retentate samples from a cold, reconstituted microfiltration run by TLC using Solvent System 1. Lane 1, BM; 2, P50; 3, R50; 4, P1X; 5, R1X; 6, P2X; 7, R2X; 8, SM; 9, P3X; 10, R3X; 11, PC; 12, P4X; 13, R4X; 14, P5X; 15, R5X.

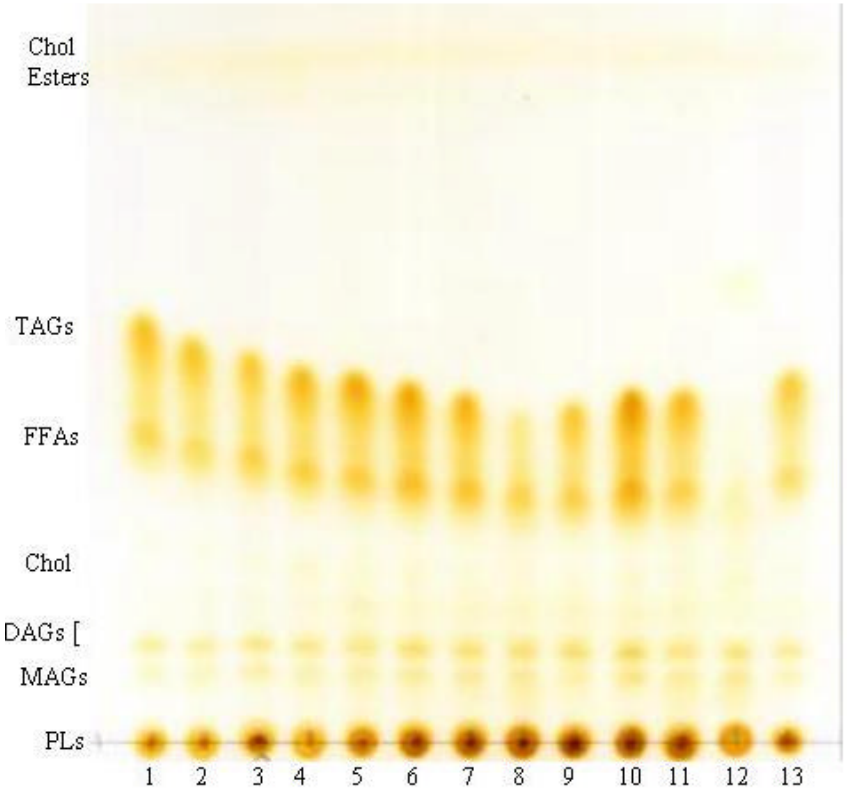


Figure 4.3.2. Nonpolar lipid profiles of the permeate and retentate samples from a cold, reconstituted microfiltration run by TLC using Solvent System 2. Lane 1, BM; 2, P50; 3, R50; 4, P1X; 5, R1X; 6, P2X; 7, R2X; 8, P3X; 9, R3X; 10, P4X; 11, R4X; 12, P5X; 13, R5X.

Cold Fresh Buttermilk

The polar and nonpolar lipid profiles from a representative processing run using cold conditions, and fresh buttermilk are given in Figures 4.3.3 and 4.3.4, respectively. Though not all standards are shown on the plates, the spots have been identified, and labeled accordingly. Based on the intensity of the spots, the fresh samples are more concentrated in nonpolar lipids than polar lipids. The Permeate samples appear slightly more concentrated in polar lipid material than do the Retentate samples; this is most evident in Figure 4.3.4 where the PLs have remained at the source.

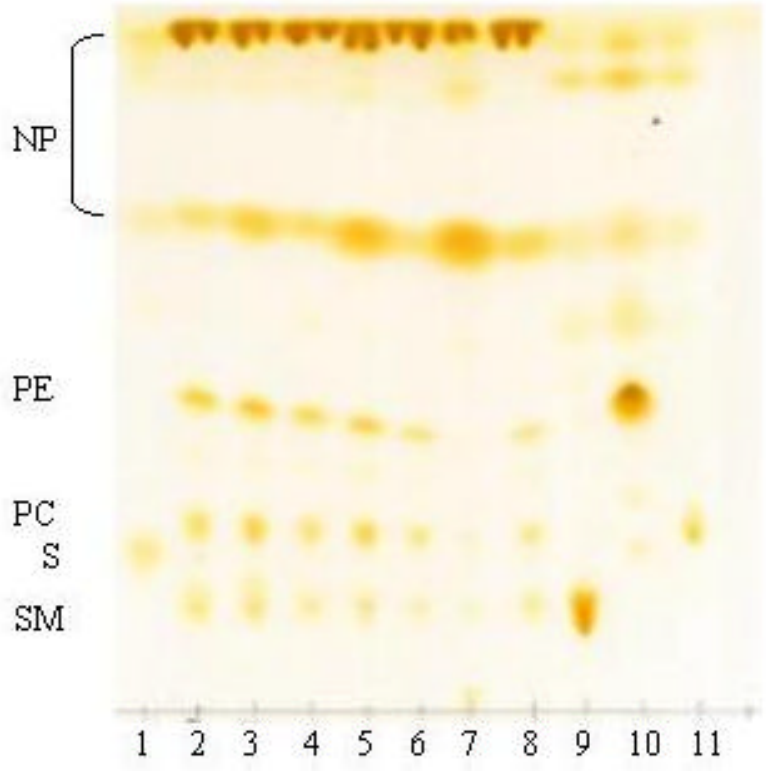


Figure 4.3.3. Polar lipid profiles of the permeate and retentate samples from a cold, fresh microfiltration run by TLC using Solvent System 1. Lane 1, S standard; 2, BM; 3, P1X; 4, R1X; 5, P3X; 6, R3X; 7, P5X; 8, R5X; 9, SM; 10, PE; 11, PC.

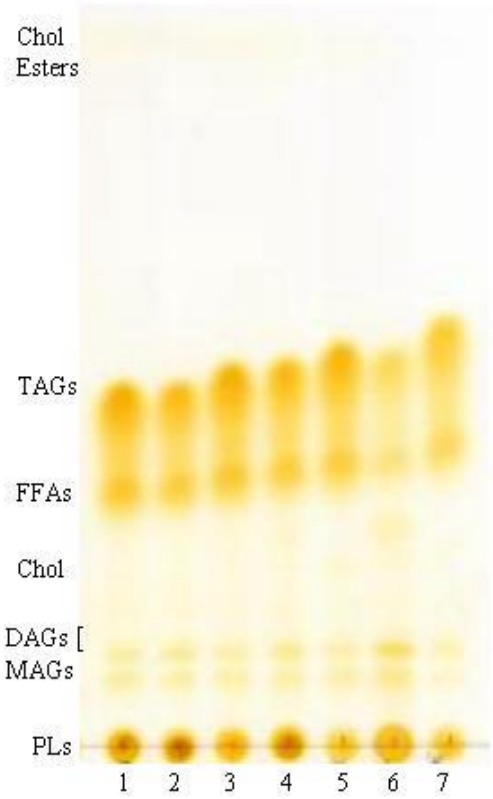


Figure 4.3.4. Nonpolar lipid profiles of the permeate and retentate samples from a cold, fresh microfiltration run by TLC using Solvent System 2. Lane 1, BM; 2, P1X; 3, R1X; 4, P3X; 5, R3X; 6, P5X; 7, R5X.

HOT RECONSTITUTED BUTTERMILK POWDER

The polar and nonpolar lipid profiles from the processing runs using hot conditions, and reconstituted buttermilk powder are given in Figures 4.3.5 and 4.3.6, respectively. Though not all standards are shown on the plates, the spots have been identified, and labeled accordingly. Lipid profiles indicated that the nonpolar lipids, particularly cholesterol, and the mono and di glycerides, were retained in higher concentrations than with cold conditions.

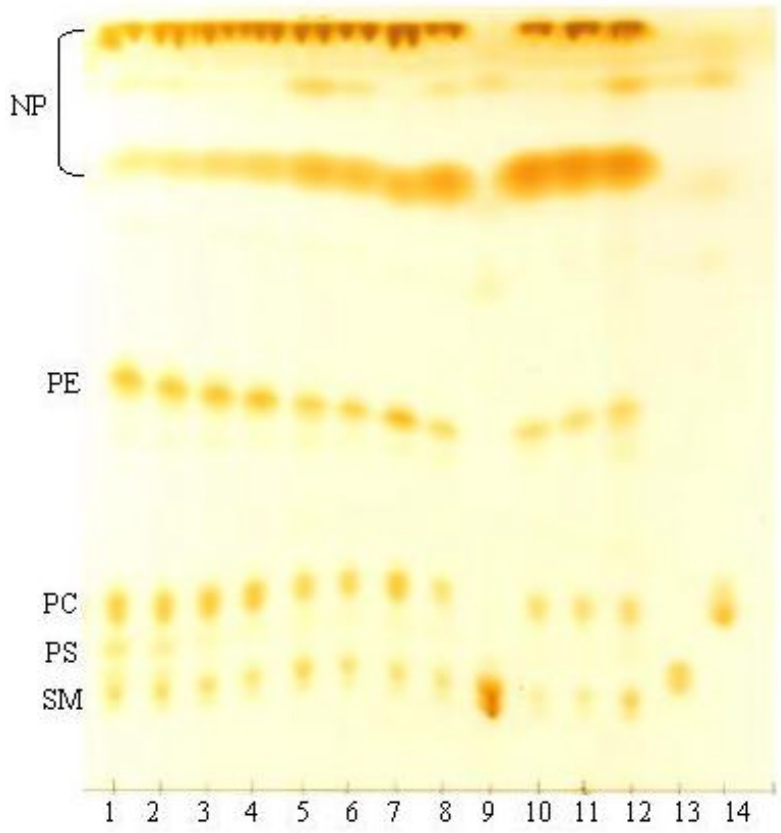


Figure 4.3.5. Polar lipid profiles of the permeate and retentate samples from the hot, reconstituted buttermilk microfiltration runs by TLC using Solvent System 1. Lane 1, BM-A; 2, BM-B; 3, P1X-A; 4, P1X-B; 5, R1X-Pooled; 6, P3X-A; 7, P3X-B; 8, R3X-Pooled; 9, SM; 10, P5X-A; 11, P5X-B; 12, R5X-Pooled; 13, PS; 14, PC.

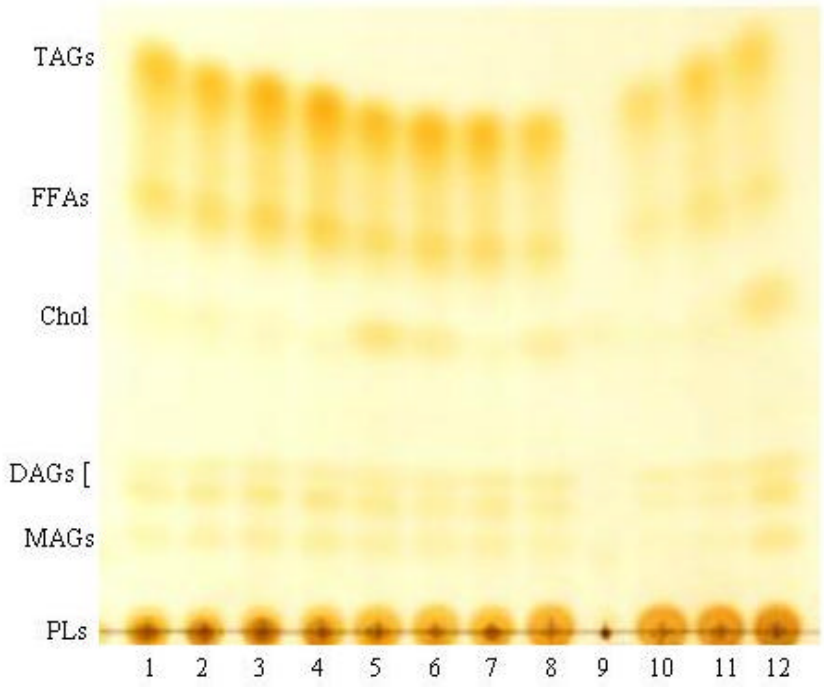


Figure 4.3.6. Nonpolar lipid profiles of the permeate and retentate samples from the hot, reconstituted buttermilk microfiltration runs by TLC using Solvent System 2. Lane 1, BM-A; 2, BM-B; 3, P1X-A; 4, P1X-B; 5, R1X-Pooled; 6, P3X-A; 7, P3X-B; 8, R3X-Pooled; 9, SM; 10, P5X-A; 11, P5X-B; 12, R5X-Pooled.

Hot, Fresh Buttermilk

The polar and nonpolar lipid profiles from a representative processing run using hot conditions, and fresh buttermilk are given in Figures 4.3.7 and 4.3.8, respectively. Though not all standards are shown on the plates, the spots have been identified, and labeled accordingly. All samples appear more concentrated with nonpolar lipid material than polar lipid material. Compared to buttermilk the Retentate samples are not as concentrated, and the Permeate samples appear to be slightly more concentrated with polar lipid material than the Retentate samples, most evident in Figure 4.3.8 at where the PLs have remained at the source.

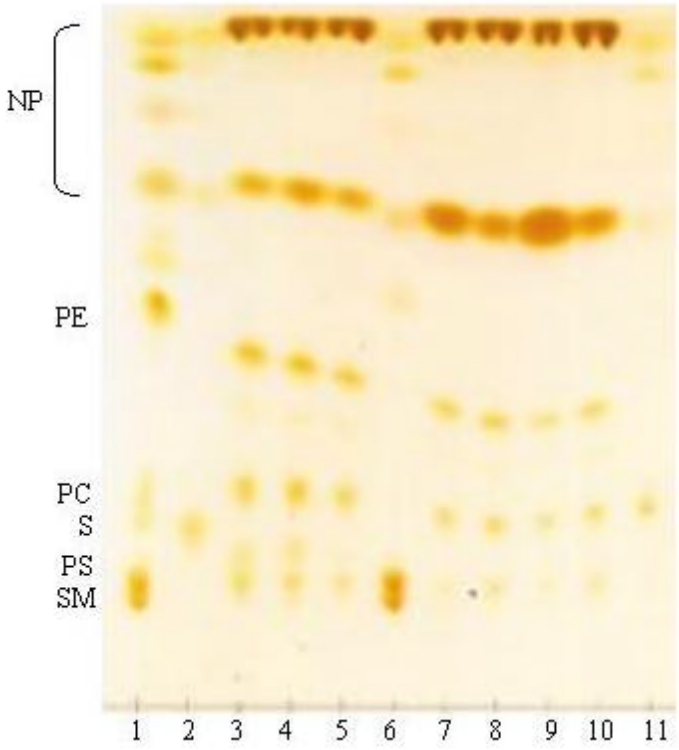


Figure 4.3.7. Polar lipid profiles of the permeate and retentate samples from a hot, fresh buttermilk microfiltration run by TLC using Solvent System 1. Lane 1, all standards; 2, S; 3, BM; 4, P1X; 5, R1X; 6, SM; 7, P3X; 8, R3X; 9, P5X; 10, R5X; 11, PC.

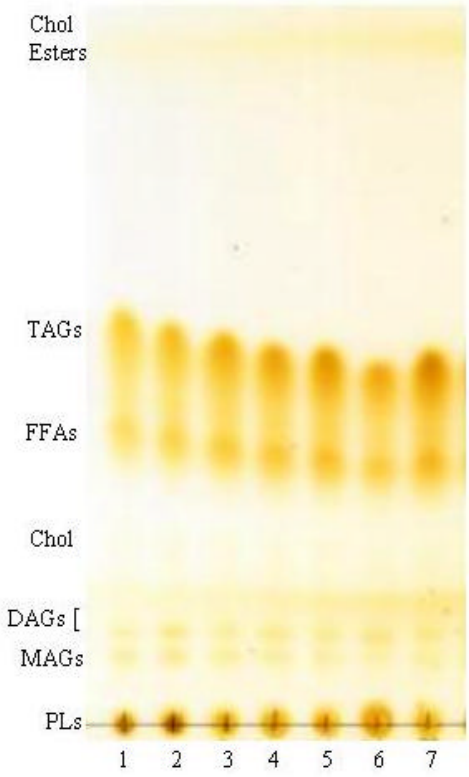


Figure 4.3.8. Nonpolar lipid profiles of the permeate and retentate samples from a hot, fresh buttermilk microfiltration run by TLC using Solvent System 2. Lane 1, BM; 2, P1X; 3, R1X; 4, P3X; 5, R3X; 6, P5X; 7, R5X.

4.4. Microfiltration – Statistical Analysis

Cold Reconstituted Buttermilk

Protein

Analysis of Variance shows how the percent protein varied significantly between the samples ($p = 0.000$, $a = 0.05$). Though the microfiltration process significantly altered the protein content among the samples, not all of the samples differed significantly from one another. The intervals given in Figure 4.4.1 show which samples vary significantly from one another; if the interval contains zero, then they are not significantly different from one another. The protein content of the Retentates from 1X,

2X, 3X, 4X, and 5X, all significantly differ from buttermilk. Figure 4.4.1 gives the Individual 95% Confidence Intervals from the analysis. It is evident how the protein content is at a maximum in the R3X sample, and how the R3X, 4X, and 5X are not significantly different from one another.

	BM	P1X	P2X	P3X	P4X	P50	P5X	R1X	R2X	R3X	R4X	R50
P1X	-2.65 22.63											
P2X	-18.35 6.92	-28.34 -3.07										
P3X	-37.99 -12.71	-47.98 -22.7	-32.27 -7									
P4X	-39.15 -13.87	-49.14 -23.86	-33.43 -8.16	-13.8 11.48								
P50	2.06 27.34	-7.93 17.35	7.78 33.05	27.41 52.69	28.57 53.85							
P5X	-43.46 -16.65	-53.44 -26.63	-37.74 -10.93	-18.1 8.71	-16.94 9.87	-58.16 -31.35						
R1X	-30.72 -5.44	-40.71 -15.43	-25 0.27	-5.37 19.91	-4.21 21.07	-45.42 -20.14	-1.43 25.38					
R2X	-39.98 -14.71	-49.97 -24.69	-34.27 -8.99	-14.63 10.65	-13.47 11.81	-54.68 -29.41	-10.7 16.11	-21.9 3.37				
R3X	-60.2 -34.93	-70.19 -44.92	-54.49 -29.21	-34.85 -9.58	-33.69 -8.42	-74.9 -49.63	-30.92 -4.11	-42.12 -16.85	-32.86 -7.58			
R4X	-56.86 -31.58	-66.85 -41.57	-51.14 -25.87	-31.51 -6.23	-30.35 -5.07	-71.56 -46.28	-27.57 -0.76	-38.78 -13.5	-29.51 -4.24	-9.29 15.98		
R50	-20.84 4.43	-30.83 -5.55	-15.13 10.15	4.51 29.79	5.67 30.95	-35.54 -10.27	8.44 35.25	-2.76 22.51	6.5 31.78	26.72 52	23.38 48.65	
R5X	-59.26 -33.99	-69.25 -43.98	-53.55 -28.27	-33.91 -8.64	-32.75 -7.48	-73.96 -48.69	-29.98 -3.17	-41.18 -15.91	-31.92 -6.64	-11.7 13.58	-15.04 10.23	-51.06 -25.78

Table 4.4.1. Intervals obtained from Analysis of Variance using Tukey's pairwise comparisons for % Protein from the cold reconstituted buttermilk microfiltration runs. Intervals containing zero are not significantly different from one another.

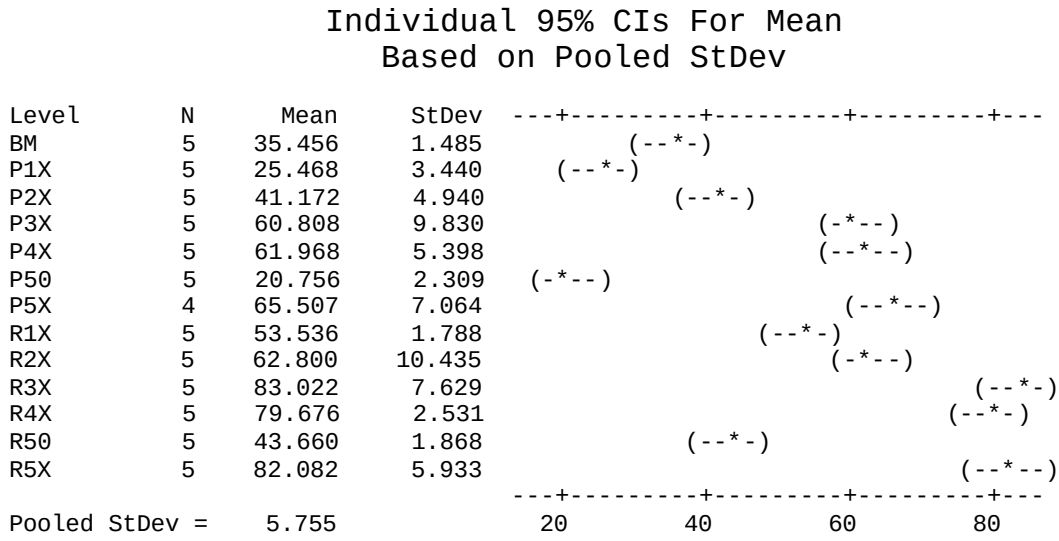


Figure 4.4.1. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Protein from the cold reconstituted buttermilk microfiltration runs.

Fat

Analysis of Variance shows how the percent fat varied significantly between the samples ($p = 0.000$, $a = 0.05$). Table 4.4.2 gives the intervals between the samples; and Figure 4.4.2 gives the Individual 95% Confidence Intervals from the analysis. It is clear that the fat content is at a maximum at the R3X point, and that the R3X, and R5X samples contain significantly more fat than does buttermilk.

	BM	P1X	P2X	P3X	P4X	P50	P5X	R1X	R2X	R3X	R4X	R50
P1X	-1.707 3.489											
P2X	-2.96 1.94	-3.999 1.197										
P3X	-4.138 1.058	-5.17 0.307	-3.628 1.568									
P4X	-3.56 1.636	-4.592 0.885	-3.05 2.146	-2.161 3.316								
P50	-0.765 4.135	-1.804 3.392	-0.255 4.645	0.627 5.823	0.049 5.245							
P5X	-4.688 0.508	-5.72 -0.243	-4.178 1.018	-3.289 2.189	-3.866 1.611	-6.373 -1.177						
R1X	-4.09 0.81	-5.129 0.067	-3.58 1.32	-2.698 2.498	-3.276 1.92	-5.775 -0.875	-2.148 3.048					
R2X	-4.24 0.66	-5.279 -0.083	-3.73 1.17	-2.848 2.348	-3.426 1.77	-5.925 -1.025	-2.298 2.898	-2.6 2.3				
R3X	-6.14 -1.24	-7.179 -1.983	-5.63 -0.73	-4.748 0.448	-5.326 -0.13	-7.825 -2.925	-4.198 0.998	-4.5 0.4	-4.35 0.55			
R4X	-4.61 0.29	-5.649 -0.453	-4.1 0.8	-3.218 1.978	-3.796 1.4	-6.295 -1.395	-2.668 2.528	-2.97 1.93	-2.82 2.08	-0.92 3.98		
R50	-3.225 1.675	-4.264 0.932	-2.715 2.185	-1.833 3.363	-2.411 2.785	-4.91 -0.01	-1.283 3.913	-1.585 3.315	-1.435 3.465	0.465 5.365	-1.065 3.835	
R5X	-5.65 -0.75	-6.689 -1.493	-5.14 -0.24	-4.258 0.938	-4.836 0.36	-7.335 -2.435	-3.708 1.488	-4.01 0.89	-3.86 1.04	-1.96 2.94	-3.49 1.41	-4.875 0.025

Table 4.4.2. Intervals obtained from Analysis of Variance using Tukey's pairwise comparisons for % Fat from the cold reconstituted buttermilk microfiltration runs. Intervals containing zero are not significantly different from one another.

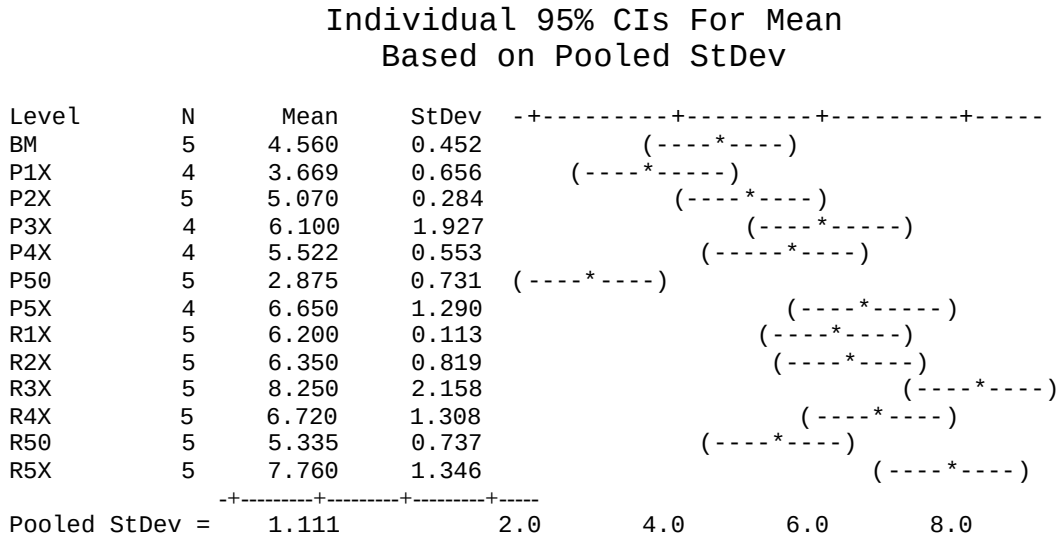


Figure 4.4.2. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Fat from the cold reconstituted buttermilk microfiltration runs.

Ash

Analysis of Variance shows how the percent fat varied significantly between the samples ($p = 0.000$, $a = 0.05$). Table 4.4.3 gives the intervals between the samples; and Figure 4.4.3 gives the Individual 95% Confidence Intervals from the analysis. The ash content of none of the Retentate samples varied significantly from buttermilk; the P2X, 3X, 4X and 5X samples all contain significantly more ash than buttemilk.

	BM	P1X	P2X	P3X	P4X	P50	P5X	R1X	R2X	R3X	R4X	R50
P1X	-1.6752											
	0.3											
P2X	-1.8656	-1.178										
	0.1096	0.7972										
P3X	-2.441	-1.7534	-1.563									
	-0.3459	0.3417	0.5321									
P4X	-4.3432	-3.6556	-3.4652	-2.9956								
	-1.7302	-1.0426	-0.8522	-0.2909								
P50	-1.453	-0.7654	-0.575	-0.1195	1.2648							
	0.5222	1.2098	1.4002	1.9756	3.8778							
P5X	-7.1157	-6.4281	-6.2377	-5.7789	-4.3839	-6.6503						
	-5.0207	-4.3331	-4.1427	-3.5706	-1.6791	-4.5553						
R1X	-0.9936	-0.306	-0.1156	0.3399	1.7242	-0.5282	5.0147					
	0.9816	1.6692	1.8596	2.435	4.3372	1.447	7.1097					
R2X	-1.1384	-0.4508	-0.2604	0.1951	1.5794	-0.673	4.8699	-1.1324				
	0.8368	1.5244	1.7148	2.2902	4.1924	1.3022	6.9649	0.8428				
R3X	-1.468	-0.7804	-0.59	-0.1345	1.2498	-1.0026	4.5403	-1.462	-1.3172			
	0.5072	1.1948	1.3852	1.9606	3.8628	0.9726	6.6353	0.5132	0.658			
R4X	-1.5162	-0.8286	-0.6382	-0.1827	1.2016	-1.0508	4.4921	-1.5102	-1.3654	-1.0358		
	0.459	1.1466	1.337	1.9124	3.8146	0.9244	6.5871	0.465	0.6098	0.9394		
R50	-0.8018	-0.1142	0.0762	0.5317	1.916	-0.3364	5.2065	-0.7958	-0.651	-0.3214	-0.2732	
	1.1734	1.861	2.0514	2.6268	4.529	1.6388	7.3015	1.1794	1.3242	1.6538	1.702	
R5X	-1.4626	-0.775	-0.5846	-0.1291	1.2552	-0.9972	4.5457	-1.4566	-1.3118	-0.9822	-0.934	-1.6484
	0.5126	1.2002	1.3906	1.966	3.8682	0.978	6.6407	0.5186	0.6634	0.993	1.0412	0.3268

Table 4.4.3. Intervals obtained from Analysis of Variance using Tukey's pairwise comparisons for % Ash from the cold reconstituted buttermilk microfiltration runs. Intervals containing zero are not significantly different from one another.

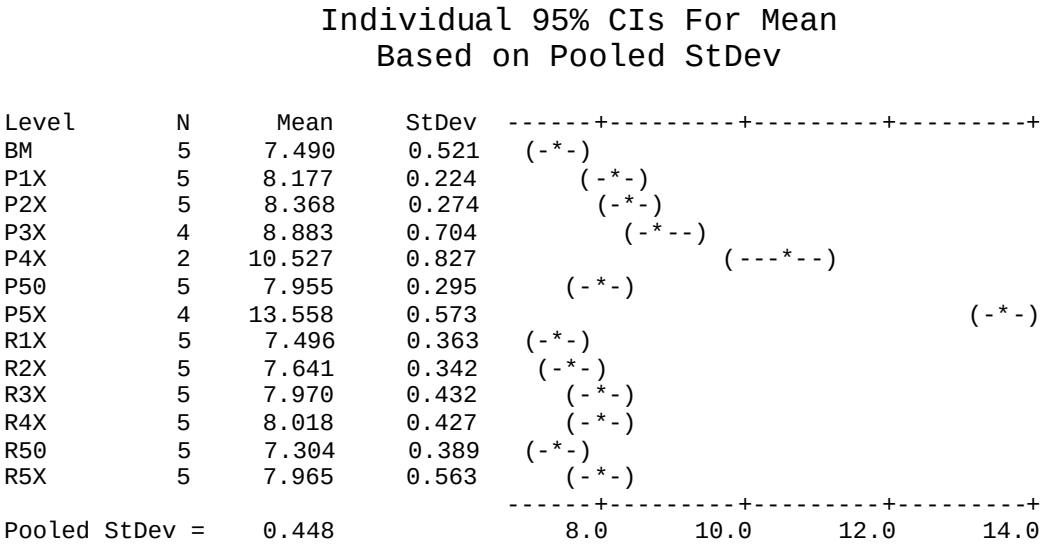


Figure 4.4.3. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Ash from the cold reconstituted buttermilk microfiltration runs.

Total Solids

Analysis of Variance shows how the percent total solids varied significantly between the samples ($p = 0.000$, $a = 0.05$). Table 4.4.4 gives the intervals between the samples; and Figure 4.4.4 gives the Individual 95% Confidence Intervals from the analysis. For both the Retentate and Permeate samples, as diafiltration increased, the total solids content decreased.

	BM	P1X	P2X	P3X	P4X	P50	P5X	R1X	R2X	R3X	R4X
P1X	5.814										
	6.607										
P2X	7.846	1.658									
	8.639	2.406									
P3X	8.998	2.81	0.777								
	9.791	3.557	1.525								
P4X	9.478	3.29	1.258	0.107							
	10.271	4.038	2.006	0.855							
P50	2.959	-3.229	-5.261	-6.412	-6.893						
	3.752	-2.481	-4.513	-5.664	-6.145						
P5X	9.659	3.471	1.439	0.288	-0.193	6.326					
	10.452	4.219	2.187	1.036	0.555	7.074					
R1X	5.79	-0.398	-2.43	-3.581	-4.062	2.457	-4.243				
	6.583	0.35	-1.682	-2.833	-3.314	3.205	-3.495				
R2X	7.759	1.571	-0.461	-1.612	-2.093	4.426	-2.274	1.595			
	8.552	2.319	0.287	-0.865	-1.345	5.174	-1.526	2.343			
R3X	8.611	2.423	0.391	-0.761	-1.241	5.278	-1.422	2.447	0.478		
	9.404	3.17	1.138	-0.013	-0.494	6.025	-0.675	3.194	1.225		
R4X	8.988	2.8	0.768	-0.383	-0.864	5.655	-1.045	2.824	0.855	0.003	
	9.781	3.548	1.515	0.364	-0.117	6.402	-0.298	3.571	1.603	0.751	
R5X	9.116	2.928	0.896	-0.255	-0.736	5.783	-0.917	2.952	0.983	0.131	-0.246
	9.909	3.676	1.643	0.492	0.011	6.53	-0.17	3.699	1.731	0.879	0.502

Table 4.4.4. Intervals obtained from Analysis of Variance using Tukey's pairwise comparisons for % Total Solids from the cold reconstituted buttermilk microfiltration runs. Intervals containing zero are not significantly different from one another.

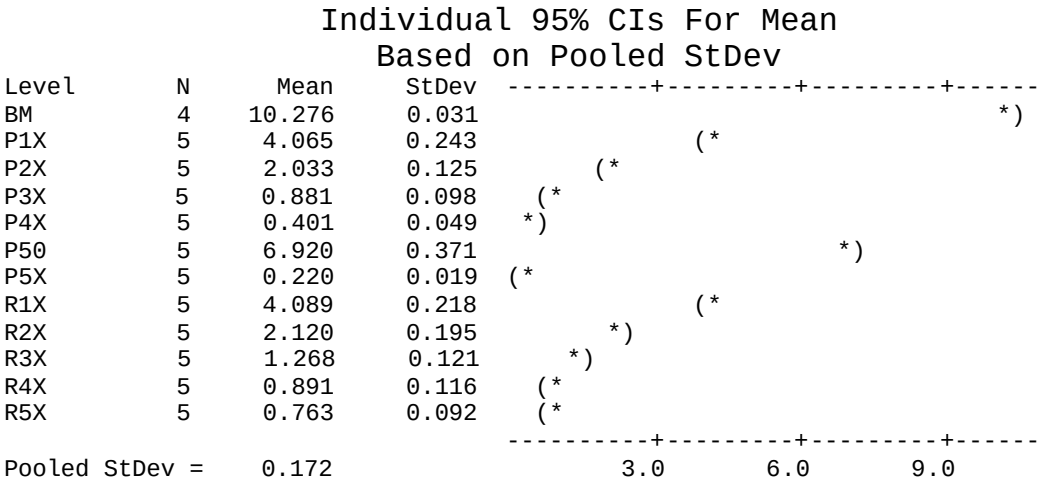


Figure 4.4.4. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Total Solids from the cold reconstituted buttermilk microfiltration runs.

Protein

Analysis of Variance showed how the percent protein of the samples varied significantly between the cold reconstituted buttermilk powder microfiltration runs, and the cold fresh buttermilk microfiltration runs ($p = 0.000$, $a = 0.05$). Figure 4.4.5 gives the Individual 95% Confidence Intervals from the analysis. It is clear that the protein content differed significantly between the cold reconstituted and cold fresh microfiltration runs.

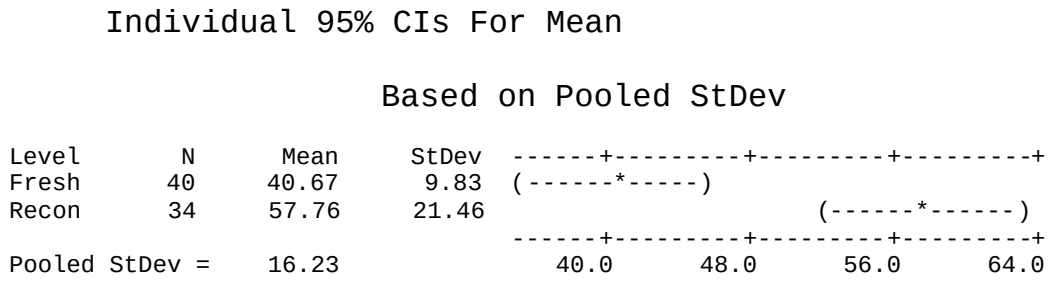


Figure 4.4.5 Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Protein from the cold reconstituted buttermilk, and fresh buttermilk microfiltration runs.

Fat

Analysis of Variance showed how the percent fat of the samples varied significantly between the cold reconstituted buttermilk powder microfiltration runs, and the cold fresh buttermilk microfiltration runs ($p = 0.000$, $a = 0.05$). Figure 4.4.6 gives the Individual 95% Confidence Intervals from the analysis. It is clear that the fat content differed significantly between the cold reconstituted and cold fresh runs.

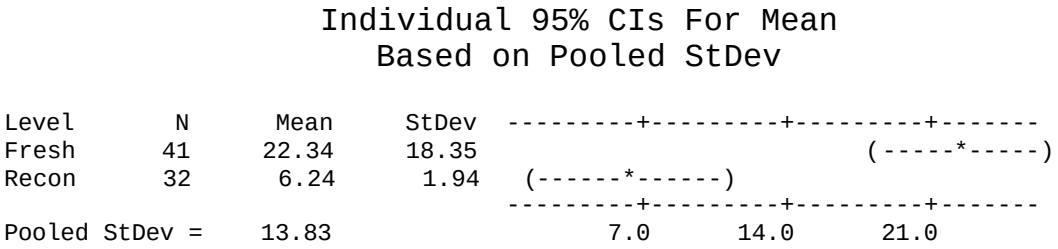


Figure 4.4.6. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Fat from the cold reconstituted buttermilk, and fresh buttermilk microfiltration runs.

Ash

Analysis of Variance showed how the percent ash of the samples did not vary significantly between the cold reconstituted buttermilk powder microfiltration runs, and the cold fresh buttermilk microfiltration runs ($p = 0.904$, $a = 0.05$). Figure 4.4.7 gives the Individual 95% Confidence Intervals from the analysis.

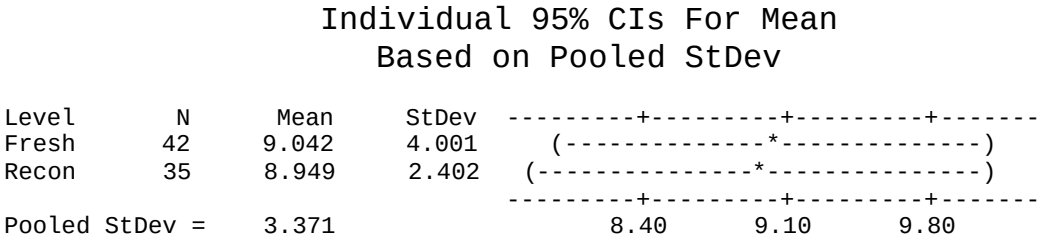


Figure 4.4.7. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Ash from the cold reconstituted buttermilk, and fresh buttermilk microfiltration runs.

Total Solids

Analysis of Variance showed how the percent total solids of the samples did not vary significantly between the cold reconstituted buttermilk powder microfiltration runs, and the cold fresh buttermilk microfiltration runs ($p = 0.908$, $a = 0.05$). Figure 4.4.8 gives the Individual 95% Confidence Intervals from the analysis.

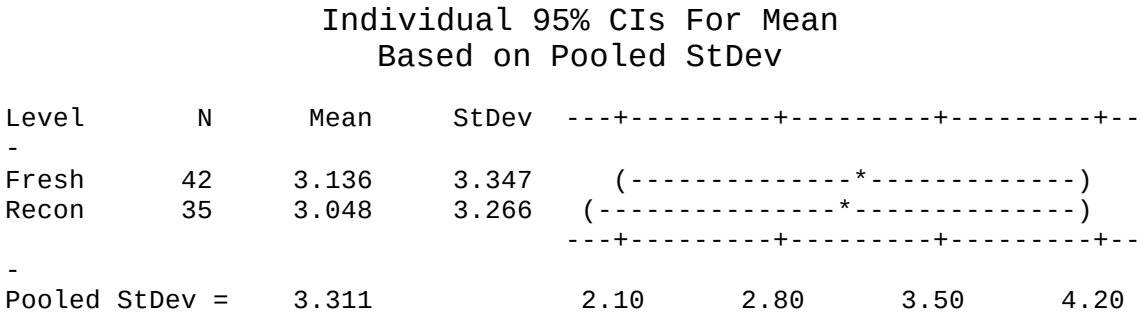


Figure 4.4.8. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Total Solids from the cold reconstituted buttermilk, and fresh buttermilk microfiltration runs.

COLD RECONSTITUTED VERSUS HOT RECONSTITUTED BUTTERMILK

Protein

Analysis of Variance showed how the percent protein of the samples did not vary significantly between the hot reconstituted buttermilk powder microfiltration runs, and the cold reconstituted buttermilk microfiltration runs ($p = 0.076$, $a = 0.05$). Figure 4.4.9 gives the Individual 95% Confidence Intervals from the analysis.

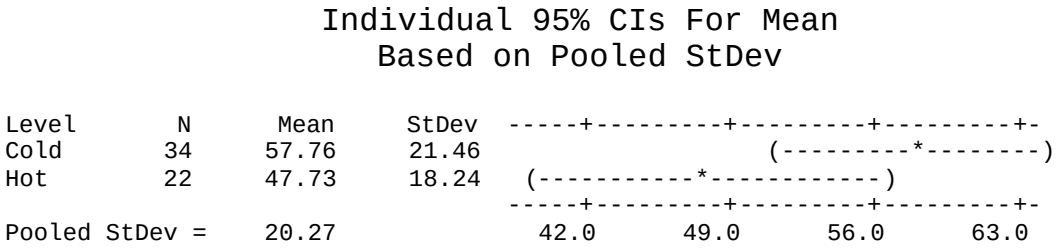


Figure 4.4.9. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Protein from the cold reconstituted buttermilk, and hot reconstituted buttermilk microfiltration runs.

Fat

Analysis of Variance showed how the percent fat the samples did not vary significantly between the hot reconstituted buttermilk powder microfiltration runs, and the cold reconstituted buttermilk microfiltration runs (p = 0.260, = 0.05). Figure 4.4.10 gives the Individual 95% Confidence Intervals from the analysis.

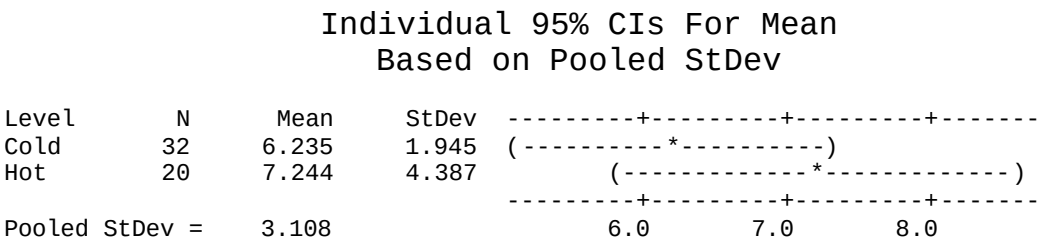


Figure 4.4.10. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Fat from the cold reconstituted buttermilk, and hot reconstituted buttermilk microfiltration runs.

Ash

Analysis of Variance showed how the percent ash the samples did not vary significantly between the hot reconstituted buttermilk powder microfiltration runs, and the cold reconstituted buttermilk microfiltration runs (p = 0.005, = 0.05). Figure 4.4.11 gives the Individual 95% Confidence Intervals from the analysis.

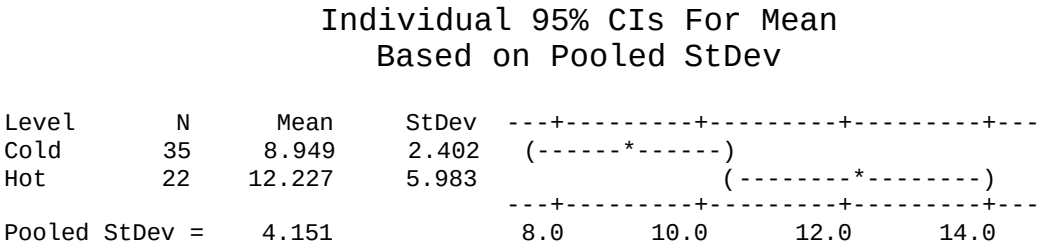


Figure 4.4.11. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Ash from the cold reconstituted buttermilk, and hot reconstituted buttermilk microfiltration runs.

Total Solids

Analysis of Variance showed how the percent total solids the samples varied significantly between the hot reconstituted buttermilk powder microfiltration runs, and the cold reconstituted buttermilk microfiltration runs (p = 0.845, = 0.05). Figure 4.4.12 gives the Individual 95% Confidence Intervals from the analysis.

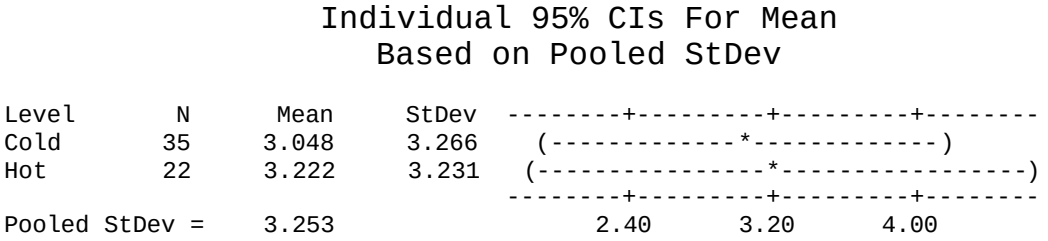


Figure 4.4.12. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Total Solids from the cold reconstituted buttermilk, and hot reconstituted buttermilk microfiltration runs.

Cold Fresh versus Hot Fresh Buttermilk

Protein

Analysis of Variance showed how the percent protein of the samples did not vary significantly between the cold fresh buttermilk microfiltration runs, and the hot fresh buttermilk microfiltration runs ($p = 0.097$, $a = 0.05$). Figure 4.4.13 gives the Individual 95% Confidence Intervals from the analysis.

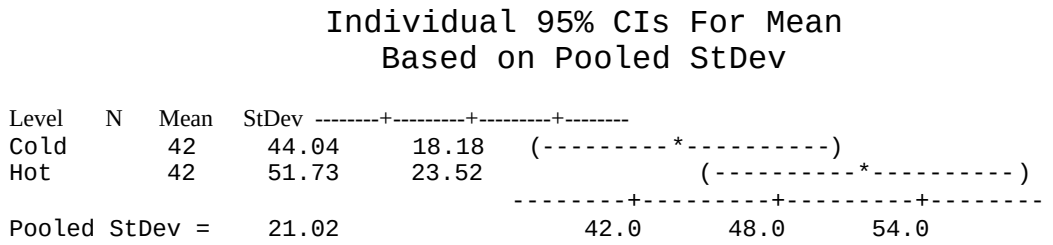


Figure 4.4.13 Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Protein from the cold reconstituted buttermilk, and hot reconstituted buttermilk microfiltration runs.

Fat

Analysis of Variance showed how the percent fat of the samples varied significantly between the cold fresh buttermilk microfiltration runs, and the hot fresh buttermilk microfiltration runs ($p = 0.000$, $a = 0.05$). Figure 4.4.14 gives the Individual 95% Confidence Intervals from the analysis.

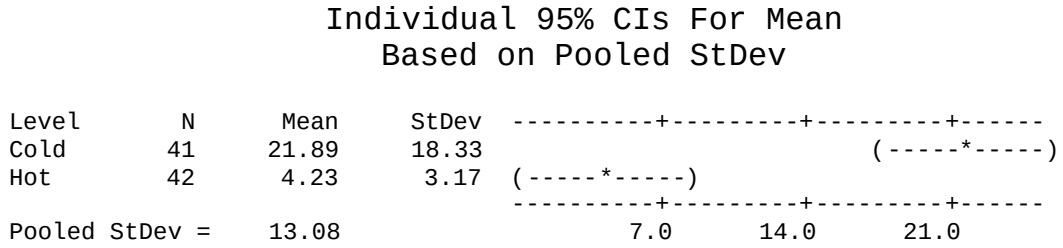


Figure 4.4.14. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Fat from the cold fresh buttermilk, and hot fresh buttermilk microfiltration runs.

Ash

Analysis of Variance showed how the percent ash of the samples did not vary significantly between the cold fresh buttermilk microfiltration runs, and the hot fresh buttermilk microfiltration runs ($p = 0.193$, $a = 0.05$). Figure 4.4.15 gives the Individual 95% Confidence Intervals from the analysis.

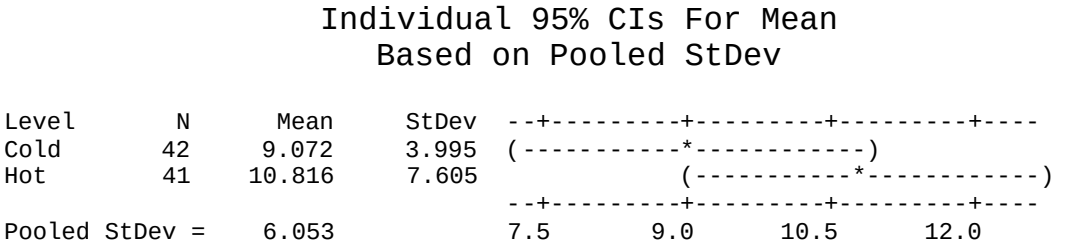


Figure 4.4.15. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Ash from the cold fresh buttermilk, and hot fresh buttermilk microfiltration runs.

Total Solids

Analysis of Variance showed how the percent total solids of the samples did not vary significantly between the cold fresh buttermilk microfiltration runs, and the hot fresh buttermilk microfiltration runs ($p = 0.739$, $a = 0.05$). Figure 4.4.16 gives the Individual 95% Confidence Intervals from the analysis.

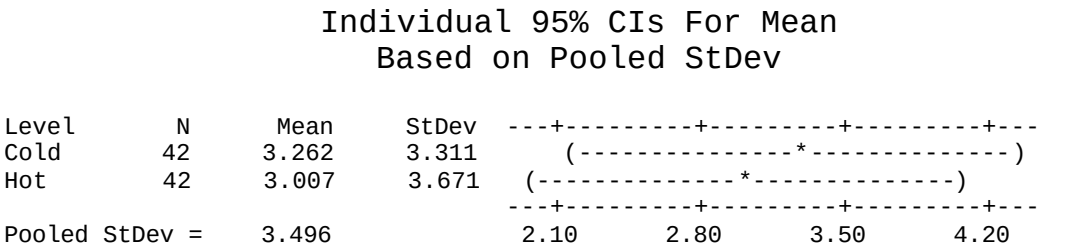


Figure 4.4.16. Individual 95% Confidence Intervals obtained from Analysis of Variance using Tukey's Pairwise comparisons for % Total Solids from the cold fresh buttermilk, and hot fresh buttermilk microfiltration runs.

4.5. Supercritical Fluid Extraction

Optimization

See Tables 3.9.1 and 3.9.2 (in Materials and Methods section) for the temperature, pressure, and flow rate conditions during the optimization of the extraction process on buttermilk powder, and retentate materials. Optimal conditions were determined to be; 375 bar, 48-60^{o}C temperature range, 20g/min, 75 min, over 3 extractions. At these conditions, the polar lipid profiles of the defatted materials were more concentrated than with the conditions using lower temperatures, and/or pressures.

Lipid Composition

Based on Analysis of Variance using Tukey's pairwise comparisons, SFE significantly reduced the total fat content in the buttermilk, 5X Retentate, and 3X Retentate powders ($p = 0.000$, $a = .05$). Figure 4.5.1 shows the individual 95% confidence intervals for the % fat before and after SFE. All replicated runs were combined; the average fat compositions of all samples before and after SFE, including the % fat retained are given in Table 4.5.1; Figure 4.5.2 shows these data graphically with standard deviations.

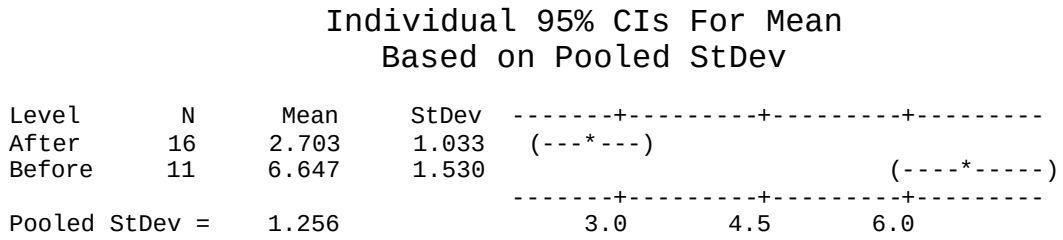


Figure 4.5.1. The individual confidence intervals for the comparison of % Fat before and after SFE are shown.

Sample	% Fat Before SFE	% Fat After SFE	% Fat Retained
Buttermilk Powder	4.965	1.705	34.34
5X Retentate Powder	7.062	1.910	27.05
3X Retentate Powder	8.975	3.417	38.08

Table 4.5.1. The average fat before and after SFE, including the % fat retained. SFE significantly reduced the total fat content in all samples. The 3X Retentate showed the greatest retention of fat, the composition of which is primarily polar lipids (shown in the lipid profiles in this section).

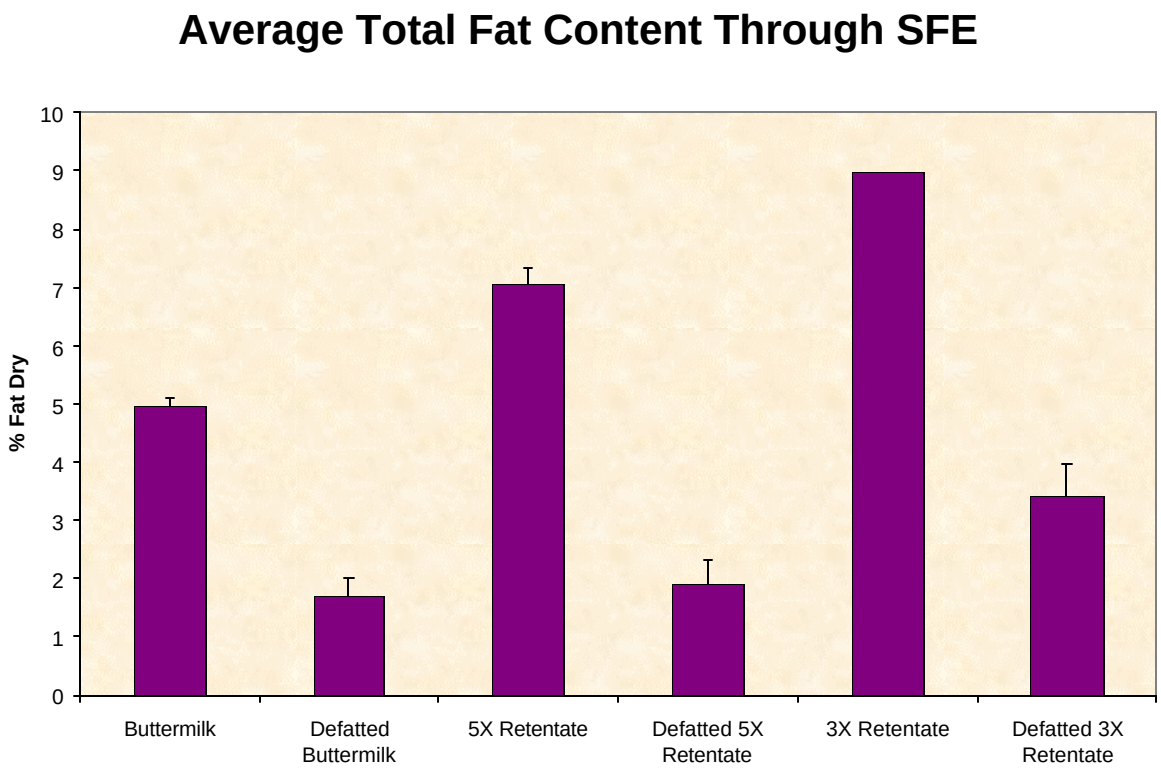


Figure 4.5.2. The average total fat content before and after SFE are shown with standard deviations.

SFE LIPID PROFILES BY TLC

The polar and nonpolar lipid profiles from TLC of SFE trials of buttermilk from 4/16, and 4/22 are given in Figures 4.5.3 and 4.5.4, respectively. Though not all standards are shown on the plates, the spots have been identified, and labeled accordingly.

The Defatted materials showed an increase in concentration of the polar lipids, and a slight decrease in nonpolar lipids; the fat removed by SFE contained only nonpolar lipids. The defatted buttermilk from trial 4/16 is more concentrated with SM, PS, S, and PE, and less concentrated with PC than it is from trial 4/22. Compared to buttermilk, both defatted trials are more concentrated in all polar lipids except for PE.

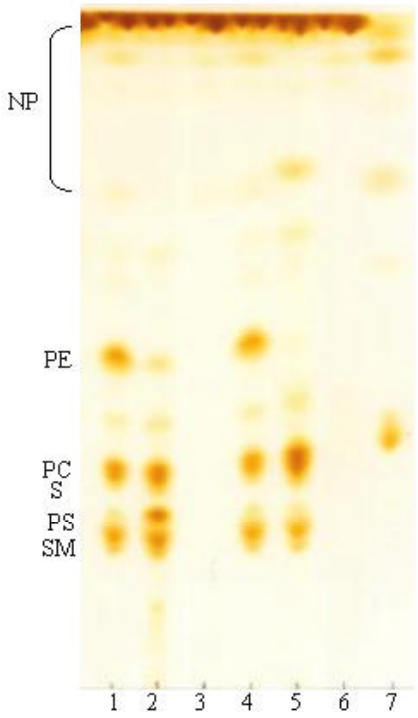


Figure 4.5.3. Polar lipid profiles from the SFE trials using prepared buttermilk powder (mixed in a 1:1 ratio with celite® 566) from 4/16, and 4/22, using Solvent System 1. Lane 1, BM; 2, DFBM 4/16; 3, Fat extracted 4/16; 4, BM; 5, DFBM 4/22; 6, Fat extracted 4/22; 7, PC.

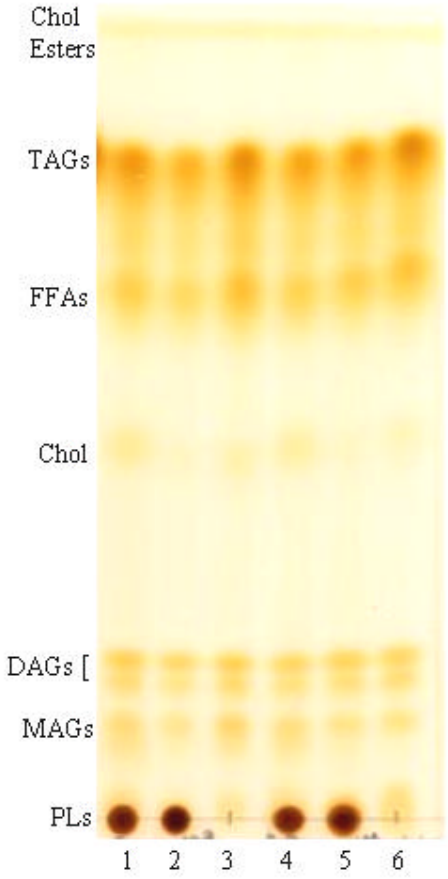


Figure 4.5.4. Nonpolar lipid profiles from the SFE trials using prepared buttermilk powder from 4/16, and 4/22, using Solvent System 2. Lane 1, BM; 2, DFBM 4/16; 3, Fat extracted 4/16; 4, BM; 5, DFBM 4/22; 6, Fat extracted 4/22.

The polar and nonpolar lipid profiles of a representative SFE of 5X Retentate are shown in Figures 4.5.5 and 4.5.6, respectively. The Defatted 5X Retentate is more concentrated in polar lipids, and less concentrated in nonpolar lipids than before SFE; the lipid removed was exclusively nonpolar. Compared to buttermilk and the 5X Retentate, the defatted 5X Ret is more concentrated in all polar lipid species. There was no difference between the lipid profiles of the 5X Retentate analyzed with and without celite® 566.

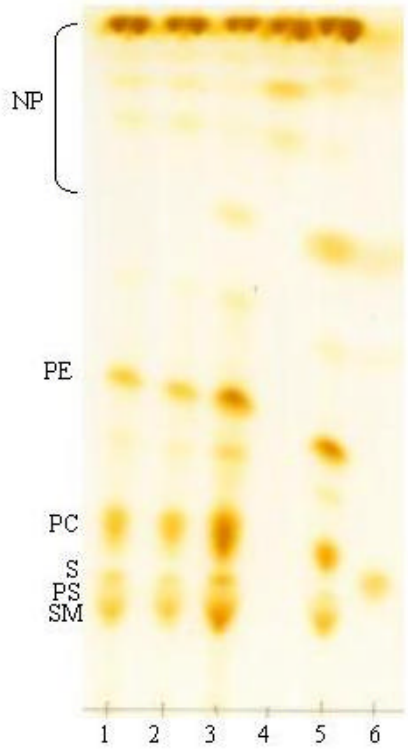


Figure 4.5.5. Polar lipid profiles from the SFE trial with 5X Retentate from 5/15 using Solvent System 1. Lane 1, 5X Ret; 2, 5X Ret with celite®; 3, DF 5X Ret 5/15; 4, fat extracted; 5, BM; 6, S.

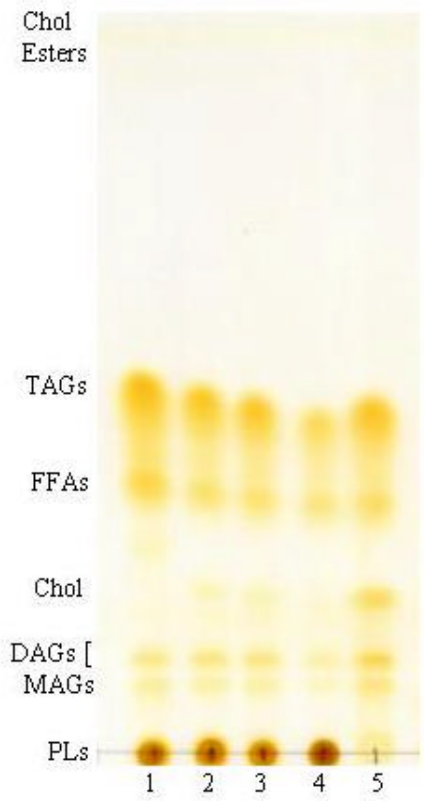


Figure 4.5.6. Nonpolar lipid profiles from SFE with 5X Retentate from 5/15 using Solvent System 2. Lane 1, BM; 2, 5X Ret; 3, 5X Ret with celite®; 4, DF 5X Ret 5/15; 5, fat extracted.

The polar and nonpolar lipid profiles of all three SFEs of 3X Retentate (trials A, B, and C) are shown in Figures 4.5.7 and 4.5.8, respectively. All three Defatted 3X samples have increased concentrations of polar lipids, and decreased concentration of nonpolar lipids compared to before SFE, and buttemrilk. The extracted lipid was composed exclusively of nonpolar lipids. In the Defatted 3X from trial A, there is a spot present that is slightly polar (above PE) that is not present in the other two trials.

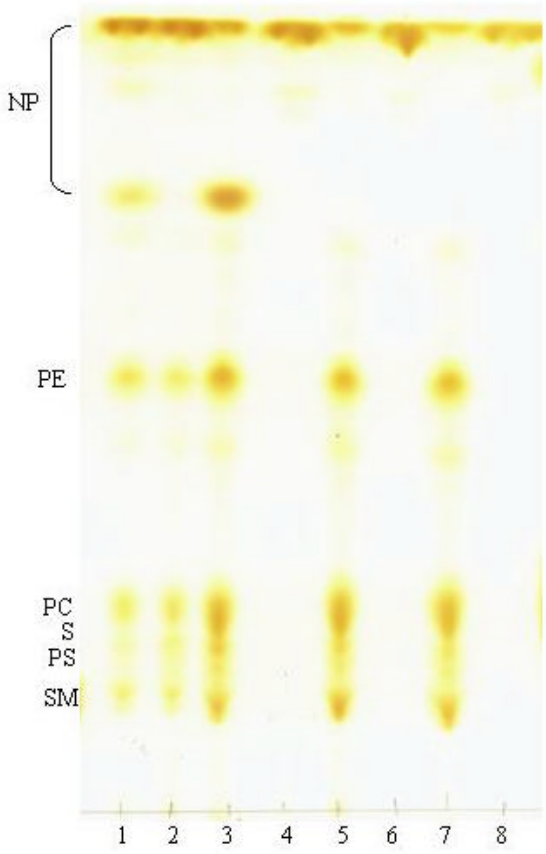


Figure 4.5.7. Polar lipid profiles from SFEs with 3X Retentate, trials A, B, and C, using Solvent System 1. Lane 1, BM; 2, 3X Ret; 3, DF 3X Ret-A; 4, extracted fat-A; 5, DF 3X Ret-B; 6, extracted fat-B; 7, DF 3X Ret-C; 8, extracted fat-C.

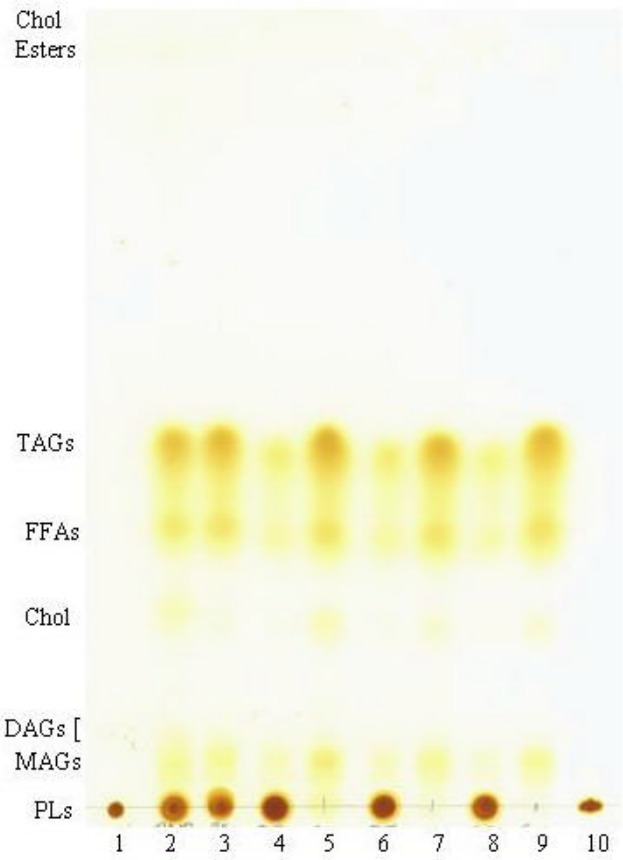


Figure 4.5.8. Nonpolar lipid profiles from SFEs with 3X Retentate, trials A, B, and C, using Solvent System 2. Lane 1, PE; 2, BM; 3, 3X Ret; 4, DF 3X Ret-A; 5, extracted fat-A; 6, DF 3X Ret-B; 7, extracted fat-B; 8, DF 3X Ret-C; 9, extracted fat-C; 10, SM.

PARTICLE SIZE DISTRIBUTION ANALYSIS

Particle size analysis describes the ranges of particle diameters as a distribution in terms of number percent, and volume percent. Number percent describes the mean diameter of all particles (quantitative), while volume percent describes the mean diameter of the major space-occupying particles (qualitative). Figures 4.5.9 and 4.5.10 show the particle size distributions for buttermilk and 3X Retentate powder before and after SFE by number percent, and volume percent, respectively. The average mean particle diameters for these samples are given in Table 4.5.2.

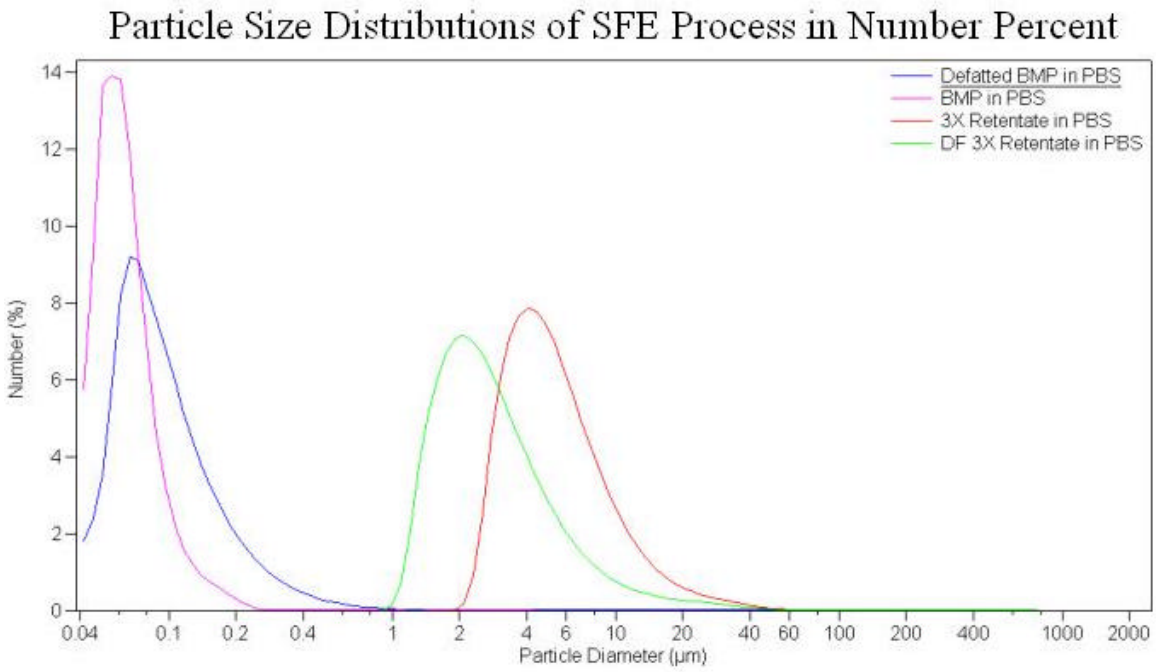


Figure 4.5.9. Number Percent particle size distribution of buttermilk and 3X Retentate powder before, and after (defatted) SFE.

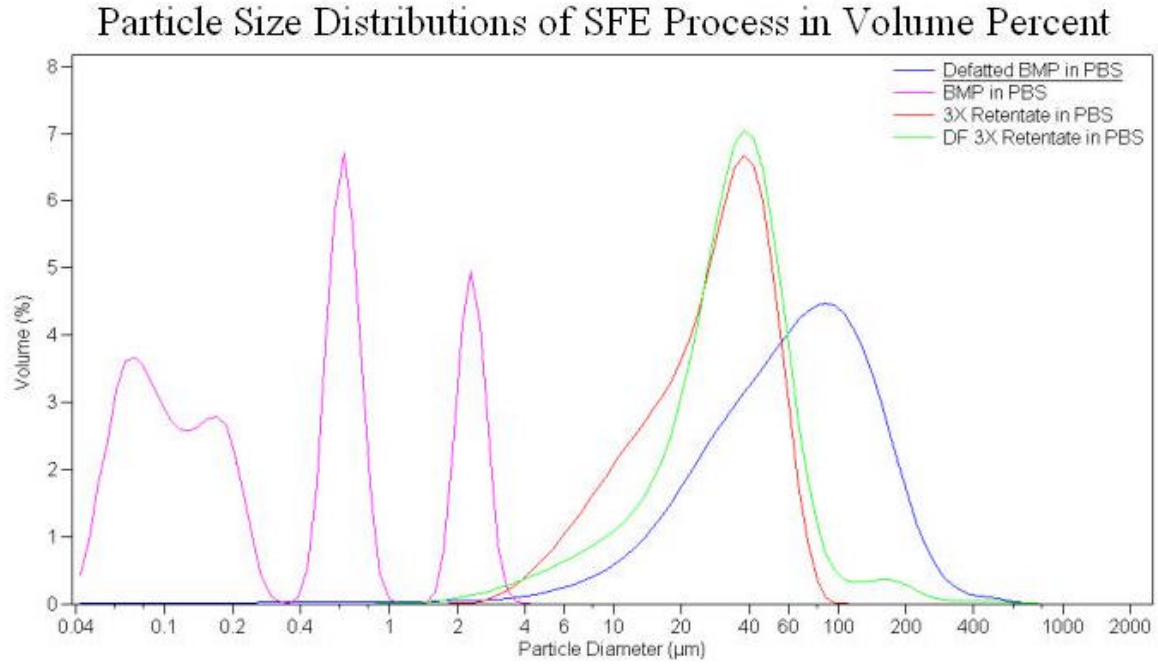


Figure 4.5.10. Volume Percent particle size distribution of buttermilk and 3X Retentate powder before, and after (defatted) SFE.

	Number % (μm)	Volume % (μm)
Buttermilk	0.0687	7.250
Defatted Buttermilk	0.1165	79.615
3X Retentate	6.536	29.925
Defatted 3X Retentate	3.794	37.85

Table 4.5.2. The average mean particle diameters of buttermilk, and 3X Retentate powders before, and after SFE are shown. Means are taken from both the Number % and Volume % distributions.

For buttermilk, after SFE the majority of the volume-occupying particles are much larger, while the average particle size increased as well. The number percent distributions show that the SFE process increased the mean particle diameter within the buttermilk powder, 0.0687 μm to 0.1165 μm. For the 3X Retentate, after SFE an increase is seen in the mean size of the volume-occupying particles, along with a narrower distribution the number percent distributions show that the SFE process decreased the mean particle diameter within the 3X Retentate powder from 6.536 mm to 3.794 mm.

Chapter 5.0

Discussion

5.1. Microfiltration

The objective of the microfiltration process was to maximally concentrate the polar lipids of the MFGM, namely sphingomyelin, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, on a pilot plant scale (20 L) using an in-house manufactured stainless steel shell and tube module containing a GEA Niro brand ceramic membrane (Hudson, WI) with a pore size of 0.8µm. To determine the most efficient system to achieve this, a factorial design with two variables was constructed. The first variable was the origin of the buttermilk, reconstituted from powder, and fresh. Fresh buttermilk would be ideal for large dairy operations where all processed dairy products are present. In this instance, buttermilk is assumed to be readily available, and the process for MFGM lipid enrichment should start with this raw material. However, considering large operations in California, it is likely that dried buttermilk be a reasonable starting material for MFGM material fractionation. In this case, it may be possible to use buttermilk that has been stored for some time, and which market value may not be at an optimal price.

The analysis of the Microfiltration design for this work focused on two aspects related to the process: the changes in the composition of the total protein, fat, ash, and total solids; and the specific compositional profiles of the proteins and lipids in the fractions. From the former, an optimum point in the microfiltration process was determined, and from the latter the variables in the design were chosen that maximized the retention of the MFGM lipids.

The R3X point was chosen as the optimum point of concentration for the microfiltration process using cold conditions and reconstituted buttermilk because it showed the highest concentrations of both total protein, and total fat. When compared to buttermilk, the percent protein in the 3X Retentate was increased from 35.46% to 83.02%, while the percent fat was increased from 4.56% to 8.25 %; both significant increases. Compared to buttermilk, the concentration of ash in the 3X Retentate increased slightly, but not significantly from 7.49% to 7.97%. Refer to Table 4.1.1 for all compositional data. Because the percent total solids describes the aqueous samples during the diafiltration process, a steady decrease was seen through the process for both the Permeate and Retentate samples; this trend was seen in all microfiltration systems.

In the microfiltration system using cold conditions and fresh buttermilk, the protein and fat concentrations were at their maximum at the R5X diafiltration point. From buttermilk, the increase in protein was from 31.60% to 40.76%; for fat the increase was from 10.15% to 58.28%; refer to table 4.1.2. These retentions of protein and fat differ significantly from the cold reconstituted system ($p=0.000$). From these data, the cold fresh microfiltration system retained the total lipid material more efficiently than did the cold reconstituted system, but the lipid profiles by TLC indicated that the cold reconstituted system retained the polar lipids of the MFGM more efficiently, and contained them in higher concentrations in the end product.

Figures 4.3.1 and 4.3.3 show the polar lipid profiles for the cold reconstituted and cold fresh samples respectively. Because equal amounts of total lipid were spotted for TLC, the intensity of the spots indicated the abundance of a particular compound, and samples could be compared quantitatively. All samples from the cold reconstituted

system are more concentrated in the polar lipids than are the cold fresh samples; because of the increased total fat in the fresh buttermilk, a smaller fraction of the total fat is composed of the MFGM lipids. It is also evident that the cold reconstituted system retained the polar lipids more effectively than did the fresh samples. It appears in the profiles that in the fresh system, the MFGM lipids permeated more than in the reconstituted system. The nonpolar lipid profiles for these runs, given in Figures 4.3.2 and 4.3.4, show how the fresh processing samples composed more abundantly of nonpolar lipids than polar lipids.

The second variable considered was the temperature of processing. In normal industrial operations, milk and milk products are handled either at a cold temperature of $4^{o}C$, or at a processing temperature of $50^{o}C$. When cold temperatures are used, the aim is conservation, while high temperatures have the objective of low viscosity and high efficiency. Because it is generally recognized that lipids become more fluid under warm conditions, we hypothesized that using cold conditions would retain the lipid materials more effectively. Though the processing time with cold conditions is much slower than with hot conditions (approximately 24 hours versus 8 hours), the lipid profiles indicated the cold conditions retained the polar lipids of the MFGM more efficiently, particularly with the cold reconstituted buttermilk system. In the hot reconstituted system, lipid profiles indicated that the nonpolar lipids, particularly cholesterol, and the mono and di glycerides, were retained in higher concentrations than with cold conditions; these profiles are shown in Figures 4.3.6 and 4.3.2, respectively. A similar trend was seen in the hot fresh buttermilk processing runs. In the reconstituted buttermilk system, the heated conditions could be increasing the permeability of the MFGM and any MFGM

complexes that formed during previous processing of the powder. The phospholipid bilayer structure of the MFGM, like other biological membrane systems, is naturally less rigid in structure than other lipids, such as cholesterol and saturated glycerides. Perhaps the increased temperature was such that more rigid lipids were retained due to their stable structures, and lipids with lower T_ms, such as the MFGM and unsaturated fats, were easily permeated through the membrane.

We have shown that the polar MFGM lipids retained most effectively in the cold reconstituted system; in the fresh system they showed more permeability through the membrane despite the better retention of total fat. We propose two explanations for this occurrance. Firstly, the fresh buttermilk is much higher in total fat than is the reconstituted buttermilk, 4.56% versus 10.15%; the extra fat is primarily composed of nonpolar fat, such as glycerides, free fatty acids, and cholesterol. The portion of fat contributed by the polar MFGM lipids is minimal in comparison to the nonpolar fats. The fat retained in the fresh system is likewise heavily composed of these nonpolar fats. Because the reconstituted buttermilk is previously skimmed of excess fat and spray dried during production, much of the nonpolar lipids are removed, leaving the polar MFGM lipids as a greater representative of the total fat, and remaining more concentrated in the final Retentate fraction.

Secondly, we propose that the cold reconstituted system retained the MFGM lipid more efficiently due to the processing of the buttermilk powder. The spray drying processing of the reconstituted buttermilk could affect the retention of the polar MFGM lipids positively if the structure of the MFGM complex were affected. The heat treatment could cause the formation of high molecular weight complexes through crosslinking of

proteins or other components with the MFGM. This would increase the total size of the MFGM, thereby increasing its retention in the filtration process.

Protein profiling by SDS-PAGE provides information on how the protein species are fractionating during the microfiltration process. Because equal amounts of protein were separated through the matrix, the intensity of the bands indicates the relative amount of that particular species. Though comparisons can be made between samples of the same protein, different proteins within a given sample cannot be compared with each other because all proteins do not equally respond to dye treatments. The presence of proteins specifically found in the MFGM is an indication that there is MFGM material in the sample; however, it cannot be assumed by this alone, even if they are not naturally found in the serum. During the microfiltration process, the MFGM proteins may become dissociated, allowing them to permeate through the system. Since we're detecting the lipids of the MFGM, their absolute identification was done using lipid profiling by TLC.

In the cold reconstituted system, SDS-PAGE shown in Figure 4.2.1, it is clear that with increased diafiltration, the caseins decreased; this phenomenon was observed in the fresh samples as well. Specifically, ß-casein and ?-casein are markedly different between the hot and cold runs. This is in agreement with the documented solubilization of ß-casein at a low temperature, and casein micelle disruption at a low temperature in microfiltration systems (Walstra, 1999). The proteins of the MFGM are more concentrated in the Retentate samples than in the Permeate samples, indicating the presence of the MFGM. A similar, more distinct result is seen in the cold, fresh buttermilk system in Figure 4.2.2. While the band at ~80K remains relatively stable between the Permeate and Retentate samples, the bands at ~48K and ~70K are clearly

increasing with concentration in the Retentate samples with increased diafiltration. Having already shown that the cold, fresh system did not retain the polar lipids of the MFGM as well as the cold reconstituted system, the clear MFGM protein retention in the fresh system is not indicative of the degree to which the MFGM lipids were retained. Based on the protein profiles alone, it appears that the fresh system retained the MFGM more efficiently, but the lipid profiles indicated otherwise. Again, the MFGM lipid profiles do not have to correlate to the MFGM protein profiles as they are not obligately linked. The hot conditions seem to have decreased the retentions of the MFGM proteins in both the reconstituted and fresh buttermilks; these are shown in Figures 4.2.3 and 4.2.4, respectively.

The microfiltration system using cold conditions and reconstituted buttermilk diafiltered to a 3X factor concentrated the polar lipids of the MFGM most efficiently; however, approximately half of the remaining lipid was nonpolar in nature. These nonpolar lipids; composed primarily of triacylglycerides, cholesterol, and free fatty acids; are not necessarily desirable constituents of a final product produced for human consumption. Diets high in these types of fats are associated with increased health risks (Conklin, 2002; Kromhout, 2001 (A); Kromhout, 2001 (B)), therefore creating a lipid-enriched product in which only 50% or less of the lipids are deemed beneficial may prove counterproductive to any regimen designed to promote health. An additional nontoxic processing step to remove the nonpolar lipids by an extraction procedure would leave the remaining fraction enriched exclusively in the polar MFGM lipids.

5.2. Supercritical Fluid Extraction

SFE proved to be a successful method to increase the concentration of the polar MFGM lipids (namely SM, PC, and PE) in buttermilk, and microfiltered buttermilk containing increased fat. Because the fat therein is composed of various polar and nonpolar species, removing exclusively nonpolar lipids from the powders left the remaining fraction consisting primarily of polar MFGM lipids. Based on quantitative TLC lipid profiles, the Defatted materials after SFE showed an increase in concentration of the polar lipids, and a decrease in nonpolar lipids; the fat removed contained only nonpolar lipids. The 3X Retentate showed the greatest concentration of polar lipids after SFE, as well as showing the greatest percent of lipid retained after SFE, 38.08%; the compositions are given in Table 4.5.1. The 5X Retentate contained less total lipid than the 3X Retentate, a likely result of excessive diafiltration, and retained 27.05% of the total lipid after SFE. These factors caused the polar lipid concentration in the defatted 5X product to be less relative to the 3X Retentate. Though the buttermilk powder retained 34.34% fat after SFE (more than the 5X Ret), it is clear from the nonpolar lipid profiles in Figure 4.5.4 that much of the fat retained was nonpolar in nature. It appears that the SFE process was not as thorough with the buttermilk as with the 5X and 3X Retentates. Both Defatted 5X and 3X Retentates showed a dramatic reduction in nonpolar lipids; these nonpolar profiles are given in Figures 4.5.6 and 4.5.8, respectively. Turner and Mathiasson (2000) demonstrated that the presence of lactose could interfere with the SFE process. Since our microfiltration system is such that lactose would permeate, the lower extraction efficiency seen in the buttermilk could be explained by the presence of lactose.

Although SFE removed the majority of the nonpolar lipid material, some nonpolar lipids remained in the powders, evident in all Defatted samples. After three successive extractions, additional extractions did not remove significantly more lipid material. This suggests that the lipids with a greater solubility in SC-CO_2 under the conditions used had been removed by completion of the third extraction cycle, and the remaining lipids were not soluble enough to be extracted. A fourth extraction cycle removed slightly more lipid, but it was not significant enough to warrant the increased processing. It was demonstrated by Markom et al. (2001), who extracted triglycerides from crude palm oil, that triglycerides rich in saturated fatty acids with lower molecular weights (C12:0, C14:0, and C16:0) were more soluble in SC-CO_2 than were triglycerides rich in unsaturated fats with higher molecular weights (C18:1, C18:3, C20:0) (Markom et al., 2001). Fatty acid analysis of our fractions would reveal if the nonpolar lipids remaining after SFE were composed of primarily unsaturated, long chain fatty acids. If this were the case, the presence of these lipids in our fractions may not necessarily be negative, as numerous unsaturated long-chain fatty acids are not only required in our diets, they may lend health benefits as well (Conklin, 2002; Kromhout, 2001 (A); Kromhout, 2001 (B)). The lipid extracted by SFE versus the lipids extracted from the defatted materials was white to yellow in color and solid, versus semi-liquid and clearer. Alone, this suggests that the removed lipids' composition was largely saturated in nature while the lipid retained was highly unsaturated, or of a different structure (phospholipids and sphingolipids).

The removal of nonpolar lipids from the powders changed the composition of the particle sizes of all samples. For buttermilk, the majority of the volume-occupying

particles after SFE are much larger; the average total particle size increased as well. This would occur if the lipids removed were mostly small particles. Their removal would allow the larger particles to become more dominant.

In the 3X Retentate, the majority of the small particles (<0.8μm), including micelles, would have been previously removed during the microfiltration process. Similarly to BM, after SFE an increase is seen in the mean size of the volume-occupying particles, along with a narrower distribution. This effect could be caused by SFE removing nonpolar lipids that exist as part of a large complex with MFGM. Number % wise, this also explains the decrease seen in the mean particle size.

5.3. Conclusions and Future Research

Microfiltration and SFE were successful methods to fractionate buttermilk to increase the concentration of the polar MFGM lipids without utilizing harmful substances. Microfiltration succeeded in concentrating the total lipid material, including the MFGM. The most efficient fractionation was seen with the microfiltration system using reconstituted buttermilk and a processing temperature of 4^{o}C, with diafiltration to three times the starting volume. After concentration and spray drying of the final Retentate fraction, SFE using SC-CO_2 further increased the concentration of the polar MFGM lipids by removing the majority of the nonpolar lipid material. These findings offer numerous directions for future research.

Gas chromatography mass spectroscopy (GC-MS) analysis of our fractions would reveal more quantitatively the degree to which the MFGM lipids were concentrated, as well as the fatty acid compositions of the lipids therein. This analysis would also more

accurately describe the fractionation process of our microfiltration system, and the solubility of the lipids in our SFE system.

After the microfiltration process upon the freezing and thawing of the liquid Retentate fractions it was observed that a distinct separation occurred, giving two layers. The top layer appeared as a hazy, translucent liquid with slight foaming at the surface; the bottom layer contained a thick, white, precipitate, almost sludgy in consistency. While preliminary investigations indicated that there was no distinct partitioning of the lipids between the layers, the underlying reasons for the observed phenomenon are unrevealed. Perhaps the freezing and thawing process altered the molecular structure of some of the particles, affecting their solubilities. A thorough investigation into the composition of the layers may reveal the freezing and thawing process as a unique fractionating step in isolating specific components, not necessarily lipids, of the Retentate mixture.

A similar microfiltration study using whey cream as a source of the MFGM may provide interesting results as well. In the buttermilk system, the removal of the casein proteins during microfiltration was not complete. Further diafiltration would produce a retentate fraction with significantly less caseins; however, disadvantages such as increased processing time, and an increased loss of MFGM product would occur. Alternatively to milk cream, whey cream is naturally devoid of caseins, while both creams contain the MFGM. If whey cream churns similarly to produce a fluid equivalent to buttermilk with high amounts of the MFGM material, processing methods like those presented here may yield a fraction equally concentrated in the MFGM lipids, and less total protein with no caseins? If using whey cream as a starting source instead of milk cream appeared successful, would the two fractions behave the same metabolically in

living systems? These questions warrant investigation from a processing standpoint as well as a biological one.

Using supercritical carbon dioxide as the only solvent was appropriate for the extraction of nonpolar lipids in our system, as we desired our end product to be a complex buttermilk fraction consisting of various proteins and lipids, not a pure extraction of the polar MFGM lipids. Whether this is most effective way to fractionate the system to maximize their *in vivo* metabolic effects is moot until biochemical research provides substantial evidence. SFE is capable of extracting lipids more specifically by employing a succession of extraction steps utilizing various solvent systems. For example, a primary set of extractions with SC-CO_2 would remove nonpolar lipids, followed by a secondary set of extractions using more polar solvents (and/or cosolvents) to remove the remaining polar lipids. As ethanol is highly polar (as well as a GRAS substance), it would be a good selection as an additional solvent in a system such as this. Extraction models such as this have been successful in selective isolations of multiple lipid types (Montanari et al., 1999).

It has been shown that endospores from bacteria, including *Bacillus* and *Clostridium species*, have been deactivated by treatment with SC-CO_2 at pressures and temperatures less severe than our selected conditions (Enomoto et al., 1997; Spilimbergo et al., 2002; Smelt and Rijke, 1997). Because our fractions are intended for use in further applications (both research and dietary, assuring that our final fractions are free of microbes and endospores is ideal. This is particularly important for our processing procedures as our microfiltration system likely retains bacteria and spores due to the 0.8µm pore size. Further research demonstrating that our SFE procedures adequately

deactivate spores and kill microbes should be done to ensure the microbial quality of our final fraction.

Product development research can build tremendously off of this groundwork as well. The application of a functional food such as this can be targeted towards mass consumer products in the marketplace, as well as towards specialty markets such as the medical, pharmaceutical, and naturopathic industry. Anticancer research will undoubtedly seek for their applications as chemotherapeutics, as they already have begun to do. The unique lipids and proteins in dairy products can be developed into supplements targeted towards those with intolerance to dairy, or incorporated into other more widely consumed foods. Similarly to the widespread consumption of herbs such as ginsing and ginko biloba, perhaps MFMG lipids will become a dietary mainstay for a group of people seeking a specific nutritional affect from their consumption. From a marketing standpoint, many fats may be recognized as being "Fabulous Fats" and "Functional Fats", not "Fattening Fats".

However, claiming these lipids as endowing health-enhancing effects on their consumers is premature, if not reckless at this stage. While numerous health benefits may result in response to their dietary supplementation, a deeper knowledge into their metabolic mechanisms of action from countering perspectives is important before a substantiated claim can be made. For example, while seeking to show that dietary supplementation can reduce specific types of carcinogenesis, equal attention should be focused on their effects on plaque rupture in atherosclerotic plaques, and on aging and age-related diseases. Ideally, our understanding of their metabolic effects may imply that

tailoring dietary manipulations designed to reduce or increase sphingomyelin and ceramide production may lead to an increased control we can claim over our health.

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Appendix A

Product Specifications for Land O'Lakes Spray Dried Buttermilk Powder (Product code: 26048, St. Paul, MN, U.S.A.)

Analysis	Typical	Specification
Protein	32.0%	30.0%
Moisture	3.2%	4.0% Maximum
Carbohydrate	49%	47.0% Maximum
Butterfat	5.5%	4.5% Minimum
Minerals (Ash)	8.5%	
Titratable acidity	0.12%	0.18% Maximum
Density (g/ml)	0.65 - 0.75	
Solubility Index (ml)	0.20	1.25 Maximum
Scorched Particles	Disc A (7.5 mg)	Disc B (15.0 mg) Maximum
Color	Cream	
Flavor	Clean with no off flavor	
Salmonella	Negative	Negative
Standard Plate Count	< 3,000 per g	50,000 per g Maximum
Coliform	< 10 per g	10 per g Maximum

Product is packaged in 50 pound bags.

This product meets the standards of the American Dry Milk Institute and the U.S. Department of Agriculture for Extra Grade Sweet Cream Buttermilk Powder.

Appendix B

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a method for separating proteins in a sample based on their molecular weights; this is done by the application of an electrical field to the gel system. In this system, the resolving matrix is polyacrylamide, and is stacked in two distinct layers. The first layer is the stacking gel, a non-restrictive large pore gel that ensures the samples all begin migration through the resolving layer simultaneously. The resolving gel can vary in its composition of polyacrylamide; common analyses use 8% to 15% polyacrylamide in the resolving gel. Separation of proteins in this system is accomplished because of two main principles: unfolding the proteins, and denaturing the proteins. SDS is an ionic detergent that dissociates and unfolds proteins into their subunits by binding the polypeptides; this results in a complex with a fairly constant charge to mass ratio, negatively charged. Denaturing conditions in the electrophoretic system are achieved by using the denaturing agent beta-mercaptoethanol; this allows the electrophoretic migration rate through the gel matrix to be determined by the size of the complexes. When the electrical field is applied, the charged particles migrate through the gel at a rate determined by their size. The molecular weights of the separated proteins can be determined by comparison to standard protein markers of known molecular weights. When the samples have migrated through the length of the gel, it is visualized after staining procedures. Depending on what type of proteins are present, different

staining procedures can be used. Commonly used staining methods use Coomassie Brilliant Blue, Silver Stain reagent, and PAS reagent.

Recipes and Reagents Used for SDS-PAGE Analysis

Sample Buffer

4.0 ml de-ionized water
1.0 ml 0.5 M Tris-HCl (pH 6.8)
0.8 ml glycerol (Fisher Scientific, Tustin, CA, U.S.A.) (Catalog # G33-1)
1.6 ml 10% SDS in de-ionized water (Fisher, BP-166-500)
0.2 ml 1% bromophenol blue in de-ionized water
0.4 ml β-mercaptoehtanol (Sigma, M-6250)

Mix all ingredients until dissolved; store at 4°C for up to two months.

RESOLVING GEL BUFFER

36.3 g Tris Base (Fisher, BP152-1, M.W. 121.1) dissolved in 150 ml de-ionized water.
Adjust the pH to 6.8 by the addition of HCl
Bring the final volume up to 200 ml
Store at 4°C for up to three months in reduced light conditions

Resolving Gel (12% Acrylamide*)

5 ml 1.5 M Tris-HCl buffer, pH 8.8
8.8 ml de-ionized water
0.2 ml 10% SDS in de-ionized water (Fisher, BP-166-500)
6 ml 40% acrylamide (Fisher, BP-1410)
0.1 ml 10% Ammonium Per-Sulfate (APS) in de-ionized water (Sigma, A-3678)
0.01 ml TEMED (N,N,N,N,'-Tetra-methyl-ethylenediamine) (Bio-Rad, 161-0800)

Mix everything but the APS in a 125 ml side arm flask, and de-gas under vacuum for 10 minutes. Add the remaining ingredients just prior to pouring the gel.

* For % acrylamide gels other than 12%, more or less acrylamide should be used, compensating for the adjusted volume by increasing/decreasing the amount of water.

Stacking Gel Buffer

2.0 g Tris Base (Fisher, BP-152-1, M.W. 121.1) dissolved in 40 ml de-ionized water.
Adjust the pH to 6.8 by the addition of HCl
Bring the final volume up to 50 ml
Store at 4°C for up to three months in reduced light conditions

Stacking Gel (4% acrylamide)

2.5 ml 0.5M Tris-HCl (pH 6.8) buffer
6.4 ml de-ionized water
0.1 ml 10% SDS in de-ionized water (Fisher, BP-166-500)
1.0 ml 40% acrylamide (Fisher, BP-1410)
0.05 ml APS (Sigma, A-3678)
0.005 ml TEMED (Bio-Rad, 161-0800)

TANK BUFFER

6 g Tris Base
28.8 g glycine (Sigma, G-7126)
2.0 g SDS

Dissolve ingredients in 2 L de-ionized water. A 10X solution can be prepared, and stored at room temperature for up to six months.

COOMASSIE STAIN

Brilliant Blue R Concentrate (Sigma, BP-8647) is diluted 1:1 with de-ionized water before use.

COOMASSIE DESTAIN SOLUTION

40% methanol, 10% acetic acid, 50% de-ionized water

BlueRanger® Prestained Protein Molecular Weight Marker Mix (Pierce, IL; # KL1120)

Component	Post-stained molecular weight (K)
Myosin	208
Phosphorylase B	112
Bovine Serum Albumin	85
Ovalbumin	48
Carbonic Anhydrase	32
Trypsin Inhibitor	26
Lysozyme	18

Appendix C

Protocol for Manual SFE Run*

Step 1 – Water Bath

The water bath should be turned on and allowed to reach the temperature set point of 5°C before proceeding; if a lower temperature set point is desired the water bath should be filled with coolant instead of water to prevent freezing. The low temperature point is important as they circulate to keep the pump heads cold. Improperly cooled pump heads result in poor pumping efficiency.

Step 2 – BPR

In the **BPR Panel**, set the back pressure regulator control point with the pressure desired for the run, and the valve temperature. The valve temperature need not be as high as the temperature for the run conditions, it functions to keep your sample in the supercritical state until reaching the cyclone. Activate the BPR by clicking start. The alarm point has been preset.

Step 3 – Temperatures

In the **TCM Panel**, set the temperature conditions for the run. On the LED readout, the heat Exchanger is Zone 1, the sample vessel heater is Zone 2, the sample vessel interior is Zone 3, and the cyclone is Zone 4. Set each temperature set point where you would like for the run, then push start for each. For the vessel interior you need only turn on the monitor as there is further control other than the vessel heather; you only activate this to monitor the inside temperature. The alarm points have been preset. Because the temperatures are controlled by a PID program, your set point may not be the actual resulting temperature—you may have to enter a set point value higher than what you actually want to obtain an actual temperature closer to what you what. You may like to allow temperature to preheat 5-10 min before proceeding.

Step 4 – Pumping

After making sure the CO_2 valve is open on the tank, start the flow of solvent(s). In the **Dual Pump Panel** select the flow rate you would like to use, and click start. If you are using the slave pump for a co-solvent, make sure the master pump profile is a flow profile, then enter the total flow of all solvents into the master pump flow box. Click on the "enable slave" check box and enter the % of the master's flow would like the slave to pump. The alarm values for pumps are preset. Once all values are set, click start, and go to lunch.

STEP 5 – THE RUN

As a general rule, you want about 2-3 flushes of volume through the vessel for 1 run. This will depend on your selected flow rate. During the duration of the run the machine should be checked periodically to ensure that everything is working smoothly.

All fittings must be tight to prevent any leaks during the run, which would cause a drop in pressure, potentially freezing your product in the vessel. Also, should the CO_2 tank become low the pressure will drop; if this happens the tank needs to be changed. A trouble-shooting guide is included with the manual in the likely event that further problems are encountered.

Step 6 – Shut down

Now that your run is completed, you have to depressurize the system. To do this, go through the above procedures in reverse order. That is, stop the pumping and close the CO_2 valve, turn off the heaters, and then stop the BPR. Once the BPR has been stopped you can bleed off the pressure by opening the BPR's needle valve to a specified number of counts; 4000 counts is a good bleed of position, but if all the CO_2 rushing out at once scares you, go lower. Make sure the cyclone pressure does not exceed its maximum.

* Protocol for a manual SFE run is adopted from general instructions provided by Thar Designs (Pennsylvania, U.S.A.).