THE EFFECTS OF MICROFLUIDIZATION AND HOMOGENIZATION ON THE
COMPOSITION AND STRUCTURE OF LIPOSOMAL AGGREGATES FROM
WHEY BUTTERMILK AND COMMERCIAL BUTTERMILK

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Agriculture, with specialization in Dairy Products Technology

by

Tracey Mai Truc Nguyen

August 2013

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COMMITTEE MEMBERSHIP

TITLE:	The effects of microfluidization and homogenization on the composition and structure of liposomal aggregates from whey buttermilk and commercial buttermilk
AUTHOR:	Tracey Mai Truc Nguyen
DATE SUBMITTED:	August 2013
COMMITTEE CHAIR:	Rafael Jimenez-Flores, PhD. Professor of Dairy Science
COMMITTEE MEMBER:	Phillip S. Tong, PhD. Professor of Dairy Science
COMMITTEE MEMBER:	Jennifer Carroll, PhD. Associate Professor of Chemistry
COMMITTEE MEMBER:	John Walker, PhD. Professor of Statistics

ABSTRACT

The effects of microfluidization and homogenization on the structure of liposomal aggregates from whey buttermilk and commercial buttermilk

Tracey Mai T. Nguyen

Milk derived ingredients from the production of cheese and butter can be used as vehicles for nutrients. Buttermilk is a nutritious product of milk that comes from the churning of cream into butter. One of the advantages of buttermilk is that it is enriched in milk fat globule components, such as phospholipids and forms emulsions with fat when treated with high shear. The objective of this work was to explore the effects of shear on regular buttermilk and whey buttermilk in terms of liposomal aggregate size and chemical composition. The effects of microfluidization at 2000 psi and homogenization at 2000 psi/500 psi on the particle size distribution of liposomal aggregates between whey buttermilk (WBM) at pH 4.6 and 6.8 and commercial sweet buttermilk (SBM) at pH 4.60 were compared with whey protein isolate (WPI) at pH 4.6. At pH 6.80, WPI and SBM are too soluble in water to measure particle size but WBM is not as soluble. From this investigation, the mean particle diameter of the SBM aggregates at pH 4.6 decreased after the first pass through the microfluidizer and the same is true, after homogenization. SBM aggregates at pH 4.6 had a significantly larger mean particle diameter before treatments in both shear processes compared to WPI at pH 4.6 and WBM at pH 4.6 and WBM at pH 6.8 ($p < 0.0001$). WPI at pH 4.6 and WBM at both pH showed no significant differences in their mean particle size in both homogenized and microfluidized treatments. WPI and SBM samples resulted in significant particle diameter differences from before to after homogenizing at pH 4.6. SBM at pH 4.6 had significantly larger

average particle diameter than WBM at pH 4.6 ($p < 0.0002$), WPI at pH 4.6 ($p < 0.0002$) and WBM at pH 6.8 ($p < 0.0045$) before microfluidization at pass 0.

WBM and WPI across all treatments showed very similar tendencies in small particle size attributes and some similarities in protein composition. In addition, the small aggregate size of WBM is suggested to be influenced by the presence of phospholipids and thus, creating significantly smaller mean particles compared to SBM even before inducing high shear. In contrast, treated and untreated SBM differed from WBM in phospholipid composition in both homogenization and microfluidization techniques. WBM samples contained more phospholipids than SBM, whereas WPI samples contained very low concentrations of phospholipids. Through HPLC analysis, WPI, SBM, and WBM showed different profiling of the phospholipid classes. These differences may be due structural changes of the aggregates from shearing, initial thermal treatments or hydrophobic and/or protein-phospholipid interactions between the aggregates. SBM samples also exhibited different protein profiling than WBM and WPI samples. This study suggests that high shear and presence of phospholipids impact the size distribution of liposomal aggregates through structural alterations. The aggregates can be utilized as a novel ingredient and in the processing of dairy foods to deliver nutrition.

Keywords: milk fat globule membrane (MFGM), phospholipids, microfluidization, homogenization, delivery system.

ACKNOWLEDGMENTS

Sometimes life takes a turn and takes you places that change your life forever. My life changed when it took a turn and journeyed me to Cal Poly in beautiful San Luis Obispo. Many people along this journey have touched my life and shaped me into the person I am today.

I would like to thank my committee chair and advisor, Dr. Rafael Jimenez-Flores for his mentorship and guidance through this journey. Thank you for giving me a wonderful opportunity to grow not only as a person, but also as a young scientist. I would like to thank Dr. Jennifer Carroll for all her support and guidance through this project. Thank you for giving me the confidence to learn your HPLC system and for conquering my fears of breaking it. I would like to thank Dr. Phillip Tong for sharing his knowledge and advice. I have enjoyed our insightful conversations and appreciate your encouragements. I would like to thank Dr. John Walker for his enthusiasm and help through the analysis of this project. Thank you for patiently teaching me statistics and for your continued support.

I would also like to thank Dr. Chad Immoos for his collaboration in this project. I wish to thank Dr. Nana Farkye for teaching me dairy chemistry and for his words of wisdom. I enjoy our conversations and appreciate all your positive encouragements.

This project would not have been successful without the help and support of the DPTC staff. I would like to thank Sean Vink for all his help and contribution throughout this project. Thank you for taking the time to help me with processing and troubleshooting the particle size analyzer. Thank you Matt Arnold for your assistance in tracking down laboratory equipments (they tend to have feet and walk away when you're

not looking). Thank you Dr. Vandna Sikand for your encouragements and company in the lab. Thank you Kristen Herbaugh for being so understanding and assisting me whenever I needed help. Thank you Katy Lees for the company, good conversations, and generous quantities of delicious food. I look forward to seeing your farmstead cheese in supermarket shelves in the near future. Thank you Andrea Laubscher for your wonderful friendship and for everything, in every sense of the word. This project would not have been possible without your generous help. I truly appreciate your genuine and tireless efforts training me on equipment use, locating lab ware that mysteriously walked away, rush ordering supplies when I needed it quickly, helping me run and fix my experiments when they've failed, and for always reassuring me that everything will turn out well in the end.

My experiences wouldn't be complete without the unfailing friendship of the DPTC graduate students. I would like to thank Hadi Eshpari for his kindness, wisdom, and words of encouragements to never give up. Thank you Emily Kong for being my neighbor, partner in crime, and going through graduate school with me from the very beginning. Thank you Lauren Collinsworth for your enthusiasm and for bringing laughter to the grad room. Thank you Melissa Hsu for your charisma and entertaining conversations. Thank you Mariela Poveda for your strength and the wonderful memories. Thank you Valerie Arechiga, Corbin Kembel, and Garrett Walsh for being the trio of fun and entertainment at the DPTC. Thank you Cecilia Ribeiro for your kindness, support and help. Thank you Dan Zhao for your company and for all the chocolate milk. Thank you all for the unforgettable memories, laughter, and support.

I would like to thank Dr. Nosang Myung and Dr. Sandra Hernandez for the wonderful opportunity to research under your direction as an undergraduate. I learned so much working with you and from the time spent in your lab. You have inspired me to become the person I am today.

To my friends at home: Caroline Chang, Yuh Jiun Tan, and Veronica Nyaung, thank you for your friendship, love, support, and kind words of encouragements all these years. No matter the distance or how much time has passed, I can always count on you to be there for me. Thank you Ann Tran, Henry Pham, and Agyn Atienza (aka the gang) for your continued support and encouragements along this journey to find my true passion.

I would like to thank Kim, Steve, and Nolan Ramseyer and Lauren Cadish for their endless love, motivation, positive attitudes, and for believing in me in my moments of doubt. Thank you so much for taking part in this journey with me.

To my family: Mom, Dad, and sister, Jackie. I am where I am today because of your unconditional love and immeasurable support. Thank you for empowering me to become a strong person and to never give up, for encouraging me to stay true to myself and chase after my dreams, for sharing my joys during the best of times, and for lifting my spirits during rough times. Words cannot express my gratitude. I love you and appreciate all that you have done to help me succeed all these years.

And finally, to Niles Ramseyer. Thank you for being so supportive and embarking on this incredible journey with me. Thank you for reading and editing my thesis, for being my strength in my time of weakness, and for believing that I can do anything I put my mind to. You bring out the best in me.

TABLE OF CONTENTS

LIST OF TABLES	xiii
LIST OF FIGURES	xiv
CHAPTER	
1.0 Introduction.....	1
2.0 Literature Review.....	4
2.1 Origin and Function of MFGM.....	4
2.2 Composition of MFGM.....	6
2.2.1 Proteins	6
2.2.2 Lipids	9
2.3 Sources of MFGM.....	12
2.4 Health Benefits and Food Applications of MFGM.....	14
2.5 Physiochemical Effects of Processing on Phospholipids and MFGM.....	18
2.5.1 Homogenization.....	18
2.5.2 Microfluidization	20
2.5.3 Temperature and pH	21
2.6 Techniques of Analysis	22
2.6.1 Particle Size Distribution	22
2.6.2 Phospholipid Analysis	23
2.6.3 Protein Analysis	26

2.7	Justification for Present Research	27
3.0	Materials and Methods.....	30
3.1	Powders	30
3.2	Microfluidization: Sample Preparation, Treatments and Sample Collection.....	31
3.2.1	Sample Preparation	31
3.2.2	Microfluidization Treatment and Sample Collection	32
3.3	Homogenization: Sample Preparation, Treatments and Sample Collection.....	33
3.3.1	Sample Preparation	33
3.3.2	Homogenization Treatment and Sample Collection	34
3.4	Physical Properties of Liposomal Aggregates	35
3.4.1	Particle Size Distribution	35
3.5	Chemical Composition.....	36
3.5.1	Fat Extraction by Mojonnier Method	36
3.5.2	Phospholipid Solid Phase Extraction (SPE)	38
3.5.3	Phospholipid Profiling and Quantification by High Pressure Liquid Chromatography (HPLC).....	40
3.6	Protein Composition.....	42
3.6.1	Protein Quantification – Bicinchoninic Acid (BCA) Assay	42

3.6.2	Sodium Dodocyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	42
3.7	Experimental Design	43
3.8	Statistical Analysis	44
4.0	Results and Discussion	46
4.1	BCA Protein Assay	46
4.2	Physical Properties of Liposomal Aggregates	47
4.2.1	Particle Size Analysis	47
4.2.2	Confirmation with Confocal Microscopy	56
4.3	Chemical Composition.....	58
4.3.1	Fat Extraction by Mojonnier Method	58
4.3.2	Phospholipid Solid Phase Extraction (SPE)	61
4.3.3	Phospholipid Profiling and Quantification by HPLC	63
4.4	Protein Analysis by SDS-PAGE	71
5.0	Conclusions.....	78
5.1	Limitations of this Study	79
6.0	Directions for Future Research	81
	REFERENCES	82
	APPENDICES	
	Appendix A: Randomization for Homogenization	88

Appendix B: Randomization for Microfluidization.....	89
Appendix C: Homogenization Paired Data JMP Output and Model Assumptions	90
Appendix D: Microfluidization Repeated Measures Data SAS Output	93
Appendix E: Homogenization and Microfluidization Combined Paired Data JMP Output and Model Assumptions	98
Appendix F: HPLC Calibration Curves	102

LIST OF TABLES

Table 2.1. Protein composition of bovine milk fat globule membrane modified from Singh (2006) and Keenan and Dylewski (1995).	7
Table 2.2. Lipid composition of bovine milk fat globule membrane adapted and modified from Singh (2006) and Keenan and Dylewski (1995).	10
Table 2.3. Composition of dairy powders.	14
Table 2.4. Phospholipid and protein composition in various dairy products adapted from Elias-Argote (2011).	14
Table 4.1. BCA protein assay of 5% (w/v) reconstituted dairy powders.	47
Table 4.2. Least Square Means for Homogenized Samples.	51
Table 4.3. Mean Particle Diameter for Microfluidized Samples.	53
Table 4.4. Least Square Means for Homogenized and Microfluidized Samples.	55
Table 4.5. Fat content of WPI, SBM, and WBM by Mojonnier Extraction.	60
Table 4.6. Phospholipid concentration (%) of WPI, SBM, and WBM by SPE.	62
Table 4.7. Linear equations of phospholipid standards and their respective retention time.	64
Table 4.8. Average percentage of phospholipid content in fat globules.	67
Table 4.9. Compositional summary of WPI, WBM, and SBM products.	77

LIST OF FIGURES

Figure 1.1. Production trends of dry buttermilk from 2009 to 2013 in the United States adapted from the USDA (2013).	2
Figure 2.1. A representation of the milk fat globule membrane adapted from Lopez et al., (2010)	4
Figure 2.2. A diagram of the synthesis and secretion of milk constituents from apical membrane of the epithelial cell of a mammary gland adapted from Keenan et al., (1988).	5
Figure 2.3. Chemical structures of polar phospholipids of the MFGM (Contarini & Povolo, 2013).....	11
Figure 2.4. Butter manufacturing flow diagram adapted and modified from O'Mahony (1988).	13
Figure 2.5. Typical structure of a liposomal vesicle adapted from McClements (2005).	17
Figure 2.6. Homogenizer mechanism diagram adapted from Spence et al. (2010).	18
Figure 2.7. A representation of the MFGM in homogenized milk (Fox & McSweeney, 1998).	20
Figure 2.8. Microfluidizer mechanism diagram modified from Spence et al., (2010).	21
Figure 2.9. Daily fluid milk consumption per capita in the United States (Stewart, Dong, & Carlson, 2013). Whole milk refers to milk containing $\geq 3.25\%$ fat and lower fat milk refers to 2%, 1%, and skim milks.	28

Figure 2.10. Annual dry buttermilk consumption (pounds) per capita in the United States (USDA, 2012).....	29
Figure 3.1. Sample Preparation for Microfluidization.....	32
Figure 3.2. Avestin EmusliFlex™-C5 microfluidizer unit. A) sample cylinder; B) air motor; C) pressure gauge; D) interaction chamber; E) air pressure gauges from source.	33
Figure 3.3. Pilot plant scale homogenizer.....	34
Figure 3.4. Mojonniier fat extraction flow diagram.....	37
Figure 3.5. Solid phase extraction process flow diagram.	39
Figure 3.6. Shimadzu Prominence HPLC unit with a CAD detector. A) degassing unit; B) pump; C) autosampler; D) communication bus module; E) column oven; F) CAD detector.....	40
Figure 4.1. BSA standard curve of absorbance at 562 nm vs. concentration (mg/mL). $R^2 = 0.957$	46
Figure 4.2. Particle Size Distribution of WPI, SBM, and WBM before and after each pass through the microfluidizer. (a) WPI at pH 4.60; (b) SBM at pH 4.60; (c) WBM at pH 4.60 and 6.80.	48
Figure 4.3. Particle Size Distribution of WPI, SBM, and WBM before and after homogenization with 3 repetitions per treatment structure. (a) WPI at pH 4.60; (b) SBM at pH 4.60; (c) WBM at pH 4.60; (d) WBM at pH 6.80.....	49
Figure 4.4. Mean particle diameter plot for microfluidized samples.....	54
Figure 4.5. Confocal images of WPI, SBM, and WBM at various conditions.	57

Figure 4.6. Images of WPI, WBM, and SBM samples after microfluidization. (a) WPI at pH 6.8; (b) WPI at pH 4.6; (c) WBM at pH 6.8; (d) WBM at pH 4.6; (e) SBM at pH 6.8; (f) SBM at pH 4.6.	59
Figure 4.7. Percentage of total fat (% per 1 g solid or 10 g fluid) in WPI, WBM, and SBM under various conditions per day.	60
Figure 4.8. Percentage of phospholipids (% per 1 g solid) in WPI, WBM pellets (WBMP), and SBM pellets (SBMP) at pH 4.6 per day. MF = Microfluidization and Homog = Homogenization.	63
Figure 4.9. Average concentration of Phospholipid classes (mg/mL per 1000 mg solid).	66
Figure 4.10. HPLC Chromatogram of phospholipids extracted from WPI, WBM, and SBM powder. 1. WPI powder; 2. WBM powder; 3. SBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS = Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin.	66
Figure 4.11. Average percentage of phospholipid classes in relation to total mg of phospholipids in each sample.	68
Figure 4.12. HPLC Chromatogram of phospholipids extracted from SBM. 1. SBM Pellet from Microfluidization at pH 4.60; 2. SBM Pellet from Homogenization at pH 4.60; 3. SBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid	

peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS =
 Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin..... 70

Figure 4.13. HPLC Chromatogram of phospholipids extracted from WBM. 1. WBM Pellet from Microfluidization at pH 4.60; 2. WBM Pellet from Homogenization at pH 4.60; 3. WBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS = Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin..... 70

Figure 4.14. SDS-PAGE (15% acrylamide gel) of reconstituted WPI, SBM and WBM under (a) reduced and (b) non-reduced conditions. Lane 1. Protein standards; Lane 2. WPI untreated at pH 6.8; Lane 3. SBM untreated at pH 6.8; Lane 4. WBM untreated at pH 6.8; Lane 5. WPI microfluidized at pH 4.60; Lane 6. SBM microfluidized at pH 4.60; Lane 7. WBM microfluidized at pH 4.60; Lane 8. WPI homogenized at pH 4.60; Lane 9. SBM homogenized at pH 4.60; Lane 10. WBM homogenized at pH 4.60. Protein Bands: XO = xanthine oxidase; BTN = butyrophilin; Pas 6/7 = periodic acid Schiff 6/7; α -CN = α -casein; β -CN = β -casein; κ -CN = κ -casein; β -LG = β -lactoglobulin; α -LA = α -lactalbumin..... 72

Figure 4.15. Trace Quantity (intensity x mm) of SDS-PAGE under reducing conditions in (a) WPI, (b) SBM, and (c) WBM samples..... 75

Figure 4.16. Trace Quantity (intensity x mm) of SDS-PAGE under non-reducing conditions in (a) WPI, (b) SBM, and (c) WBM samples..... 76

1.0 Introduction

Milk is defined as a fluid secreted by all female mammalian species to supply nutrients and provide immunological and anti-bacterial protection to the neonate (Fox & McSweeney, 1998). Milk is widely known as a nutritious and beneficial beverage with an excellent source of protein, calcium, vitamins, and minerals. Despite the nutritious benefits of milk, the per capita consumption of fluid milk in the United States has declined since 1970 from 0.96 cup-equivalents to 0.61 cup-equivalents per day (Stewart, Dong, & Carlson, 2013). According to the USDA, about 20,834 million pounds of conventional fluid milk products were sold in May of 2013 in the United States, year to date. Compared to last year, these numbers represent a 1.6% decrease in fluid milk sales (USDA, 2013).

Despite the fall in fluid milk consumption, the overall dairy consumption per capita still remains steady at 1.5 cup-equivalents per day, with a rising consumption of cheese (Stewart, Dong, & Carlson, 2013). Additionally, the production and consumption of dry buttermilk has been increasing every year since 2009 (**Figure 1.1**) (USDA, 2013). Buttermilk is traditionally made from the churning of sweet cream into butter. This process releases a liquid co-product known as buttermilk. There are many other sources of buttermilk that exist such as cultured buttermilk and whey buttermilk (Sodini, Morin, Olabi, & Jimenez-Flores, 2006). Buttermilk is also considered a nutritious dairy ingredient that is saturated in milk fat globule membrane (MFGM) components. These components consist of phospholipids and proteins that have been attributed to be advantageous and beneficial. The MFGM constituents, especially phospholipids, have been shown to possess abilities that prevent colon cancer, suppress *H. pylori* infections,

and inhibits intestinal absorption of cholesterol (Spitsberg, 2005) (Parodi, 1997) (McDaniel, Maier, & Einstein, 2002) (Sprong, Hulstein, & Van Der Meer, 2002).

Figure 1.1. Production trends of dry buttermilk from 2009 to 2013 in the United States adapted from the USDA (2013).

Over the last several decades, chronic ailments and diseases have been increasing worldwide. There is a great need for cures and preventative measures for diseases like hypercholesterolemia, diabetes, and cancer. Further research is needed to study how nature's most nutritious beverage can help prevent and cure diseases. Research on the development of novel ingredients and innovative techniques can also help put nutrition and beneficial elements back into our bodies. Phospholipids from the MFGM can be utilized as a delivery system for bioactive components such as active drugs, flavor compounds, and vitamins, for functional use in the nutraceutical, pharmaceutical, and food industries.

This thesis focuses on structural and compositional comparisons of liposomal aggregates from commercial sweet buttermilk and whey buttermilk prepared by microfluidization and homogenization. The induction of high shear structurally effects

the formation of liposomes. By utilizing two shearing types, a comparison can be made on which mechanisms will produce smaller liposomes for more functional and practical applications in food products. The investigation of aggregate size is important not only in functionality, but also texturally. This research takes a look at liposomal aggregates from sweet buttermilk and whey buttermilk at the physicochemical level to assess its size distribution, phospholipid profiling, and protein composition. Ideas from this investigation contributes to current knowledge and research of utilizing liposomes as vehicles for delivering nutrients.

2.0 Literature Review

2.1 Origin and Function of MFGM

Bovine milk contains 3-5% fat that is dispersed in the form of small droplets. Roughly 97-98% of the total lipids in milk are composed of triacylglycerols (TAG) and less than 1% represents phospholipids. The fat droplets range in diameter from 0.1 μm to 20 μm and average 3.5 μm (Fox & McSweeney, 1998). Many factors influence the fat globule size distribution and composition including cow breed, stage of lactation, diet, and seasons. Lipids are insoluble in water and great tension to exist between the dispersed lipids and water causing coalescence, or fusion of lipids, to occur. Emulsifiers are surface active agents used to prevent coalescence by forming a film around the fat globules, which reduces the surface tension and protects the lipids from enzymatic reactions. In unprocessed milk, the milk fat globule membrane (MFGM) exists as a natural emulsifier and provides a protective layer around the fat globules (Singh, 2006).

Figure 2.1. A representation of the milk fat globule membrane adapted from Lopez et al., (2010)

The MFGM is a tri-layer membrane originating from the rough endoplasmic reticulum (rER) and the apical membrane of epithelial secretory cell of the mammary gland (**Figure 2.1**) (Fox & McSweeney, 1998). Milk fat globules are synthesized in the rER in the form of small droplets and fuse together to give rise to larger droplets (Singh, 2006). The droplets are primarily made of TAGs and are protected by a surface coat derived from the rER. This surface coat is comprised of cholesterol and major phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS) (Fox & McSweeney, 1998). It provides stability to the fat globule and is the most inner layer of the MFGM. Once the fat globules are secreted from the rER, it travels through the cytoplasm towards the apical membrane. During secretion, the outer apical lipid bilayer surrounds the fat globules and is released from the cell (Singh, 2006).

Figure 2.2. A diagram of the synthesis and secretion of milk constituents from apical membrane of the epithelial cell of a mammary gland adapted from Keenan et al., (1988).

There are three mechanisms in which the secretory vesicles containing fats, proteins and other milk constituents interact with the apical plasma membrane. According to Dylewski and Keenan (1983), the secretory vesicles form a chain of fused vesicles to release contents outside of the cell. Alternatively, individual vesicles may incorporate its membrane with the apical membrane in order to discharge its contents by exocytosis. Frank et al. (1976) and Dylewski & Keenan (1983) described the third and most accepted mechanism as a direct envelopment or “budding” of the fat globule. As the globule journeys to the apical pole it is engulfed by the plasma membrane and buds from the cell. **Figure 2.2** shown above illustrates these mechanisms.

2.2 Composition of MFGM

2.2.1 Proteins

Bovine milk contains approximately 2.0-3.5% protein (Fox, 2003) (Fox & McSweeney, 1998). Milk proteins are synthesized in the ER, transported to the Golgi Apparatus (GA), and integrated in the secretory vesicle along with lactose and other milk components (Keenan, Mather, & Dylewski, 1988). Protein accounts for 25-60% of the total dry weight of the MFGM (Keenan & Dylewski, 1995). The MFGM is comprised of over 40 proteins and some principle proteins are xanthine oxidase (XO), butyrophilin (BTN), and periodic acid/Shiff 6 and 7 (PAS 6 and 7) (Singh & Ye, 2010). A summation of the major and minor MFGM proteins is listed along with their molecular weights in **Table 2.1**.

Xanthine oxidase has a molecular weight of 150 kDa and accounts for 10- 20% of the total MFGM protein (Singh, 2006). Its complex is part of the iron-sulfur-molybdenum flavin hydroxylase family, which converts xanthine to uric acid (Singh, 2006) (Keenan,

Mather, & Dylewski, 1988). At least 60% of XO is associated with the MFGM derived from the apical membrane as it is removed from washing with non-ionic detergents or high salt concentrations (Keenan, Mather, & Dylewski, 1988). XO can act as an antimicrobial agent by producing free radicals and hydrogen peroxide in vivo (Keenan, Mather, & Dylewski, 1988).

Table 2.1. Protein composition of bovine milk fat globule membrane modified from Singh (2006) and Keenan and Dylewski (1995).

Protein	Molecular Weight (kDa)
Mucin I (MUC I)	160-200
Xanthine Oxidase (XO)	150
PAS III	95-100
CD36 or PAS IV	76-78
Butyrophilin (BTN)	67
Adipophilin (ADPH)	52
PAS 6/7	48-54
Fatty acid binding protein (PABP)	13
BRCA1	210

Butyrophilin has a molecular weight of 67 kDa and is described as an insoluble, hydrophobic glycoprotein that has a high affinity for binding with lipids (Keenan, Mather, & Dylewski, 1988). It is mainly found on the apical surface of the MFGM and plays a key role on the enclosure and stability of the fat globules during secretion (Keenan, Mather, & Dylewski, 1988). BTN is highly associated with the MFGM and has strong interactions with other proteins, making it difficult to purify by salts and active detergents (Keenan & Dylewski, 1995). Thus, sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gels are used to successfully separate and elute this protein from the membrane.

PAS 6 and PAS 7 are two types of glycoproteins with molecular weights ranging from 48-54 kDa (Singh, 2006). The amino acid sequences of these proteins are the same; however, they differ in the glycosylation with carbohydrates (Hvarregaard, Anderson, Berglund, Rassmussen, & Petersen, 1996). Their exact structure and function is not yet known but can be easily visualized in SDS-PAGE gels with periodic acid/Schiff's reagent or by staining with Coomassie Blue.

Milk contains two major heterogeneous proteins – caseins and whey proteins – which interact with the surface of the MFGM. Milk also contains blood serum albumin (BSA), immunoglobulins, lactotransferrin, and enzymes (i.e. lipases), but these components are not investigated in this research. The biological function of casein is to provide calcium from mother to neonate. Caseins are structurally complex and consist of several components including α -, β -, γ -, and κ -casein. Together, these components represent the entire composition of the protein. Caseins associate with the MFGM by disulphide bonding between the κ -casein and MFGM proteins or by sulphydryl-disulphide (SH-SS) interchanges with the MFGM proteins (Singh & Ye, 2010). They mainly exist in the form of spherical micelles, which range from 50-500 nm in diameter (Fox & McSweeney, 1998). Cheese is manufactured by precipitating caseins from milk by acidification or rennet (enzyme) coagulation. Caseins coagulate at the isoelectric point of 4.6 at 20 °C (Fox, 2003). At these conditions, caseins readily form large aggregates and precipitate from solution.

Whey (serum) proteins represent about 20% of the total proteins in bovine milk (Fox, 2003). Whey proteins are described as the globular proteins, which are roughly 3.6 nm in diameter, that remain in the milk serum after the precipitation of caseins at pH 4.6

at 20 °C (Fox & McSweeney, 1998)(Whitney, 1988). Like caseins, whey proteins contain several components: β -lactoglobulin (β -Lg) and α -lactalbumin (α -La). β -Lg has a molecular weight of 18 kDa and represents approximately 50% of whey proteins in the milk of bovine, sheep, and goat (Fox, 2003). However, it is not present in human, mouse or guinea pig milk. β -Lg is also complex in structure and contains an abundant amount of sulfur amino acids, cysteine, and two intramolecular disulfide bonds (Fox, 2003). These bonds play a role in the association of whey proteins with the MFGM. The disulfide bonds can participate in SH-SS interchanges when denatured with heat, which will be discussed later in Section 2.5.3. Lastly, α -La accounts for about 20% of whey protein and is the principle protein in human, mouse, and guinea pig milk (Fox & McSweeney, 1998). It is a less complex globular protein that has a molecular weight of about 14 kDa (Fox, 2003). It is rich in some amino acids, including tryptophan and methionine, and contains four intramolecular disulfide bonds (Fox & McSweeney, 1998). Bovine α -La is synthesized in the mammary gland and is a component of lactose synthetase, which plays an important role in the production of lactose (Fox, 2003) (Whitney, 1988).

2.2.2 Lipids

Lipids account for approximately 2-6% of the total weight of the milk fat globule and is reported to contain 0.5-1.1 mg of lipid/ mg of protein (Singh, 2006) (Keenan, Mather, & Dylewski, 1988). The MFGM is comprised of neutral lipids (triglycerides, diglycerides, sterols, and free fatty acids) and polar phospholipids that surround the fat globule for stability and prevent enzymatic degradation by lipases (Rombaut & Dewettinck, 2006). Triglycerides make up the majority of the milk fat globule core and

part of the membrane, while diglycerides, sterols, and free fatty acids make up less than 10% of the MFGM (**Table 2.2**).

Table 2.2. Lipid composition of bovine milk fat globule membrane adapted and modified from Singh (2006) and Keenan and Dylewski (1995).

Constituent Class	% of total lipids
Triglycerides	62
Diglycerides	9
Sterols	0.2-2.0
Free fatty acids	0.6-6.0
Phospholipids	26-31
Sphingomyelin	22
Phosphatidylcholine	36
Phosphatidylethanolamine	27
Phosphatidylinositol	11
Phosphatidylserine	4
Lysophosphatidylcholine	2

Phospholipids are amphiphilic molecules with a hydrophilic head and a hydrophobic tail. The major classes of phospholipids in order of abundance are PC, PE, SM, PI, and PS (**Table 2.2**). Specifically, the MFGM is rich in PC, PE, and SM in a 2:2:1 ratio (Fox & McSweeney, 1998). PC, PE, PI, and PS are all part of the glycerophospholipid family consisting of a glycerol backbone with two fatty acids esterified on the sn-1 and sn-2 positions and a phosphate residue with different organic groups on the sn-3 position (**Figure 2.3**) (Fox, 2003) (Rombaut & Dewettinck, 2006). In contrast, SM is part of the sphingolipid family consisting of ceramide, which contains a long-chain sphingosine backbone attached to phosphocholine (Dewettinck, et al., 2008). SM and PC are largely situated outside of the membrane while PE, PS, and PI are generally concentrated on the inner surface (Rombaut & Dewettinck, 2006). These polar

phospholipids contain phosphorous, which acts as an emulsifier that keeps the globule in suspension due to its positive and negative charge (Fox & McSweeney, 1998).

Figure 2.3. Chemical structures of polar phospholipids of the MFGM (Contarini & Povo, 2013)

The MFGM contains an essential number of phospholipids that can provide health benefits. Some studies claim that PC and PS are “brain-specific” nutrients and aid in enhancing memory performance in older adults with Alzheimer’s disease and recollection of words, while others show inconclusive results (McDaniel, Maier, & Einstein, 2002). SM, for example, is known for its contribution to a number of health advantages and has been greatly studied. In a study by Noh and Koo (2004), milk SM was found to be effective in inhibiting the intestinal absorption of cholesterol and other lipids in adult male rats. They suggest that the inhibitory effects are associated with the saturation of

longer chain fatty acid groups of SM derived from milk, which cause the rate of luminal lipolysis, micelle solubility, and transfer of lipids to the enterocyte to decrease (Noh & Koo, 2004). SM also exerts anti-carcinogenic activity by inhibiting cell growth, induction of differentiation, and apoptosis (Parodi, 1997). Mice with multiple intestinal neoplasia (Min) were fed SM and showed a reduced numbers of colon tumors and aberrant crypt foci (Parodi, 1997). Animal and in vitro studies have also shown that bovine fat and shingolipids enhance resistance to certain types of food-borne gastrointestinal pathogens (Sprong, Hulstein, & Van Der Meer, 2002).

2.3 Sources of MFGM

Cream, whole milk, and buttermilk are excellent sources of MFGM constituents and can be extracted using several methods of isolation (Singh, 2006) (Fox & McSweeney, 1998). Buttermilk is considered the most important source of MFGM material because it is enriched in essential phospholipids and proteins (Jimenez-Flores & Brisson, 2008). Buttermilk is the liquid by-product of churning cream into butter (**Figure 2.4**). Butter churning involves a phase inversion from oil-in-water emulsion of cream or whole milk to a water-in-oil emulsion (Fox & McSweeney, 1998). This is achieved by mechanical agitation to disrupt the MFGM and expose globular fats, followed by kneading or working to release fat liquids and subsequently form clumps of fat known as butter (Fox & McSweeney, 1998). The fat liquid is referred to as sweet buttermilk or cultured (sour) buttermilk if inoculated with lactic acid bacteria. The MFGM material is largely lost in the buttermilk, thus making it a great source of phospholipid.

Figure 2.4. Butter manufacturing flow diagram adapted and modified from O’Mahony (1988).

In this research, sweet buttermilk and whey buttermilk powder was used to compare liposomal aggregates. The compositions of the ingredients used in this project are shown in **Table 2.3**. Sweet buttermilk powder (SBM) is commercially produced, rich in phospholipids, and contains caseins and whey proteins. It is used in various food products, such as baked goods, for flavor and in salad dressings for its emulsification properties (Morin, Pouliot, & Jimenez-Flores, 2005). Whey buttermilk powder (WBM) is a novel dairy ingredient that is free of non-polar lipids and made from whey cream and treated with supercritical fluid extraction. The production of whey buttermilk powder is described in Section 3.1. In a study by Elias-Argote (2011), whey buttermilk was shown to be even more enriched with phospholipids and contain more proteins than sweet buttermilk (**Table 2.4**). Lastly, whey protein isolate (WPI) was used as the control in this research to compare the effects of phospholipids on aggregation.

Table 2.3. Composition of dairy powders.

Product	% Dry Matter			
	Protein	Fat	Lactose	Ash
Whey Protein Isolate (WPI)	93.00	0.50	1.50	2.50
Sweet Buttermilk Powder (SBM)	32.46	9.17	ND	7.36
Whey Buttermilk Powder (WBM)	72.10 ± 5.10*	20.3 ± 0.80*	4.8 ± 1.40*	2.7 ± 0.20*

* Percent standard deviation; ND = Not Determined.

Table 2.4. Phospholipid and protein composition in various dairy products adapted from Elias-Argote (2011).

Product	Phospholipids (g/100g)		Protein %	
	Wet basis	Dry basis	Wet basis	Dry basis
Raw milk	0.035	0.280	3.30	-
Skim milk	0.020	0.280	3.41	7.55
Cream	0.190	0.400	3.16	-
Isolated MFGM	0.650	6.500	1.80	60.00
Butter	0.190	0.230	0.85	-
Sweet Buttermilk	0.160	2.030	3.31	32.95
Butterserum	1.250	11.50	-	-
Fresh acid sour buttermilk	0.310	1.860	3.31	-
Acid buttermilk whey	0.100	1.840	0.99	84.70
WBP*	-	14.81	-	47.39
SPE-WBP*	-	60.59	-	72.69
Cheddar cheese	0.150	0.250	-	24.89
Cheddar cheese whey	0.020	0.260	3.49	35.20

*Abbreviations: WBP= whey buttermilk powder; SPE= solid phase extraction.

2.4 Health Benefits and Food Applications of MFGM

Over the last two decades, researchers have accumulated a great deal of knowledge on the health benefits of bovine MFGM components. The MFGM contains an abundant number of proteins and phospholipids, which can be beneficial and functional in food and nutraceutical applications (Morin, Pouliot, & Jimenez-Flores, 2005).

Membrane proteins are one contributor to functional and beneficial health including anti-

cancer effects. Studies have shown that the fatty acid binding protein (FABP), isolated from bovine MFGM, inhibits several in vivo breast cancer cell line growth (Spitsberg, 2005). Additionally, BRCA1 protein, an onco-suppressor, was found to be present in both bovine and human MFGM (Spitsberg, 2005). In a study by Wang et al. (2001), glycoproteins from bovine MFGM demonstrated inhibition of *Helicobacter pylori* infection of the gut mucosa in a mouse model. The glycoproteins used in Wang's experiments were extracted from bovine buttermilk, administered orally to mice with the *H. pylori* infection, and resulted in healing of the infection in the gastric mucosa. Another glycoprotein, BTN, was found to regulate T-cell response to the myelin oligodendrocyte glycoprotein (MOG) in mice models with inflammatory demyelinating disease of the brain, which correlates to human multiple sclerosis (Spitsberg, 2005).

The MFGM is enriched in phospholipids which also exert some anti-carcinogenic effects. As discussed previously, SM was shown to suppress and reduce colon cancer cells in mice, and SM derivatives were shown to induce apoptosis in adenocarcinoma human cell line (Parodi, 1997) (Spitsberg, 2005). Furthermore, SM was demonstrated to be an effective intestinal inhibitor of absorbing cholesterol in rats (Noh & Koo, 2004). Phospholipids also contribute to growth and development, cellular transport and absorption, and central nervous system myelination (McDaniel, Maier, & Einstein, 2002) (Spitsberg, 2005).

Membrane components of the milk fat globule MFGM material extracted from buttermilk or cream are considered an efficient emulsifying agent. Phospholipids in MFGM play an important role in stabilizing fat globules in oil-in-water emulsions and protecting the core from enzyme degradation (Singh, 2006). Phospholipids can be

incorporated in food systems as emulsifying agents since its natural function is to maintain the structure of the globules and keep them in suspension. These unique attributes of phospholipids have led to extensive research in developing technologies for isolating MFGM material for novel functional foods and nutraceutical applications.

Phospholipids derived from MFGM can be utilized as a delivery system in the food, pharmaceutical, and cosmetic industries (Thompson, Mozafari, & Singh, 2007). Delivery systems, in the form of liposomes, are commonly used to encapsulate agents such as active drugs, flavor compounds, vitamins, and enzymes (Nongonierma, Abrlova, Fenelson, & Kilcawley, 2009). Liposomes are defined as spherical vesicles, or structures, consisting of a single or multiple phospholipid bilayers surrounding an aqueous core (**Figure 2.5**) (Thompson & Singh, 2006). There have been many studies carried out on the encapsulation of active ingredients using liposomes as a mode of transport. Nongonierma et al. (2009) evaluated food grade liposomes to encapsulate commercialized enzyme extract that decrease bitterness in protein hydrolysates and cheese. Thompson and Singh (2006) (2007) looked at various techniques of preparing liposomes via microfluidization, and Benjamin et al. (2012) investigated liposomes as carriers for volatile organic compounds such as flavors. Bezelgues et al. (2008) demonstrated a two-fold incorporation of α -tocopherol, a form of Vitamin E, and lycopene in mixed micelles using MFGM material. Together, these studies confirmed functional and beneficial use of liposomes derived from bovine MFGM as vehicles for nutrient transport.

Figure 2.5. Typical structure of a liposomal vesicle adapted from McClements (2005).

Not only do MFGM proteins and phospholipids contribute health attributes and functional properties in food systems, but they also influence texture. The MFGM can be considered as a stabilizer or surfactant because it contains phospholipids to enhance the stability of an emulsion (McClements, 2005). Stabilizers are added to foods to reduce the rate of creaming, increase viscosity of a continuous phase, and modify texture by thickening or gelling. MFGM constituents can be incorporated into foods like baked goods, chocolate, spreads, and beverages for stabilization. To better understand texture, we must study how liposomal particles behave on a molecular level. Hydrophobic interactions are said to be the main driving force of liposome formation, which causes the liposomes to reduce unfavorable contact between the hydrophobic tails of phospholipids and water (McClements, 2005). Additionally, proteins also interact with each other and the MFGM via disulfide bonding and hydrophobic interactions, which causes unique texture attributes such as foaming (Bouaouina, Desrumaux, Loisel, & Legrand, 2006).

2.5 Physiochemical Effects of Processing on Phospholipids and MFGM

2.5.1 Homogenization

High pressure, two-stage valve homogenizers are generally used to manufacture fluid milk and milk products because of its ability to effectively minimize droplet size (Fox & McSweeney, 1998)(McClements, 2005). In industry, milk is homogenized in two stages by forcing liquid milk through a small orifice. In the first stage the milk is forced at a high pressure, usually 2000 psi, at 40°C (**Figure 2.6**). During this stage, the average diameter of the fat globules have significantly decreased through actions of high shear, collision, and distention, which results in high viscosity due to the small fat globules clumping together (Fox & McSweeney, 1998). In the second stage, the homogenization pressure is lowered to about 500 psi to reduce viscosity by dispersing the fat clumps (McClements, 2005).

Figure 2.6. Homogenizer mechanism diagram adapted from Spence et al. (2010).

Homogenization techniques can be utilized to generate liposomes and aggregates for use in various applications. Upon homogenization, fat globules are disrupted which result in globule size reduction and an increase in their specific surface area (Zamora, Ferragut, Guamis, & Trujillo, 2012). Milk processing triggers physicochemical interactions between MFGM constituents and whey proteins and caseins (Zamora, Ferragut, Guamis, & Trujillo, 2012). **Figure 2.7** illustrates this phenomenon of non-native MFGM protein adsorption. Ye et al. (2008) investigated changes in the surface proteins of fat globules during homogenization with heat treatments and found that the amount of native MFGM proteins was not changed during homogenization. Many studies have suggested that caseins and whey proteins do not alter the conformation of MFGM proteins but rather are adsorbed onto the surface of the MFGM (Ye, Anema, & Singh, 2008) (Zamora, Ferragut, Guamis, & Trujillo, 2012) (Bouaouina, Desrumaux, Loisel, & Legrand, 2006) (Lee & Sherbon, 2002).

Figure 2.7. A representation of the MFGM in homogenized milk (Fox & McSweeney, 1998).

2.5.2 Microfluidization

Microfluidization is another type of homogenizer and is a technique that can rapidly create high volumes of emulsions with very small globule sizes (McClements, 2005) (Thompson & Singh, 2006). A microfluidizer consists of a fluid inlet, an air motor pumping at high pressure, and an interaction chamber where two channels of fluid flow at high velocity and collide with each other (**Figure 2.8**) (McClements, 2005). When the fluids from the two channels collide, it generates intense, disruptive forces that result in very small emulsion droplets. Like homogenization, this technique triggers physicochemical interactions between MFGM components and whey proteins and caseins and has been utilized to prepare liposomes and aggregates for functional applications in different industries. This technique is also suitable for use in the food industry because it does not require the use of detergents or solvents (Thompson &

Singh, 2006). As mentioned previously, Nongonierma et al. (2009) evaluated liposome encapsulation of commercial enzymes using microfluidization, which resulted in high encapsulation capacity of the enzymes. Thompson and Singh (2006) (2007) also investigated liposomal preparation using a microfluidizer at various pressures and number of passes.

Figure 2.8. Microfluidizer mechanism diagram modified from Spence et al., (2010).

2.5.3 Temperature and pH

Temperature and pH play principle roles in the physical and chemical changes of lipids and proteins and are inversely related to each other. The pH of milk at 25 °C is usually 6.5-6.7 and its pH is heavily influenced by temperature than pH (Fox & McSweeney, 1998). With higher thermal treatments, the pH of milk will decrease due to lactose degradation to organic acids (Fox & McSweeney, 1998). Lipids do not have significant changes in pH because they are least effected by heat. When milk is heated to

approximately 70 °C, a group of immunoglobulins called cryoglobulins, which are responsible for the aggregation and creaming of fat globules, are irreversibly denatured and prevent creaming from occurring (Fox & McSweeney, 1998). Foaming may also occur with heat treatment and agitation due to changes in the MFGM (Fox & McSweeney, 1998).

In whey proteins, temperatures above 70 °C cause denaturation by exposing and activating free SH group in cysteine residues (Fox & McSweeney, 1998). This causes the release of hydrogen sulfide (H₂S), which is responsible for the undesirable cooked flavor in milk, and disulfide interchanges with β -Lg and α -La at the pH of milk, eventually resulting in aggregation and attachment on the fat globules (Fox & McSweeney, 1998) (Dalglish, Senaratne, & Francois, 1997) (De Wit, 2009). However, at acidic conditions the free SH groups are considered to be inactive (Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013). Hydrogen bonding has been suggested to be responsible for increasing the thermal stability of proteins at low pH (Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013). Caseins, on the other hand, are thermally stable. However, a component of casein, κ -caseins, form aggregates with denatured β -Lg via disulfide interchanges and hydrophobic interactions when heated at 85 °C-90 °C at pH 6.8 (Brown, 1988).

2.6 Techniques of Analysis

2.6.1 Particle Size Distribution

Particle size characterization is important in food products because it has a strong impact on food stability, viscosity, optical properties, and sensory attributes (McClements, 2005). Many industries employ particle size characterization to maintain

quality of products and to gain a better understanding of ingredients and processes (Malvern, 2012). The particle concentration is usually reported as volume percent (Volume %) or number percent (Number %), and the actual size of the particles are reported as mid-point particle radius or mid-point particle diameter (McClements, 2007). In this research, the particle size distribution is presented in Number % by mid-point particle diameter (μm).

A variety of analytical instruments are commercially available for use in industry to characterize particles, such as laser diffraction, dynamic light scattering, automated imaging, electrophoretic light scattering, and electrical pulse counting (McClements, 2007) (Malvern, 2012). A laser diffraction particle size analyzer from Beckman Coulter was used in this thesis to determine the particle diameters of each sample. This instrument uses a laser beam to scatter light through a dilute medium to determine the particle size distribution. This distribution is based on the intensity of the scattered light and the angle at which the light is scattered (McClements, 2007). The Beckman Coulter LS 230 Fluid Module particle size analyzer comes with software that contains a mathematical model called the “Mie Theory” that can calculate the scattering pattern of a dispersed particulate sample from characteristics of the particulates (McClements, 2005)(McClements, 2007).

2.6.2 Phospholipid Analysis

The Mojonnier Method is principally utilized in the dairy industry to measure fat content in milk. This method is the standard AOAC method (989.05) that uses a mixture of ethyl ether and petroleum ether to extract fat from both the liquid and solid samples into the organic layer without needing to remove moisture (Min & Boff, 2003).

Following extraction, the defatted solids are dried and expressed as percent fat by weight. This method has some limitations due to the use of ammonium hydroxide, ethers, and heat which can lead to oxidization and hydrolysis of phospholipids (Van der Meeren & Vanderdeelen, 2000)(Rombaut & Dewettinck, 2006). Cold-extraction procedures using chloroform and methanol, such as the Folch, Lees and Stanley method (1956), are more advantageous because it does not require heat and harsh chemicals that would affect the nature of the phospholipids (Rombaut & Dewettinck, 2006). However, the cold-extraction technique is very time consuming and non-lipid contaminants can be co-extracted and lead to erroneous results (Rombaut & Dewettinck, 2006). The Folch method is best utilized in small sample quantities as it requires the heavy use of toxic reagents. The Mojonnier method is best used when the lipid contents are much higher.

Prior to polar phospholipid analysis, a concentration and separation step is often carried out using solid phase extraction (SPE) to remove any contaminants, such as non-polar lipids. Since phospholipids are present in milk at very low concentrations, this extra step is necessary for the fractionation of polar lipids to improve the separation of polar lipids on thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC) (Bitman, Wood, Mehta, Hamosh, & Hamosh, 1984). The Bitman et al. (1984) method of separation was used in this thesis to separate and concentrate polar phospholipids for HPLC analysis. SPE was performed on Mojonnier fat extracted samples using small pre-packed, disposable silica cartridges. These cartridges contained silica particles that offered strong polar selectivity to phospholipids and could withstand the elution of non-polar impurities using hexane and diethyl ether.

Phospholipids can be separated and analyzed using a variety of techniques, including TLC and HPLC. In the past, TLC was commonly used to separate the phospholipid classes because it was simple, inexpensive, and resulted in good separation of lipids (Van der Meeren & Vanderdeelen, 2000). However, the quantification of phospholipids became a major problem because it required modern and expensive computer-controlled scanners. As an alternative, HPLC has become the method of choice to accurately separate and quantify phospholipids in dairy because the technique is highly flexible and easier to automate (Van der Meeren & Vanderdeelen, 2000).

A wide variety of detectors can be used for HPLC analysis, including evaporative light-scattering (ELSD), light absorption, fluorescence, refractive index (RI), flame ionization (FID) and mass spectrometry (MS). ELSD is the most commonly used detector for the analysis of phospholipids because of its compatibility with wide ranges of eluents and is uniform in response (Van der Meeren & Vanderdeelen, 2000). However, normal phase HPLC with a Charged Aerosol Detector (CAD) was used to analyze and quantify phospholipids. In recent years, a new type of detector, a CAD, was developed that involved the nebulization HPLC silica column effluent, evaporation of solvents, charging aerosol particulates and measurements of the aerosol current (Moreau, 2006). The CAD is capable of detecting common lipids in dairy, making it a valuable tool for quantitative HPLC analysis. In addition, the use of the CAD is advantageous because of its low minimum detection limits and linear mass-to-peak area relationship for various types of fats (Moreau, 2006).

For this project, the phospholipid analysis via HPLC was performed using methods described by Rombaut et al. (2005). This HPLC method uses a gradient of

chloroform, methanol , and formic acid buffer with triethylamine at pH 3 to resolve the five classes of phospholipids in less than 21 minutes and extends the life of the silica column to over 1500 runs (Rombaut, Camp, & Dewettinck, 2005) (Rombaut & Dewettinck, 2006).

2.6.3 Protein Analysis

There are many techniques used in the dairy industry for protein determination. The Kjeldahl method, which is an AOAC standard for measuring protein, is one technique that digests and breaks down organic nitrogen compounds using concentrated sulfuric acid (H_2SO_4) in the presence of a catalyst, into ammonium (NH_4^+), titrated, and calculated using a conversion factor to give crude protein content (Tremblay, Laporte, Leonil, Dupont, & Paquin, 2003). However, this technique uses highly-corrosive reagents and is very time consuming. The bicinchoninic acid (BCA) assay was invented by Paul Smith from the Pierce Chemical Company. The BCA assay is defined as a colorimetric detection that is used to determine the concentration of protein in a sample (Bainor, Chang, McQuade, Webb, & Gestwicki, 2011). This technique measures the reduction of Cu^{2+} (cuprous) to Cu^{1+} (cupric) using BCA, forming a purple-colored reaction (Olson & Markwell, 2007). The absorbance is then measured at 563 nm and is compared to a standard curve for concentration determination. The BCA assay was used in this project to initially determine the protein concentration of WPI, SBM, and WBM powders. This method was used over other protein determination methods because it is capable of producing better results with samples containing complex protein mixtures (Olson & Markwell, 2007).

Many proteins interact with each other through naturally occurring intermolecular disulfide bonds. These bonds can be broken down and studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Milk and MFGM protein composition is usually examined with 8% acrylamide SDS-PAGE under reducing conditions (Zamora, Ferragut, Guamis, & Trujillo, 2012). However, caseins and whey proteins rapidly run out of the 8% gels since they are low in molecular weight (Zamora, Ferragut, Guamis, & Trujillo, 2012). Therefore, 15% acrylamide SDS resolving gels are now commonly used to study the proteins with Coomassie Blue staining (Singh, 2006). In this thesis, 15% acrylamide SDS-PAGE were performed on each prepared sample to determine whether or not protein aggregation was effected by the treatment given.

2.7 Justification for Present Research

According to the USDA, Americans do not consume enough dairy products and are, on average, gradually drinking less fluid milk now than in the 1970s for all age groups (**Figure 2.9**) (Stewart, Dong, & Carlson, 2013). Milk is one of the most nutritious products that nature has provided. This continuous decline in fluid milk consumption may lead to potential health implications since many consumers may not know and understand its nutritional value (Stewart, Dong, & Carlson, 2013).

Figure 2.9. Daily fluid milk consumption per capita in the United States (Stewart, Dong, & Carlson, 2013). Whole milk refers to milk containing $\geq 3.25\%$ fat and lower fat milk refers to 2%, 1%, and skim milks.

In contrast, the per capita consumption of dry buttermilk in the United States has been on the rise since 2005 (**Figure 2.10**) (USDA, 2012). Buttermilk is a nutritious dairy ingredient that comes from butter manufacturing. Buttermilk is a great source for MFGM constituents because it is enriched in phospholipids and proteins. It is also widely used in the food industry for its compositional and functional attributes, such as protein solubility, emulsifying capacity, viscosity, and foaming properties (Sodini, Morin, Olabi, & Jimenez-Flores, 2006). MFGM components not only offer functional properties but also contribute to health benefits as well. Phospholipids derived from the MFGM can be utilized as vehicles for bioactive components and nutrients for food and nutraceutical applications.

Figure 2.10. Annual dry buttermilk consumption (pounds) per capita in the United States (USDA, 2012)

The purpose of this thesis is to study and compare the composition and structure of liposomal aggregates made from the phospholipids of commercial sweet buttermilk and whey buttermilk, prepared by microfluidization and homogenization. Particle size of the liposomal aggregates was investigated using a laser diffraction particle size analyzer. Advanced HPLC methodologies were applied to determine phospholipid concentration of the aggregates using a CAD in the buttermilk samples. Lastly, protein content was determined using SDS-PAGE in the liposomal aggregated samples. This thesis contributes to the foundation of understanding MFGM components, particularly phospholipids, in order to utilize them in future applications.

3.0 Materials and Methods

3.1 Powders

In this research, three dairy ingredients were used to study the effects of high shear on liposomal aggregates. Commercial sweet cream buttermilk (SBM) powder was obtained from Grassland Dairy Products Inc. (Lot# 02012581202, Greenwood, WI, USA). Hilmar™ 9000 Whey Protein Isolate (WPI) powder was obtained from Hilmar Ingredients (Lot# 12145, Hilmar, CA, USA). Whey buttermilk (WBM) powder was manufactured at Cal Poly from pasteurized whey cream (Hilmar Cheese Company, Hilmar, CA, USA), which was derived from cheese processing. The whey cream was processed by methods described by Costa et al. (2010). Whey cream was churned into butter using a rotary churn system (Blentech Corporation, Santa Rosa, CA, USA) and placed through a cream separator to remove any residual butter. The buttermilk was concentrated using a pilot plant scale, GEA-Niro R12 Membrane Filtration unit (Hudson, WI, USA). Ultrafiltration (UF) was carried out 4 times and diafiltration (DF) was carried out twice to remove any caseins, lactose, minerals and water. The concentrated whey buttermilk was spray-dried into powder and processed using supercritical fluid extraction (SFE) (Thar Designs, Inc., Pittsburgh, PA, USA) to remove non-polar lipids. The finished product was placed into sterile storage bags and stored in the refrigerator at 10 °C until needed.

3.2 Microfluidization: Sample Preparation, Treatments and Sample Collection

3.2.1 Sample Preparation

Samples are standardized to 2% (w/v) protein content by reconstituting WPI (2.8845 g/300 mL), SBM (2.5510 g/300 mL) and WBM powders (2.7574g/300 mL).

Figure 3.1 illustrates the process flow of preparing samples. Each powder was rehydrated with room temperature deionized water in 600 mL Pyrex® beakers and agitated with a 1 inch stir bar on a stir plate (Corning PC-620D, USA) at 360 rpm for 1 hour at room temperature (21.0 ± 1.03 °C). The pH of the solutions were adjusted to 4.60 using 0.1 or 1 N HCl (SA48-1, Fisher Scientific, Fair Lawn, NJ, USA) and to 6.80 using 0.1 or 1 N NaOH (SS266-4, Fisher Scientific, Fair Lawn, NJ, USA) using a pH meter (Thermo Scientific Orion® Model 410A+, USA) and reference electrode (Thermo Scientific Orion® 9157BN, USA). Each solution was heated to 70 °C using a hotplate (Corning PC-620D, USA) for 10 minutes with agitation at 360 rpm. The temperature of the solutions were monitored and read with a digital thermometer (Control Company Traceable®, Friendswood, TX, USA). The solutions were removed from the heat and allowed to cool to room temperature. Once cooled, the pH of the solutions were read and readjusted to 4.60 and 6.80 as needed.

Figure 3.1. Sample Preparation for Microfluidization.

3.2.2 Microfluidization Treatment and Sample Collection

The reconstituted solutions at room temperature were passed through a bench top scale microfluidizer (Avestin, Inc., model EmulsiFlex™ - C5, Ottawa, Canada) (**Figure 3.2**) at 2000 psi for a total of 3 passes. The first and last 10-15 milliliters of each sample were discarded to prevent sample dilution from any residual fluid left within the lines from cleaning and processing. The temperature of each sample was measured and recorded before and after each pass through the microfluidizer. The microfluidizer sample cylinder was rinsed and cleaned 5 times with deionized water before and after each sample. Samples were collected in 15 and 50 mL Falcon® Bluemax™ polypropylene conical tubes (Becton Dickson Labware, Franklin Lakes, NJ, USA) for analysis before and after each pass for a total of 3 passes. The 15 mL Falcon® tubes were stored in the refrigerator at 10 °C for particle size analysis and the 50 mL Falcon® tubes were stored in the freezer at -30 °C for phospholipid and protein analysis.

Figure 3.2. Avestin EmusliFlex™-C5 microfluidizer unit. A) sample cylinder; B) air motor; C) pressure gauge; D) interaction chamber; E) air pressure gauges from source.

3.3 Homogenization: Sample Preparation, Treatments and Sample Collection

3.3.1 Sample Preparation

For pilot-plant scale, 2% protein (w/v) solutions were made by reconstituting WPI (28.8450 g/3 L), SBM (25.5099 g/3 L) and WBM powders (27.5736 g/3 L). Each powder was rehydrated with room temperature deionized water and mixed together using a commercial blender (Waring MX1000, Torrington, CT, USA) on low speed for 1 minute and high speed for 10 seconds. Each rehydrated solution was placed in a 6 L container and the pH was adjusted to 4.60 and 6.80 using a pH meter. Each solution was transferred to a 1 gallon stainless steel pot and heated over a kitchen stove top on high heat to 70 °C for 10 minutes with continuous stirring using a wired whisk. The rehydrated mixtures were removed from the heat, transferred into clean 6 L containers, and allowed to cool to room temperature. The pH of each dairy ingredient was read and readjusted to 4.60 and 6.80 as needed.

3.3.2 Homogenization Treatment and Sample Collection

The reconstituted powders were homogenized at 2000 psi/500 psi using a pilot plant scale homogenizer (PMS Processing Machinery & Supply Co., Philadelphia, PA, USA) (**Figure 3.3**) at room temperature. The first and last 500 mL of each sample were discarded to prevent sample contamination from any residual fluid left within the lines from cleaning and processing. The temperature of each sample was measured and recorded before and after homogenization. The homogenizer sample cylinder was rinsed and cleaned 5 times with water before and after each sample. Samples were collected before and after homogenization in 15 and 50 mL Falcon® tubes for analysis. The 15 mL Falcon® tubes were stored in the refrigerator at 10 °C for particle size analysis and the 50 mL Falcon® tubes were stored in the freezer at -30 °C for phospholipid and protein analysis.

Figure 3.3. Pilot plant scale homogenizer.

3.4 Physical Properties of Liposomal Aggregates

3.4.1 Particle Size Distribution

The particle diameters (μm) of all WPI, SBM and WBM samples were determined according to a method described by Menard et al. (2010) using a laser diffraction particle size analyzer (Beckman Coulter, model LS 230 Fluid Module, CA). The fluid chamber was filled with 1 liter of room temperature deionized water. Before analysis, 16 mL of 35 mM EDTA/NaOH pH 6.8 buffer (99+% disodium salt dehydrate, Sigma) was added to the fluid chamber and allowed to adjust for electrical offsets and laser alignments prior to background measurements. Offsets and background were measured for 30 and 60 seconds, respectively. The fluid module was sonicated for 15 seconds before each runtime. The runtime was measured for 60 seconds with a pump speed of 51 and sample loading measurements were maintained at 10% obscuration. Samples with an obscuration rate less than 3% were considered soluble and measurements were not taken for those samples.

The standard mean diameters, in Number % mode, were calculated by the Beckman Coulter LS Software v. 3.29, and all measurements were performed in triplicate. The arithmetic mean diameter ($d_{1,0}$) is defined by Equation 1 as:

$$d_{1,0} = \left[\frac{\sum n_i d_i^1}{\sum n_i d_i^0} \right] \quad (\text{Eq. 1})$$

Where n_i is the number of aggregate particles of diameter (d_i).

3.5 Chemical Composition

3.5.1 Fat Extraction by Mojonnier Method

Fat was extracted from WPI, SBM, and WBM powders, as well as the 3rd pass of the microfluidized and after homogenization of SBM and WBM pellets at pH 4.60 using the AOAC International modified Mojonnier method (989.05, 1996). All reagents used in this analysis were ACS grade from Fisher Scientific (Fair Lawn, NJ, USA). For WPI and buttermilk powders, 1 gram of powder was weighed into Mojonnier extraction flasks (# 617600-0025, Kimbel/Kontes Glass, Vineland, NJ, USA) and diluted with 8.5 mL of deionized water at 38 °C. The flasks were shaken for 30 seconds to thoroughly mix, and then tempered in a 38 °C water bath for 30 minutes. To obtain the SBM and WBM pellets, 50 mL of both homogenized and microfluidized samples at pH 4.60 were centrifuged (Eppendorf Centrifuge 5810R, Germany) at 4000 rpm at 21 °C for 10 minutes. The supernatant was poured out and discarded. The pellets were collected and approximately 1 gram of each pellet was weighed out into Mojonnier extraction flasks. Each flask was diluted with 8.5 mL of deionized water at 38 °C, shaken for 30 seconds to mix, and tempered in a 38 °C water bath for 30 minutes.

For the first fat extraction, 1.5 mL of ammonium hydroxide (A669-212) was added to each flask to neutralize acids and dissolve caseins (**Figure 3.4**). Three to five drops of 1% phenolphthalein solution (SP62-1) were added to the sample portion to differentiate between the organic and inorganic layers. Next, 10 mL of 95% denatured ethyl alcohol (A407-4) were added to each flask to prevent gelation, and then shaken vigorously by hand for 15 seconds. To each flask, 25 mL of ethyl ether (E199-4) were added to dissolve the lipids followed by the addition of 25 mL of petroleum ether (E120-

4) to extract more lipids and remove any water from the organic layer. After each chemical addition, the Mojonnier flasks were vented to allow excess gas and pressure to escape. The flasks containing WPI, WBM, and SBM samples were centrifuged using a Garver Electrifuze 308M (Garver Manufacturing Inc., Union City, IN, USA) for 30 seconds. The organic layer was decanted into pre-weighed aluminum tins, which were dehydrated at 100 °C for 1 hour and stored in a dessicator prior to use.

Figure 3.4. Mojonnier fat extraction flow diagram.

During the second extraction, 5 mL of 95% denatured ethyl alcohol were added and shaken for 15 seconds. Fifteen mL of ethyl ether and petroleum ether were consecutively added and shaken for 1 minute after each addition. The flasks were centrifuged for 30 seconds and decanted into respective aluminum tins. The organic layer

was evaporated on a 60 °C hotplate and transferred into a 100 °C oven (Fisher Scientific, Isotemp Oven 750G, USA) for 30 minutes. The tins were transferred into a dessicator and allowed to cool to room temperature prior to weighing them. The fat samples were dissolved in 2 mL of HPLC grade hexane (H302-4) and stored in 4 mL amber vials (Fisher Scientific, USA) at -15 °C for further analysis.

3.5.2 Phospholipid Solid Phase Extraction (SPE)

Phospholipids were extracted from the Mojonier fat extracted samples by methods explained by Bitman et al. (1984). The reagents used for this analysis were of HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). The amber vials containing fat dissolved in hexane were removed from the freezer and allowed to come up to room temperature. Strata SI-1 Silica columns (2 g/12 mL, 55µm, 70A) were placed onto a 12-position SPE vacuum manifold system (Phenomenex, AH0-6023, USA) with 9 mL glass tubes positioned under each column. The silica cartridges were conditioned with 20 mL of chloroform (C606-4) and the extracted fat samples were loaded into the cartridges. The vials were washed with 1 mL of hexane to obtain residual lipids in the vial and added to the columns. The solvents and lipids were allowed to pass through the cartridge by gravity to enhance the retention of polar lipids. Neutral lipids were eluted from the fat samples with 40 mL of 1:1 (v/v) hexane:ethyl ether (309966, Sigma-Aldrich, St. Louis, MO, USA) and discarded. The 9 mL collection tubes were changed to clean ones to recover the polar lipid fraction. Polar lipids were eluted with 20 mL of methanol (A452-4) to remove 80% of the phospholipids and the rest were eluted with 20 mL of chloroform. **Figure 3.5** illustrates the process flow of the solid phase extraction. Each set of polar lipid fractions were pooled together and transferred into pre-weighed 50 mL

Pyrex® round bottom flasks (24/40, USA). The organic solvents were evaporated from each sample using a rotary evaporator (Labconco, Kansas City, MO, USA) connected to a pump (Cole-Palmer Instrument Company, MasterFlex Microprocessor Pump Drive, USA) in a 55 °C water bath. To evaporate any residual water, the round bottom flasks containing the extracted polar lipids were wrapped in aluminum foil and flash-frozen with liquid nitrogen. The frozen round bottom flasks were freeze-dried under vacuum using a lyophilizer (Labconco, Freezone 4.5, USA) for 24 hours or until white phospholipid residues were observed. The dried phospholipids were weighed and dissolved in 2:1 (v/v) chloroform:methanol to make 2 or 5 mg/mL of phospholipids per sample. The recovered phospholipids were transferred into clean, 4 mL amber vials and stored in the freezer at -15 °C until HPLC analysis.

Figure 3.5. Solid phase extraction process flow diagram.

3.5.3 Phospholipid Profiling and Quantification by High Pressure Liquid Chromatography (HPLC)

For phospholipid analysis, all reagents used were HPLC grade manufactured by Sigma-Aldrich (Germany) and Fisher Scientific (Fair Lawn, NJ, USA). The following pure polar lipids obtained from Sigma-Aldrich (Germany) were used as standards to identify lipids: 3-sn-Phosphatidylethanolamine (PE), L- α Phosphatidylinositol (PI), 3-sn-Phosphatidyl-L serine (PS), L- α -Phosphatidylcholine (PC), and N-Palmitoyl-D-sphingomyelin (SM).

Figure 3.6. Shimadzu Prominence HPLC unit with a CAD detector. A) degassing unit; B) pump; C) autosampler; D) communication bus module; E) column oven; F) CAD detector.

Phospholipid separation of each sample was analyzed using HPLC according to methods described by Rombaut et al. (2005). A Prominence Ultra Flow Liquid Chromatography system (LC-20AB, Shimadzu) (**Figure 3.6**) with 2 solvent lines was used to carry out the phospholipid separations. The HPLC communication bus module (CBM-20A, Shimadzu) was a communication link between the HPLC equipment and a

computer output. The CBM was connected to an auto sampler (SIL-20A HT, Shimadzu), degasser (DGU-20A₃, Shimadzu), pump (LC-20AB), column oven (CTO-20A, Shimadzu) and a Charge Aerosol Detection (CAD) detector (Corona Plus, Dionex, USA), where Nitrogen gas was used as a nebulizer at 35 psi. A 150 mm x 3 mm Prevail™ silica column with a 3 μm particle diameter (Part no. 99341, Grace Division, USA) and a 7.5 mm x 3 mm Prevail™ guard silica column with a 5 μm particle diameter (Part no. 99354, USA) were equilibrated in the column oven at 40 °C. Ten μL of sample were injected with the auto sampler for analysis. The elution of phospholipids were achieved using a linear gradient with 87.5:12:0.5 (v/v/v) chloroform:methanol:triethylamine (TEA) buffer (pH 3.0, 1 M formic acid) at t=0 minute to 28:60:12 (v/v/v) chloroform:methanol:TEA buffer at t=16 minutes (Rombaut et al., 2005). At t=17 minutes, the column was flushed and allowed to equilibrate at initial conditions prior to the next injection at t=28 minutes. The injection loop was rinsed with 2:1 (v/v) chloroform:methanol followed by an injection of the phospholipid standards after every 6th sample and the flow rate was maintained at 0.5 mL/min.

Phospholipid data was collected and quantified using the Shimadzu LC Solution software. Phospholipid peaks were identified by comparing the retention times of the pure phospholipid standards. The pure phospholipids were serially diluted (0.05 – 0.2 mg/mL) with 2:1 (v/v) chloroform:methanol. Calibration curves were created from peak area values) and a linear regression equation ($y = a + bx$) was applied for each phospholipid.

3.6 Protein Composition

3.6.1 Protein Quantification – Bicinchoninic Acid (BCA) Assay

Pierce BCA™ Protein Assay Kit (#23252, Thermo Scientific, Rockford, IL, USA) was used to determine the initial protein concentration of WPI, SBM and WBM powders. A test tube standard protocol was followed according to the manufacturer's instructions. A 5% (w/v) solution of each powder was made using room temperature deionized water. Dilutions of bovine serum albumin (BSA) standards were prepared per manufacturer's specifications with deionized water in 1.5 mL Eppendorf conical tubes. Working reagent was prepared by mixing 50 parts of Reagent A and 1 part of Reagent B. One hundred μL of each BSA standard dilutions and powder samples were pipetted into clean test tubes followed by a 2.0 mL addition of the working reagent. The test tubes were gently shaken to mix, incubated in a 37 °C water bath for 30 minutes, and allowed to cool to room temperature. The absorbance for each sample was measured using a spectrophotometer (Molecular Devices, SpectraMax Plus, USA) set to 562 nm. To determine the protein concentration of WPI, SBM, and WBM, a standard curve was created with the average absorbance of the BSA standard vs. its concentration ($\mu\text{g/mL}$). All powder samples and standards were prepared and quantified in duplicate.

3.6.2 Sodium Dodocyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on WPI, SBM, and WBM samples before and after homogenization and before and after the 3rd pass of microfluidization under reducing and non-reducing conditions. SDS-PAGE equipments, regents, and prestained protein standards (161-0318, broad range) were of electrophoresis grade and obtained from

BioRad Laboratories, Inc. (Hercules, CA, USA). A 1:6 dilution of each sample was prepared with 2x Laemmli sample buffer (161-0737) in 1.5 mL Eppendorf tubes. Five percent of beta-mercaptoethanol (BP176-100, Fisher Scientific, Fair Lawn, NJ, USA) was added to the sample buffer for reducing conditions. The samples were heated at 95 °C for 4 minutes to break down proteins and allowed to cool to room temperature. Handcasted, 15% acrylamide SDS gels were prepared and mounted into an electrophoresis cell. The chambers of the cell were filled with 1x Tris-Glycine-SDS running buffer (161-0772). Fifteen µL of protein standard and sample were loaded into each individual well. The electrophoresis cell was connected to a power supply unit (PowerPAC 300) and the gels were run at a constant 200 V for 40-50 minutes or until the protein bands reached the bottom of the gels. The gels were washed with deionized water 3 times prior to staining with Coomassie Brilliant Blue R-250 (161-0436). The gels were stained for about 6-8 hours at room temperature on a rotatory incubator (Max 2000 E-class, Barnstead, IA, USA), rinsed 3 times with deionized water and destained with destaining solution. Prior to imaging, the gels were rinsed an additional 3 times with deionized water. The images were captured with a BioRad Imaging Universal Hood II in EPI white mode and analyzed with Quantity One 1-D Analysis software v4.6.3 for protein band quantification. The trace quantity (peak area x density in units of intensity x mm) parameter was used to compare the protein band intensities in each sample.

3.7 Experimental Design

The experimental design of this thesis consisted of 2 processing methods: microfluidization and homogenization. The microfluidization method was conducted using a 3x2x4 factorial in a randomized complete block design with a block on day (1, 2,

3, 4, and 5). The 3 treatment factors were milk powders (WPI, SBM, and WBM), pH (4.6 and 6.8), and number of passes through the microfluidizer (0, 1, 2, and 3) with 24 treatment combinations per day. The homogenization method was conducted using a 3x2x2 factorial in a randomized complete block design with a block on day (1, 2, and 3). The 3 treatment factors were milk powders (WPI, SBM and WBM), pH (4.6 and 6.8), and time point (before and after) with 12 treatment combinations per day. Treatments were randomly assigned to the experimental unit using JMP Pro 9 (SAS Institute Inc.). All experiments were performed on different days for both processing methods. The responses were particle diameter (μm) from particle size analysis, concentration of phospholipids using HPLC, and trace quantity of protein band intensities by SDS-PAGE.

The particle sizes for WPI and SBM samples were not measured during particle size analysis because they were soluble at pH 6.8. Therefore, the experimental design of this study was adjusted to one treatment factor with four levels: SBM at pH 4.6, WPI at pH 4.6, WBM at pH 4.6, and WBM at pH 6.8.

3.8 Statistical Analysis

All experiments were performed using a randomized complete block design with a block on days. Microfluidization and homogenization processes were used to produce liposomal aggregates. The treatment factors for microfluidization were milk powder-pH combination (WPI4.6, SBM4.6, WBM4.6, and WBM6.8), and number of passes (0, 1, 2, and 3). The treatment factors for homogenization were the same as for microfluidization except time point (before and after) replaced number of passes. Particle diameter was the response variable for the statistical analysis in this research. All ANOVA statistical analyses were carried out using JMP Pro 10. ANOVA F-tests for each analysis used a 5%

overall significant level. Each ANOVA term was checked using a Bonferroni adjusted 1.67% individual significance level. Levels of significant terms were compared using a Tukey-Kramer multiple comparison test at the 1.67% significance level. Before and after particle sizes were compared using confidence intervals adjusted to 95% overall confidence (99.37% individual confidence).

4.0 Results and Discussion

4.1 BCA Protein Assay

The BCA Protein Assay was performed to determine the initial protein concentration of 5% (w/v) reconstituted solutions of WPI, SBM, and WBM powders prior to executing the experimental design. The absorbance was taken for each sample to determine the protein concentration using a bovine serum albumin (BSA) standard curve (**Figure 4.1**). The protein concentration of the WPI, SBM, and WBM solutions ranged from 10-13% with means of 10.4%, 11.8%, and 10.9%, respectively (**Table 4.1**). SBM showed higher protein content because it contained caseins. WPI and WBM are concentrated in whey proteins and contain little to no caseins. From this assay, 2% (w/v) protein solutions of WPI, SBM, and WBM were prepared for the execution of the experimental design to mimic the protein content in milk (Sodini, Morin, Olabi, & Jimenez-Flores, 2006).

Figure 4.1. BSA standard curve of absorbance at 562 nm vs. concentration (mg/mL). $R^2 = 0.957$.

Table 4.1. BCA protein assay of 5% (w/v) reconstituted dairy powders.

Repetition	Sample	Concentration (mg/mL)	Protein (%)
1	WPI	5.234	10.468 ± 0.00
2	WPI	5.161	10.321 ± 0.00
1	SBM	5.457	10.915 ± 0.00
2	SBM	6.354	12.708 ± 0.00
1	WBM	5.331	10.661 ± 0.00
2	WBM	5.547	11.095 ± 0.00

4.2 Physical Properties of Liposomal Aggregates

4.2.1 Particle Size Analysis

The particle size distribution was determined using the Beckman Coulter LS 230 particle size analyzer for WPI, SBM, and WBM samples that underwent treatments. EDTA, a chelating agent, was used to disrupt and dissociate casein micelles by sequestering Ca^{2+} for particle stability (Menard, et al., 2010). The liposomal aggregate dispersions were analyzed using the number-weighted average diameter ($d_{1,0}$) as a function of particle diameter (μm). The particle size distribution resembled a Gaussian curve, which is in accordance with previous particle size studies (Menard, et al., 2010) (Sodini, Morin, Olabi, & Jimenez-Flores, 2006) (Nongonierma, Abrlova, Fenelson, & Kilcawley, 2009). **Figure 4.2** represents the particle diameter distribution for microfluidized samples before and after each pass and **Figure 4.3** represents the particle diameter distribution for homogenized samples. These plots illustrate that the particle size in each treated sample may have decreased in diameter with processing.

Figure 4.2. Particle Size Distribution of WPI, SBM, and WBM before and after each pass through the microfluidizer. (a) WPI at pH 4.60; (b) SBM at pH 4.60; (c) WBM at pH 4.60 and 6.80.

Figure 4.3. Particle Size Distribution of WPI, SBM, and WBM before and after homogenization with 3 repetitions per treatment structure. (a) WPI at pH 4.60; (b) SBM at pH 4.60; (c) WBM at pH 4.60; (d) WBM at pH 6.80.

At pH 6.8, both WPI and SBM had an obscuration rate of less than 3% and were considered soluble. Their initial pH and standard deviations were 6.54 ± 0.283 and 7.32 ± 0.072 respectively, whereas, WBM had an initial pH of 5.36 ± 0.076 prior to any conditioning. As described earlier, particle size measurements could not be taken on WPI and SBM samples at pH 6.8. In addition, WPI, SBM, and WBM samples were not randomized for the homogenization or microfluidization process, which would result in confounding effects if analyzed in this way. Randomization occurred when assigning treatments to the dairy solutions and on the day of the experiment to randomize the run order. Since there were values that were not determined for WPI and SBM at pH 6.8, the statistical analysis of comparing the before and after particle diameter after each pass of microfluidization or after homogenization could not be performed for all combinations of powder type and pH. Instead, a one factor ANOVA was performed using the before and after paired differences for the four remaining combinations of powder and pH. To analyze the effect between homogenization and microfluidization, the particle diameter of the 3rd pass was taken to represent after processing for microfluidization. The particle diameter from the before (homogenization) or 0 pass (microfluidization) was subtracted from the after (homogenization) or 3rd pass (microfluidization). The resulting paired differences represent the change in particle size prior to treatment and after treatment. A positive difference indicates that there was a decrease in particle diameter and the opposite holds true for any negative differences.

The relationship between the particle size paired differences and the treatments of homogenized samples is strong with the model fitting the data well ($R^2 = 0.949$). However, the normality and equal variance model assumptions are violated due to

outliers (Appendix C). WPI, SBM, and WBM samples resulted in significant particle diameter differences ($p < 0.0001$) from before to after homogenizing. SBM at pH 4.6 had a least squares mean (LSM) of 11.614 μm and an interval of 9.527 to 13.700 at 98.75% confidence ($\alpha = 0.0125$) using the Bonferroni's correction method to account for the number of treatment levels. This indicates that there is a large significant difference in particle diameter from before to after homogenizing (**Table 4.2**). WBM at pH 4.6 and pH 6.8 had a LSM of 0.887 μm and 0.571 μm and a confidence interval between -1.200 to 2.973 and -1.516 to 2.657, respectively. There is not enough evidence to detect that there is a significant change from before homogenization compared to after for WBM at either pH. WPI at pH 4.6 exhibited a LSM of 3.499 μm and a confidence interval between 1.413 and 5.585. This shows that the change in particle diameter is relatively small, but is significantly different from before homogenization compared to after.

Table 4.2. Least Square Means for Homogenized Samples.

Treatment Level	Least Sq Mean (μm)	Std Error	Lower 98.75% CI	Upper 98.75% CI
SBM; pH 4.6 ^a	11.614	0.645	9.527	13.700
WPI; pH 4.6 ^b	3.499	0.645	1.413	5.585
WBM; pH 6.8 ^b	0.887	0.645	-1.200	2.973
WBM; pH 4.6 ^b	0.571	0.645	-1.516	2.657

^{a,b} = Levels that are not connected by same letter are significantly different.

The microfluidization treatment resulted in the reduction of particle diameters after the first pass for SBM samples, which is in accordance with liposomal particle size studies performed using a microfluidizer (Thompson & Singh, 2006) (Nongonierma, Abrlova, Fenelson, & Kilcawley, 2009). The mean particle diameters for all treatments at every pass are shown in **Table 4.3** from the SAS model analysis using an adjusted $\alpha = 0.0167$. There is a significant difference in the mean particle diameter between the treated

powders at a specific pH and the number of passes through the microfluidizer ($p < 0.0008$), which indicates that there was an interaction effect between the treatment and number of passes. Prior to microfluidization, SBM at pH 4.6 had a mean particle diameter of 11.152 μm , which was the largest size compared to all other treatments at pass 0. There are significant differences in the mean particle diameter between SBM at pH 4.6 and WBM at pH 4.6 ($p < 0.0002$), WPI at pH 4.6 ($p < 0.0002$), and WBM at pH 6.8 ($p < 0.0045$) at pass 0. This significance means that SBM at pH 4.6 at pass 0 had a mean particle diameter that is much larger than all other samples at pass 0. There are significant differences in the mean particle diameter between pass 0 and the other passes only for SBM at pH 4.6 ($p < 0.0001$). Changes in mean particle diameter after pass 1 were smaller and not statistically significant. More replications may be needed to detect significant differences after more than 1 pass through the microfluidizer.

Table 4.3. Mean Particle Diameter for Microfluidized Samples.

Treatment	Pass #	Mean Particle Diam. (μm)	Standard Error
SBM; pH 4.6 ^a	0	11.152	0.821
SBM; pH 4.6 ^b	1	4.189	0.693
SBM; pH 4.6 ^b	2	3.315	0.403
SBM; pH 4.6 ^b	3	2.717	0.370
WBM; pH 4.6 ^b	0	2.880	0.821
WBM; pH 4.6 ^b	1	1.955	0.693
WBM; pH 4.6 ^b	2	1.479	0.403
WBM; pH 4.6 ^b	3	1.469	0.370
WBM; pH 6.8 ^b	0	2.925	0.821
WBM; pH 6.8 ^b	1	1.980	0.693
WBM; pH 6.8 ^b	2	1.871	0.403
WBM; pH 6.8 ^b	3	1.376	0.370
WPI; pH 4.6 ^b	0	4.995	0.821
WPI; pH 4.6 ^b	1	3.038	0.693
WPI; pH 4.6 ^b	2	2.585	0.403
WPI; pH 4.6 ^b	3	2.501	0.370

^{a, b} = Levels that are not connected by same letter are significantly different.

A graphical representation of the mean particle diameters after each pass through the microfluidizer in all treatments is shown in **Figure 4.4**. From this plot, WBM at pH 4.6 and 6.8 are almost identical in the mean particle size before and after each pass. WPI at pH 4.6 showed a slightly larger mean particle size than both WBM treatments, but the differences were statistically insignificant. However, there are significant differences between WPI at pH 4.6, SBM at pH 4.6, WBM at pH 4.6, and WBM at pH 6.8 at pass 0 ($p < 0.0001$).

Figure 4.4. Mean particle diameter plot for microfluidized samples.

In comparison of both methods of high shear processing, the particle size paired differences and the treated samples exhibited a strong relationship ($R^2 = 0.885$) and normally distributed residuals. The equal variance assumption is violated due to large spreading of data and outliers (Appendix E). Some treated samples also resulted in significant particle diameter differences ($p < 0.0001$) from before to after homogenizing and microfluidizing. Homogenized and microfluidized SBM at pH 4.6 had intervals between 8.595 to 14.632 and 6.096 to 10.773, respectively, at Bonferroni adjusted 99.37% confidence ($\alpha = 0.0063$). This indicates that there is detectable significant difference in the mean particle diameter from before to after homogenizing and microfluidizing (**Table 4.4**). However, there is not enough evidence to identify a significant difference between the two processing methods. Homogenized WBM samples at pH 4.6 and pH 6.8 had a confidence interval between -2.448 to 3.589 and -2.132 to 3.905, respectively. Microfluidized WBM samples at pH 4.6 and pH 6.8 had a confidence

interval between -0.927 to 3.749 and -0.789 to 3.887, respectively. This indicates that there is not enough evidence to detect a significant change from before processing compared to after at either pH in either processes. Homogenized and microfluidized WPI samples at pH 4.6 exhibited a confidence interval between 0.480 to 6.518 and 0.156 to 4.832. This indicates that the change in particle diameter is very small, but is significantly different from before processing compared to after. Also, there were no detectable differences in processing techniques on the particle size change for any treatment.

Table 4.4. Least Square Means for Homogenized and Microfluidized Samples.

Treatment Level	Least Sq Mean (μm)	Std Error	Lower 99.37% CI	Upper 99.37% CI
SBM; pH 4.6; Homog ^a	11.614	1.002	8.595	14.632
SBM; pH 4.6; MF ^a	8.434	0.776	6.096	10.773
WPI; pH 4.6; Homog ^b	3.499	1.002	0.480	6.518
WPI; pH 4.6; MF ^b	2.494	0.776	0.156	4.832
WBM; pH 6.8; MF ^b	1.549	0.776	-0.789	3.887
WBM; pH 4.6; MF ^b	1.411	0.776	-0.927	3.749
WBM; pH 6.8; Homog ^b	0.887	1.002	-2.132	3.905
WBM; pH 4.6; Homog ^b	0.571	1.002	-2.448	3.589

^{a,b} = Levels that are not connected by same letter are significantly different.

Abbreviations: Homog = Homogenization; MF = Microfluidization.

In summary, WBM and WPI contain the smallest change in particle size in both processes at either pH. All WBM and WPI samples had significantly smaller change in particle size than SBM. The particle size of WBM did not change significantly during either process. Although WBM particle size did not change significantly, it is still an important factor in this study because its size and structure may possibly be influenced by the presence of phospholipids. WBM is a nutritious ingredient that is comprised of MFGM constituents and is more saturated in phospholipids than SBM and may contribute to its small size. In a study by Sodini and colleagues (2006), WBM also

exhibited smaller mean particle size compared to various types of sweet buttermilks and whey powder at various pH. They found that there were no significant differences between WBM at initial pH and at pH 4.0.

In this project, SBM was found to have the largest particle size and mean difference in both processes at pH 4.6. This is possibly due to caseins and whey proteins intermolecularly interacting with each other via disulfide and hydrogen bonding while being adsorbed onto the surface of liposomes forming large aggregates when treated with high shear. From the results above, WPI and WBM were not significantly different from each other given the processing and pH treatments. They both exhibited similar mean particle size trends suggesting that they share common characteristics since they consist of the same materials. One of the main differences between WPI and WBM are the presence of phospholipids in WBM which could possibly contribute to its small structure.

4.2.2 Confirmation with Confocal Microscopy

Confocal microscopy was utilized in this project to capture the presence of the aggregates and explore their structures. These images are represented in **Figure 4.5** at the various treatments and conditions. Protein structures were stained green and phospholipids were stained red. These images were taken to validate the presence of aggregates, in which are the specks of color within the images, and confirm what the particle size analyzer is detecting.

WPI; pH 6.8; No treatment

WPI; pH 4.6; Homog.

WPI; pH 4.6; MF

SBM; pH 6.8; No treatment

SBM; pH 4.6; Homog.

SBM; pH 4.6; MF

WBM; pH 6.8; No treatment

WBM; pH 4.6; Homog.

WBM; pH 4.6; MF

Figure 4.5. Confocal images of WPI, SBM, and WBM at various conditions.

4.3 Chemical Composition

4.3.1 Fat Extraction by Mojonnier Method

Fat was extracted from samples obtained after homogenization and after the 3rd pass of microfluidization using the standard Mojonnier ether method. Due to the complexity of the experimental design, analyses of all samples collected in this study were not executed in full. Instead, specific homogenized and microfluidized samples were chosen across all repetitions for fat extraction to explore how processing and pH might influence phospholipid ratios. Fat was also extracted from WPI, SBM, and WBM powders and used as a reference in this project. Samples that were treated at pH 4.6 resulted in the precipitation, or sedimentation, of liposomal aggregates. The precipitated samples were centrifuged to obtain the aggregates in pellet form. **Figure 4.6** illustrates samples at pH 4.6 with pellets and at pH 6.8 without pellets. Fat was also extracted from the WBM and SBM supernatants at pH 4.6.

Figure 4.6. Images of WPI, WBM, and SBM samples after microfluidization. (a) WPI at pH 6.8; (b) WPI at pH 4.6; (c) WBM at pH 6.8; (d) WBM at pH 4.6; (e) SBM at pH 6.8; (f) SBM at pH 4.6.

Unprocessed WPI powder resulted in an average of 0.179% total fat per 1 gram of powder (**Table 4.5**). Since this product contains very little fat, all WPI treated samples were excluded from further fat analysis. Homogenized and microfluidized WBM and SBM at pH 6.8 did not precipitate because they were soluble. For that reason, fat was extracted from the samples as they were without centrifugation and resulted in fat content of 0.254% and less than 0.1%, respectively, per 10 grams of fluid. The extracted supernatant of homogenized and microfluidized WBM and SBM samples at pH 4.6 also had a fat content of less than 0.1% per 10 grams of fluid. This information suggests that there were residual lipids that can be found in the supernatant of precipitated liposomal aggregates. The magnitude of the total fat content in these samples was not sufficient to extract and measure phospholipid content. **Figure 4.7** best illustrates the insignificant amount of total fat in WPI, supernatant of WBM and SBM at pH 4.6, and WBM and

SBM at pH 6.8 per repetition. Thus, samples that contain less than 1% total fat were excluded from further lipid analysis.

Table 4.5. Fat content of WPI, SBM, and WBM by Mojonnier Extraction.

Sample	Fat % per 1 g solid or 10 g fluid			
	Days			Avg. (%)
	1	2	3	
WPI; no treatment*	0.165	0.194	ND	0.179
WBM; no treatment*	27.375	27.426	ND	27.400
WBMSN; pH 4.6; MF**	-0.017	0.021	ND	0.002
WBMP; pH 4.6; MF*	8.451	10.101	8.540	9.031
WBM; pH 6.8; MF**	0.220	0.284	0.256	0.254
WBMP; pH 4.6; Homog*	10.968	9.899	9.269	10.045
WBM; pH 6.8; Homog**	0.256	0.220	0.202	0.226
SBM; no treatment*	8.989	8.835	ND	8.912
SBMSN; pH 4.6; MF**	-0.024	0.019	ND	-0.003
SBMP; pH 4.6; MF*	0.348	5.486	4.379	3.404
SBM; pH 6.8; MF**	0.032	0.091	0.059	0.061
SBMP; pH 4.6; Homog*	3.893	4.131	3.954	3.993
SBM; pH 6.8; Homog**	0.115	0.042	-0.025	0.044

* Solid product; ** Fluid product. ND = Not determined.

Figure 4.7. Percentage of total fat (% per 1 g solid or 10 g fluid) in WPI, WBM, and SBM under various conditions per day.

Untreated WBM powder contained about 27.4% total fat per 1 gram of powder, while processed WBM pellets at pH 4.6 contained about 9-10% per approximately 1 gram of solid (pellet). Untreated SBM powder contained 8.9% fat per 1 gram of powder, while homogenized and microfluidized SBM pellets contained about 3-4% total fat per approximately 1 gram of solid. WBM is comprised of higher fat content because it has been concentrated through membrane ultrafiltration (Costa, Elias-Argote, Jimenez-Flores, & Gigante, 2010). Since SBM was not processed and concentrated further, it contains less fat than WBM. WPI is manufactured through the use of membrane filtration and can contain residual lipids. The extracted 0.179% total lipids from WPI represent the residual lipids that were left behind in the product post manufacture.

4.3.2 Phospholipid Solid Phase Extraction (SPE)

Next, solid phase extraction was performed on the Mojonier fat extracted samples to purify polar phospholipids utilizing methods described by Bitman et al (1984). Non-polar lipids were eluted by gravity with 1:1 (v/v) hexane:diethyl ether using silica-based cartridges, and polar lipids were eluted with equal volumes of methanol and chloroform. The chloroform and methanol organic phase of all phospholipid extracted samples were evaporated off with a rotary evaporator and then freeze dried for 24 hours to remove any residual water.

The untreated WPI powder contained about 0.254% phospholipids per 1 gram of powder (**Table 4.6**), which is not consistent with the untreated WPI powder that had a total fat content of 0.179%, as previously mentioned. This could be due to human and equipment errors when weighing the phospholipid crude, or dust particles may have entered the sample after SPE, evaporation, and/or freeze drying. Untreated WBM powder

contained about 1.67% phospholipids per 1 gram of powder, while homogenized and microfluidized WBM pellets at pH 4.6 contained 0.63% and 0.32% per approximately 1 gram of solid, correspondingly. Untreated SBM powder contained about 1.10% phospholipids per 1 gram of powder, while homogenized and microfluidized SBM pellets at pH 4.6 contained 0.29% and 0.67% phospholipids per approximately 1 gram of solid.

Table 4.6. Phospholipid concentration (%) of WPI, SBM, and WBM by SPE.

Sample	Phospholipid (%) per 1 g solid			Avg. (%)
	Day			
	1	2	3	
WPI; no treatment	0.289	0.219	ND	0.254
WBM; no treatment	1.577	1.755	ND	1.666
WBMP; pH 4.6; MF	0.360	0.282	0.306	0.316
WBMP; pH 4.6; Homog	0.678	0.499	0.703	0.627
SBM; no treatment	0.996	1.196	ND	1.096
SBMP; pH 4.6; MF	0.074	0.450	0.345	0.290
SBMP; pH 4.6; Homog	0.331	0.832	0.847	0.670

ND = Not determined.

Untreated WBM and SBM powder both consists of approximately 1%-2% phospholipid content, which is in accordance with literature (Fox & McSweeney, 1998) (Keenan, Mather, & Dylewski, 1988). Homogenized and microfluidized WBM and SBM pellets all resulted in lower amounts of phospholipid content because the samples were diluted to create 2% (w/v) protein solutions. **Figure 4.8** best illustrates the phospholipid concentration in each sample per repetition. In addition, only 50 mL of sample was collected per treatment combination for lipid analysis, while the rest were use in particle size and protein analysis or discarded in excess. Theoretically, if total lipids were extracted from the entire final volume of the processed and treated samples at either pH, regardless of processing techniques, the phospholipid concentration should essentially

make up approximately 1% of the total fat. Microfluidized WBM and SBM pellets at pH 4.6 resulted in lower concentrations of phospholipids than homogenized samples due to structural changes to the aggregates during treatments with high shear. Structural alterations took place during induction of high shear causing proteins to surround the phospholipids. The protein layer acts as a protective coat making phospholipid extraction difficult to fully obtain phospholipids from the treated samples.

Figure 4.8. Percentage of phospholipids (% per 1 g solid) in WPI, WBM pellets (WBMP), and SBM pellets (SBMP) at pH 4.6 per day. MF = Microfluidization and Homog = Homogenization.

4.3.3 Phospholipid Profiling and Quantification by HPLC

Lastly, the extracted phospholipid samples were analyzed using HPLC in order to determine concentrations of the polar phospholipid classes. The phospholipid crude were diluted to 2 mg/mL in 2:1 (v/v) chloroform:methanol and stored in a freezer prior to analysis. It is important to investigate and quantify the various phospholipid classes because they are considered as bioactive compounds, which are responsible for providing structure to the milk fat membrane or liposomal aggregates, and contribute great nutritional value (Menard, et al., 2010).

To determine the concentration of phospholipid classes in each sample, five standard calibration curves were created with serially diluted phospholipid standards. The calibration curves were graphed as a function of phospholipid peak area. The peak areas for each of the phospholipid classes were manually integrated from chromatogram outputs at their respective retention times using the Shimazu LC Solutions software. Calculations were then carried out using the appropriate linear equation (**Table 4.7**). HPLC analysis occurred over three consecutive days, in which are independent of and not related to the days of the experimental design. Standards were run each day, prior to sample analysis, to achieve accurate readings.

Table 4.7. Linear equations of phospholipid standards and their respective retention time.

Day	Phospholipid Class	Retention Time (min)	Linear Equation*	R ²
1	PE	7.484	$PE_{conc} = 9.880 \times 10^{-8} * \text{Peak Area} - 0.016$	0.995
1	PI	8.438	$PI_{conc} = 3.991 \times 10^{-8} * \text{Peak Area} - 0.046$	0.989
1	PS	9.036	$PS_{conc} = 6.604 \times 10^{-8} * \text{Peak Area} - 0.019$	0.980
1	PC	9.847	$PC_{conc} = 3.275 \times 10^{-8} * \text{Peak Area} - 0.021$	0.989
1	SM	11.896	$SM_{conc} = 3.920 \times 10^{-8} * \text{Peak Area} - 0.016$	0.958
2	PE	7.456	$PE_{conc} = 8.332 \times 10^{-8} * \text{Peak Area} - 0.001$	0.996
2	PI	8.381	$PI_{conc} = 3.167 \times 10^{-8} * \text{Peak Area} - 0.028$	0.999
2	PS	9.033	$PS_{conc} = 5.381 \times 10^{-8} * \text{Peak Area} - 0.008$	0.997
2	PC	9.992	$PC_{conc} = 2.81 \times 10^{-8} * \text{Peak Area} - 0.019$	0.995
2	SM	12.107	$SM_{conc} = 2.964 \times 10^{-8} * \text{Peak Area} - 0.011$	0.999
3	PE	7.452	$PE_{conc} = 6.019 \times 10^{-8} * \text{Peak Area} - 0.013$	0.996
3	PI	8.378	$PI_{conc} = 2.413 \times 10^{-8} * \text{Peak Area} - 0.031$	0.998
3	PS	9.102	$PS_{conc} = 4.225 \times 10^{-8} * \text{Peak Area} - 0.015$	0.997
3	PC	10.091	$PC_{conc} = 2.196 \times 10^{-8} * \text{Peak Area} - 0.019$	0.997
3	SM	12.212	$SM_{conc} = 2.172 \times 10^{-8} * \text{Peak Area} - 0.007$	0.999

* All standard curves are in Appendix F.

The untreated WPI powder samples did not result in any phospholipid content after HPLC analysis, as shown in **Figure 4.9**. In **Table 4.8**, WPI samples consisted of 0% PE, and negative percentages for PI, PS, PC, and SM on average. The negative percentages correspond to the lack of peaks. Negative values occur when peak areas are integrated below baseline. The negative values represent no presence of phospholipids and resulted in negative numbers due to human errors when visually identifying peaks. **Figure 4.10** shows a representational chromatogram overlay of WPI powder in comparison to WBM and SBM powder, phospholipid standards, and 2:1 (v/v) chloroform:methanol as baseline. In this figure, WPI is illustrated as the first chromatogram (1), which does not show any peaks or any presence of phospholipids. These results do not correlate with the 0.254% average phospholipid concentration obtained by the SPE method. It is possible that the concentration of phospholipids within the WPI samples were so small that the CAD was not able to detect its presence during analysis.

Figure 4.9. Average concentration of Phospholipid classes (mg/mL per 1000 mg solid).

Figure 4.10. HPLC Chromatogram of phospholipids extracted from WPI, WBM, and SBM powder. 1. WPI powder; 2. WBM powder; 3. SBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS = Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin.

Table 4.8. Average percentage of phospholipid content in fat globules.

Phospholipid Class	Average percent (%) per total mg phospholipids						
	Dry Powder			Microfluidization		Homogenization	
	No Treatment			pH 4.6		pH 4.6	
	WPI	WBM	SBM	WBMP	SBMP	WBMP	SBMP
PE	0.000	13.329	6.573	30.260	14.380	15.052	4.521
PI	-1.157	0.898	0.991	-0.040	1.569	-0.435	-0.419
PS	-0.275	1.760	2.423	-0.100	0.000	-0.084	-0.248
PC	-0.353	2.820	2.355	2.770	7.686	2.273	0.983
SM	-0.196	1.347	0.414	0.000	2.490	0.019	-0.111
Total PL (mg)	2.550	16.700	11.000	3.333	5.133	1.700	3.900

As mentioned previously, WBM powder that was untreated contained the highest phospholipid concentration compared to SBM powder and this is reflected in the HPLC results. **Figure 4.11** is a visual representation of **Table 4.8**. WBM and SBM powder both contained the five phospholipid classes with PE being the majority of the phospholipid in each sample. **Figure 4.10** illustrates and **Table 4.8** confirms that WBM powder contains about 1% SM of the total phospholipid content and SBM powder contains less than 0.5%. WBM and SBM powders both contain about the same concentration of PI and PC, 0.90%-0.99% and 2.36%-2.84% of the total phospholipid content, respectively. However, SBM powder contains 2.42% PS, while WBM contains only about 1.76%.

Figure 4.11. Average percentage of phospholipid classes in relation to total mg of phospholipids in each sample.

According to **Figure 4.9** and **Figure 4.11**, Homogenized SBM pellet at pH 4.6 resulted in less phospholipid content within the polar classes than the microfluidized SBM pellet. Similarly, homogenized WBM pellets at pH 4.6 also exhibited lower phospholipid content within each class. **Figure 4.12** shows chromatogram overlays of SBM samples and **Figure 4.13** shows overlays of WBM samples. Both figures are in accordance with **Figure 4.9** and **Figure 4.11** above. **Table 4.8** shows that microfluidized WBM and SBM pellets had a higher total phospholipid yield than homogenized samples. Microfluidized WBM pellets at pH 4.6 yield an average of 3.33 mg total phospholipids, whereas homogenized WBM pellet samples only yield about 1.7 mg total. Microfluidized SBM pellet samples contained about 5.13 mg total phospholipids, while homogenized SBM pellets contained 3.90 mg total phospholipids. This difference in total phospholipid yield may be due to dilutions of the sample during treatment. As mentioned previously, if the entire volume of samples collected were analyzed, the amount of total phospholipids would be similar.

There are many factors that may also contribute to changes in the phospholipid profile. According to Keenan (1983), homogenization does not alter the phospholipid ratios in MFGM and skim milk. Across all homogenized and microfluidized samples, PE and PC remained present, even after treatment and extraction procedures. However, the presence of PI, PS, and SM in these samples were either very low or could not be detected. A study by Gallier and colleagues (2010) suggest that thermal treatment during pasteurization is responsible for the different profiling of phospholipids in milk. These differences in phospholipid profiling may possibly be due to the initial thermal treatment to 70 °C to denature the whey proteins for aggregation. Alternatively, these changes in phospholipid profiling may be also due to protein interactions during processing (i.e. homogenization or microfluidization) causing structural changes to take place. Whey proteins can interact with caseins by disulfide and hydrophobic interactions, forming aggregates that are adsorbed onto the MFGM (Brown, 1988) (Ye, Anema, & Singh, 2008). β -Lg and α -La can also interact with each other to form aggregates that are adsorbed on the membrane (Fox & McSweeney, 1998). Perhaps these protein interactions with the MFGM altered the aggregate structure and limited phospholipid extraction using the extraction methods used causing difference in phospholipid profiling.

Figure 4.12. HPLC Chromatogram of phospholipids extracted from SBM. 1. SBM Pellet from Microfluidization at pH 4.60; 2. SBM Pellet from Homogenization at pH 4.60; 3. SBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS = Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin.

Figure 4.13. HPLC Chromatogram of phospholipids extracted from WBM. 1. WBM Pellet from Microfluidization at pH 4.60; 2. WBM Pellet from Homogenization at pH 4.60; 3. WBM powder; 4. Phospholipid standards (0.1 mg/mL); 5. 2:1 (v/v) Chloroform: Methanol (Baseline). Phospholipid peaks: PE = Phosphatidylethanolamine; PI = Phosphatidylinositol; PS = Phosphatidylserine; PC = Phosphatidylcholine; SM = Sphingomyelin.

4.4 Protein Analysis by SDS-PAGE

SDS-PAGE is a great technique to study protein. Many proteins naturally interact with each other through disulfide bonds, which can be degraded by using a detergent called sodium dodecyl sulfate (SDS). SDS-PAGE was used in this project to analyze treated WPI, SBM, and WBM samples to determine if protein aggregation is effected by phospholipids and the pH treatment given under reduced and non-reduced conditions. Homogenized and the 3rd pass of microfluidized samples at pH 4.6 were compared to untreated WPI, SBM, and WBM samples at 6.8 using prestained protein standards as molecular markers. The gels were run at 200 V for 40-50 minutes and then stained with Coomassie Blue. Trace quantity of the protein bands were quantified using the QuantityOne 1-D Analysis software. Trace quantity is determined by the area under the intensity profile curve of a band and its units are intensity x mm.

Protein bands can be visualized in SDS polyacrylamide gels shown in **Figure 4.14**. Lanes 2, 5, and 6 represent WPI samples, lanes 3, 6, and 9 represent SBM samples, and lanes 4, 7 and 10 represent WBM samples in both SDS gels. The gels can be divided into three horizontal sections by molecular weight. MFGM associated proteins like xanthine oxidase (XO) and butyrophilin (BTN) are located in the first third, caseins are located in the second third, and whey or serum proteins are located on the last third of the gel. All WPI and WBM samples contain high intensity bands of whey proteins, while SBM samples contain high intensity bands of caseins. SBM and WBM both contain bands associated with MFGM proteins, whereas WPI contain very small trace amounts. In **Figure 4.14(b)**, large protein and liposomal aggregates remain within the combs of the stacking gel because the aggregates are too large to move down the gel.

Figure 4.14. SDS-PAGE (15% acrylamide gel) of reconstituted WPI, SBM and WBM under (a) reduced and (b) non-reduced conditions. Lane 1. Protein standards; Lane 2. WPI untreated at pH 6.8; Lane 3. SBM untreated at pH 6.8; Lane 4. WBM untreated at pH 6.8; Lane 5. WPI microfluidized at pH 4.60; Lane 6. SBM microfluidized at pH 4.60; Lane 7. WBM microfluidized at pH 4.60; Lane 8. WPI homogenized at pH 4.60; Lane 9. SBM homogenized at pH 4.60; Lane 10. WBM homogenized at pH 4.60. Protein Bands: XO = xanthine oxidase; BTN = butyrophilin; Pas 6/7 = periodic acid Schiff 6/7; α-CN = α-casein; β-CN = β-casein; κ-CN = κ-casein; β-LG = β-lactoglobulin; α-LA = α-lactalbumin.

Trace quantities of milk proteins in each sample are represented in **Figure 4.15** under reduced conditions and **Figure 4.16** under non-reduced conditions. Reduced samples were treated with 5% β -mercaptoethanol to break down proteins. Both treated and untreated WPI samples contain very small traces of MFGM proteins, including BTN and Pas 6/7, and equal traces of whey proteins regardless of processing treatments and pH (**Figure 4.15(a)**). Untreated SBM sample at pH 6.8 shows lower quantities of proteins compared to microfluidized and homogenized SBM samples at pH 4.6. **Figure 4.15(b)** illustrates that homogenized SBM at pH 4.6 contained higher quantities of caseins and whey proteins than the microfluidized SBM. Treated and untreated WBM at both pH displays equal trace quantities of whey proteins, but homogenized WBM samples at pH 4.6 displays slightly greater MFGM associated proteins as well as β -caseins (**Figure 4.15(c)**).

Under non-reduced conditions and across all WPI samples, the quantity of whey proteins remained the same. However, MFGM proteins that were present in the reduced samples no longer appear in the non-reduced samples. This is due to the formation of large protein aggregates. Large aggregates are too large to flow down 15% acrylamide SDS gels and therefore, remain at the top of the stacking gel as demonstrated in **Figure 4.14(b)**. Additionally, **Table 4.9** provides a compositional summary of all samples analyzed in this project and it shows that WPI contains residual phospholipids, in which may contribute to aggregate formation by phospholipid and protein interactions. In both untreated and treated SBM samples, the quantity of MFGM associated proteins, caseins, and whey proteins had decreased, which means that some proteins had interactions with each other to form large aggregates at both pH (**Figure 4.16(b)**). Untreated WBM at pH

6.8 showed no signs of MFGM related proteins or caseins (**Figure 4.16(c)**). Treated WBM samples at pH 4.6 were consistent with each other in the amount of whey proteins and caseins, but inconsistent on the low amount of MFGM associated proteins. These results compared to **Figure 4.15** indicate that processing and pH may not have an effect on protein quantities. Instead, it can be suggested that phospholipids play an interesting role in the formation of liposomal aggregates by phospholipid-protein and protein-protein interactions. Although further research is needed to fully understand how phospholipids and protein interact with each other, this suggestion is feasible because milk proteins are adsorbed onto the MFGM during homogenization or microfluidization by some means of interaction. However, the mechanism in which they occur would need further investigation.

Figure 4.15. Trace Quantity (intensity x mm) of SDS-PAGE under reducing conditions in (a) WPI, (b) SBM, and (c) WBM samples.

Figure 4.16. Trace Quantity (intensity x mm) of SDS-PAGE under non-reducing conditions in (a) WPI, (b) SBM, and (c) WBM samples

Table 4.9. Compositional summary of WPI, WBM, and SBM products.

Composition	Dry Powder			Microfluidization		Homogenization	
	No Treatment			pH 4.6		pH 4.6	
	WPI	WBM	SBM	WBMP	SBMP	WBMP	SBMP
Total Fat (%)*	0.179	27.400	8.912	8.984	3.226	10.027	3.990
PL (%)*	0.254	1.666	1.096	0.317	0.276	0.633	0.665
Protein (%)*	41.578	43.512	47.246	2.000	2.000	2.000	2.000
Avg. Total Fat (g)	0.002	0.275	0.089	0.095	0.020	0.081	0.023
Avg. Sample Wt. (g)	1.003	1.003	1.004	1.053	0.615	0.811	0.587

*All percentages are per approximately 1 gram of solid.

5.0 Conclusions

Incorporating buttermilk powder into food systems has advantages in many ways. The addition of buttermilk in baked goods, chocolate, ice cream, cheese, and dressings enhances their texture, flavor, and structure due to the abundance of MFGM components, especially phospholipids (Costa, Elias-Argote, Jimenez-Flores, & Gigante, 2010). MFGM phospholipids have been extensively studied because they possess emulsification properties and offer health benefits, such as inhibition of colon cancer and enhancing growth and development (Spitsberg, 2005). The focus of this thesis was to study the composition and structure of liposomal aggregates made from phospholipids of commercial sweet buttermilk and whey buttermilk prepared by microfluidization and homogenization. These liposomes can be utilized as delivery systems for bioactive components in many industries for various applications.

Liposomal preparation by microfluidization and homogenization resulted in analogous mean particle size differences across all samples and therefore, no significant differences were detected between the two shearing techniques. The use of homogenization and microfluidization both provoked similar changes in aggregate size, from before treatments to after, by creating smaller particle distributions in SBM at pH 4.6, and the results are in accordance with literature (Thompson & Singh, 2006) (Nongonierma, Abrlova, Fenelson, & Kilcawley, 2009). There were significant differences in the mean particle size from before processing and after in SBM and WPI at pH 4.6 using either shear technique but not in other treatments. The SBM results suggest that casein interactions with other milk proteins formed very large aggregates with phospholipids since caseins are found in abundance in SBM.

In contrast, WBM treated at pH 4.6 and pH 6.8 did not show significant differences in their mean particle size at either shear treatments. It can be suggested that the enrichment of phospholipids may contribute to the small structures of the aggregates in WBM samples. These results also are in agreement with studies by Sodini and colleagues (2006). WBM also exhibited similar characteristics as WPI since their particle sizes were not significantly different from each other. WBM contained higher concentrations of phospholipids compared to WPI, which contained trace amounts left behind from processing, and SBM. The abundance of phospholipids within WBM makes it an important and beneficial dairy ingredient.

Differences in phospholipid profiling were seen across all sample types and treatment combinations. In addition, homogenization and microfluidization treatments, as well as pH conditions, did not greatly affect the phospholipid profiling. This is possibly due to heat treatment to 70 °C at initial conditioning to denature whey proteins. Alternatively, proteins may have had interactions with phospholipids during high shear treatments causing structural changes. These changes inhibited the full extraction of phospholipids and thus, resulted in the differences of phospholipid profiling in treated samples. Furthermore, the SDS-PAGE analysis suggests that phospholipids may possibly play an important role in aggregate formation via interactions with other milk proteins.

5.1 Limitations of this Study

Inevitably, variation occurs in manufacturing and processing practices, geographic locations, seasons, and cows. The dairy powders used to conduct research in this project were manufactured from different sources and thereby, causing some variation. This is important to address because these ingredients were not created using

the same batch of milk from the same dairy source. However, the results from this research provide quality contributions and suggestive relationships between the buttermilk powders. The complexities of the experimental design led to some restrictions on the number of samples that could be analyzed and thus, limited the analysis of some parameters. In addition, data could not be collected on particle size distribution for WPI and SBM at pH 6.8 since these samples were soluble under the treated conditions. No experimental design is perfect and complications can naturally arise. Despite the complexities, the results from this investigation provided modest contributions to the existing research on the MFGM and its phospholipids to be utilized as vehicles for bioactive compounds for the delivery of nutrition and beneficial health in future applications.

6.0 Directions for Future Research

Interactions between phospholipids and proteins are important to continuously study for better understanding their structural changes and functions. Perhaps stronger relationships between high shear and the pH condition of aggregates can be detected by obtaining or manufacturing dairy ingredients in such a way that reduce variability. Modifications to phospholipid extractions can be studied to optimize extraction procedures to fully extract phospholipids from samples. Further investigation of particle size distribution, phospholipid profiling, and protein content can be performed with various concentrations of phospholipids, pH ranges, and thermal conditions. Various shear techniques, such as sonication, can be utilized to prepare small liposomal aggregate particles in addition to homogenization and microfluidization. Lastly, investigation of the encapsulation capacity of bioactive compounds, such as fat soluble vitamins and antioxidants, into liposomal aggregates can be utilized as vehicles to deliver nutrition.

REFERENCES

- Benjamin, O., Silcock, P., Leus, M., & Everett, D. W. (2012). Multilayer emulsions as delivery systems for controlled release of volatile compounds using pH and salt triggers. *Food Hydrocolloids*, 27, 109-118.
- Bezelgues, J. B., Morgan, F., Palomo, G., Crosset-Perrotin, L., & Ducret, P. (2008). Short communication: milk fat globule membrane as a potential delivery system for liposoluble nutrients. *Journal of Dairy Science*, 92, 2524-2528.
- Bitman, J., Wood, D., Mehta, N., Hamosh, P., & Hamosh, M. (1984). Comparison of the phospholipid composition of breast milk from mothers of term and preterm infants during lactation. *The American Journal of Clinical Nutrition*, 40, 1103-1119.
- Bouaouina, H., Desrumaux, A., Loisel, C., & Legrand, J. (2006). Functional properties of whey proteins as affected by dynamic high-pressure treatment. *International Dairy Journal*, 16, 275-284.
- Brown, R. J. (1988). Milk coagulation and protein denaturation. In N. P. Wong (Ed.), *Fundamentals of Dairy Chemistry* (3 ed., pp. 583-507). New York: Van Nostrand Reinhold Company.
- Contarini, G., & Povolo, M. (2013). Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. *International Journal of Molecular Sciences*, 14, 2808-2831.
- Costa, M. R., Elias-Argote, X. E., Jimenez-Flores, R., & Gigante, M. L. (2010). Use of ultrafiltration and supercritical fluid extraction to obtain a whey buttermilk

- powder enriched with milk fat globule membrane phospholipids. *International Dairy Journal*, 1-5.
- Dalgleish, D. G., Senaratne, V., & Francois, S. (1997). Interactions between a-Lactalbumin and b-Lactoglobulin in the early stages of heat denaturation. *Journal of Agriculture and Food Chemistry*, 45, 3459-3464.
- De Wit, J. N. (2009). Thermal behavior of bovine b-lactoglobulin at temperatures up to 150 °C. a review. *Trends in Food Science & Technology*, 20, 27-34.
- Dewettinck, K., Rombaut, R., Thienpont, N., Trung Le, T., Messens, K., & Van Camp, J. (2008). Nutritional and technological aspects of milk fat globule membrane material. *International Dairy Journal*, 18, 436-457.
- Dissanayake, M., Ramchandran, L., Piyadasa, C., & Vasiljevic, T. (2013). Influence of heat and pH on structure and conformation of whey proteins. *International Dairy Journal*, 28, 56-61.
- Dylewski, D. P., & Keenan, T. W. (1983). Compound exocytosis of casein micelles in mammary epithelial cells. *European Journal of Cell Biology*, 31, 114-124.
- Elias-Argote, X. E. (2011). Effects of milk processing on the milk fat globule membrane constituents. 1-236. San Luis Obispo, CA, USA.
- Folch, J., Lees, M., & Stanley, H. S. (1956). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, 226, 497-509.
- Fox, P. F. (2003). Milk proteins: general and historical aspects. In P. F. Fox, & P. L. McSweeney (Eds.), *Advanced Dairy Chemistry: Proteins* (3 ed., Vol. 1A, pp. 1-41). New York, New York, USA: Kluwer Academic/ Plenum Publishers.

- Fox, P. F., & McSweeney, P. L. (1998). *Dairy Chemistry and Biochemistry*. London, UK: Blackie Academic & Professional.
- Franke, W. W., Luder, M. R., Kartenbeck, J., Zerban, H., & Keenan, T. W. (1976). Involvement of vesicle coat material in casein secretion and surface regeneration. *Journal of Cell Biology*, 69, 173-195.
- Hvarregaard, J., Anderson, M. H., Berglund, L., Rassmussen, J. T., & Petersen, T. E. (1996). Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. *European Journal of Biochemistry*, 240, 628-638.
- Jimenez-Flores, R., & Brisson, G. (2008). The milk fat globule membrane as an ingredient: why, how, when? *Dairy Science Technology*, 88, 5-18.
- Keenan, T. W., & Dylewski, D. P. (1995). Intercellular origin of milk lipid globules and the nature and structure of the milk lipid globule membrane. In P. F. Fox (Ed.), *Advanced Dairy Chemistry: Lipids* (Vol. 2, pp. 89-130). London, UK: Chapman & Hall.
- Keenan, T. W., Mather, I. H., & Dylewski, D. P. (1988). *Fundamentals of Dairy Chemistry* (3 ed.). (N. P. Wong, Ed.) New York: Van Nostrand Reinhold Company.
- Lee, S. J., & Sherbon, J. W. (2002). Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. *Journal of Dairy Research*, 69, 555-567.
- Lopez, C., Madec, M.-N., & Jimenez-Flores, R. (2010). Lipid rafts in the bovine milk fat globule membrane revealed by the lateral segregation of phospholipids and heterogeneous distributions of glycoproteins. *Food Chemistry*, 120, 22-33.

- Malvern. (2012, May 30). *Back to basics with Malvern Instruments particle characterization white paper*. Retrieved from Malvern Instruments Ltd.: <http://www.malvern.com/malvern/pr.nsf/id/pr2437>
- McClements, D. J. (2005). *Food Emulsions* (2 ed.). Boca Raton, Florida, USA: CRC Press.
- McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47(7), 611-649.
- McDaniel, M. A., Maier, S. F., & Einstein, G. O. (2002). "Brain-specific" nutrients: a memory cure? *Psychological Science in the Public Interest*, 3, 12-38.
- Menard, O., Ahmad, S., Rousseau, F., Briard-Bion, V., Gaucheron, F., & Lopez, C. (2010). Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food Chemistry*, 120, 544-511.
- Min, D. B., & Boff, J. M. (2003). Crude Fat Analysis. In S. Nielsen, *Food Analysis* (3 ed., pp. 113-130). New York: Springer Science + Business Media, LLC.
- Morin, P., Pouliot, Y., & Jimenez-Flores, R. (2005). A comparative study of the fractionation of regular buttermilk and whey buttermilk by microfiltration. *Journal of Food Engineering*, 1-8.
- Noh, S. K., & Koo, S. I. (2004). Milk sphingomyelin is more effective than egg sphingomyelin in inhibiting intestinal absorption of cholesterol and fat in rats. *Journal of Nutrition*, 134, 2611-2616.

- Nongonierma, A. B., Abrlova, M., Fenelson, M. A., & Kilcawley, K. N. (2009). Evaluation of two food grade proliposomes to encapsulate and extract of a commercial enzyme preparation by microfluidization. *Journal of Agricultural and Food Chemistry*, 57, 3291-3297.
- O'Mahony, F. (1988). *Rural Dairy Technology: Experiences in Ethiopia* (Vol. 4). Addis Ababa, Ethiopia: ILCA. Retrieved from http://www.ilri.org/InfoServ/Webpub/fulldocs/ilca_manual4/MilkProcessing.htm
- Parodi, P. W. (1997). Cows' milk fat components as potential anticarcinogenic agents. *Journal of Nutrition*, 127, 1055-1060.
- Rombaut, R., & Dewettinck, K. (2006). Properties, analysis and purification of milk polar lipids. *International Dairy Journal*, 16, 1362-1373.
- Rombaut, R., Camp, J. V., & Dewettinck, K. (2005). Analysis of phospho- and sphingolipids in dairy products by a new HPLC method. *Journal of Dairy Science*, 88, 482-488.
- Singh, H. (2006). The milk fat globule membrane – a biophysical system for food applications. *Colloid & Interface Science*, 11, 154-163.
- Singh, H., & Ye, A. (2010). Controlling milk protein interactions to enhance the reconstitution properties of whole milk powders - a minireview. *Dairy Science Technology*, 90, 123-136.
- Spence, K., Venditti, R., & Rojas, O. (2010). Aspects of raw materials and processing conditions on the production and utilization of microfibrillated cellulous. *International Conference on Nanotechnology for the Forest Products Industry*

- (pp. 1-49). North Carolina State University. Retrieved from <http://www.tappi.org/content/events/10nano/papers/24.1.pdf>
- Spitsberg, V. L. (2005). Invited review: bovine milk fat globule membrane as a potential nutraceutical. *Journal of Dairy Science*, 88, 2289-2294.
- Sprong, R. C., Hulstein, M. F., & Van Der Meer, R. (2002). Bovine milk fat components inhibit food-borne pathogens. *International Dairy Journal*, 12, 209-215.
- Thompson, A. K., & Singh, H. (2006). Preparation of liposomes from milk fat globule membrane phospholipids using a microfluidizer. *Journal of Dairy Science*, 89, 410-419.
- Thompson, A. K., Mozafari, M. R., & Singh, H. (2007). The properties of liposomes produced from milk fat globule membrane material using different techniques. *Lait*, 87, 349-360.
- Wang, X., Hirno, S., Millen, R., & Wadstrom, T. (2001). Inhibition of *Helicobacter pylori* infection by bovine milk glycoconjugates in a BALB/cA mouse model. *FEMS Immunology & Medical Microbiology*(20), 275-281.
- Whitney, R. M. (1988). Proteins of Milk. In N. P. Wong (Ed.), *Fundamentals of Dairy Chemistry* (3 ed., pp. 81-170). New York: Van Nostrand Reinhold Company.
- Ye, A., Anema, S. G., & Singh, H. (2008). Changes in the surface protein of the fat globules during homogenization and heat treatment of concentrated milk. *Journal of Dairy Research*, 75, 347-353.
- Zamora, A., Ferragut, V., Guamis, B., & Trujillo, A. J. (2012). Changes in the surface protein of the fat globules during ultra-high pressure homogenization and conventional treatments of milk. *Food Hydrocolloids*, 29, 135-143.

APPENDICES

Appendix A: Randomization for Homogenization

Homogenization Day 1

Powders	pH	Randomization	Run Order
SBM	4.6	0.040043249	1
WBM	6.8	0.315799282	2
WPI	4.6	0.570297671	3
WPI	6.8	0.641186231	4
WBM	4.6	0.679798881	5
SBM	6.8	0.862066505	6

Homogenization Day 2

Powders	pH	Randomization	Run Order
WBM	4.6	0.003241287	1
WPI	4.6	0.089398186	2
WPI	6.8	0.476119442	3
WBM	6.8	0.697712829	4
SBM	6.8	0.726706819	5
SBM	4.6	0.739543682	6

Homogenization Day 3

Powders	pH	Randomization	Run Order
SBM	6.8	0.074157962	1
WPI	6.8	0.221086383	2
SBM	4.6	0.269736291	3
WPI	4.6	0.810364482	4
WBM	6.8	0.852048019	5
WBM	4.6	0.968896785	6

Appendix B: Randomization for Microfluidization

Microfluidization Day 1				Microfluidization Day 4			
Powders	pH	Randomization	Run Order	Powders	pH	Randomization	Run Order
WBM	4.6	0.116160437	1	WBM	6.8	0.046589733	1
SBM	6.8	0.158058539	2	SBM	6.8	0.25498164	2
WPI	6.8	0.454649064	3	WPI	4.6	0.381852791	3
WPI	4.6	0.705048944	4	SBM	4.6	0.828516249	4
SBM	4.6	0.708346708	5	WBM	4.6	0.86136854	5
WBM	6.8	0.834251298	6	WPI	6.8	0.888758399	6

Microfluidization Day 2				Microfluidization Day 5			
Powders	pH	Randomization	Run Order	Powders	pH	Randomization	Run Order
WPI	4.6	0.370113966	1	WBM	4.6	0.102348806	1
WPI	6.8	0.446552658	2	WPI	4.6	0.128427148	2
WBM	6.8	0.540073213	3	WBM	6.8	0.33353294	3
SBM	4.6	0.559263787	4	SBM	4.6	0.434078138	4
SBM	6.8	0.731676259	5	SBM	6.8	0.530879443	5
WBM	4.6	0.761249457	6	WPI	6.8	0.972914692	6

Microfluidization Day 3			
Powders	pH	Randomization	Run Order
WBM	4.6	0.206504088	1
WPI	6.8	0.42807719	2
WBM	6.8	0.43794731	3
SBM	4.6	0.469256789	4
SBM	6.8	0.826942774	5
WPI	4.6	0.841389305	6

Appendix C: Homogenization Paired Data JMP Output and Model Assumptions

Response = Difference in diameter (Before – After)

Factor = Treatment (4 levels: SBM 4.6, WBM 4.6, WBM 6.8, and WPI 4.6)

Blocking Factor = Day (3 levels: 1, 2, and 3)

Estimation Method = Restricted Maximum Likelihood (REML)

Summary of Fit

RSquare	0.94862
RSquare Adj	0.929352
Root Mean Square Error	1.174807
Mean of Response	4.1425
Observations (or Sum Wgts)	12

Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Treatment	3	3	6	57.6674	<.0001*

LSMeans Differences Tukey HSD

$\alpha=0.050$

LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	SBM4.6	WBM4.6	WBM6.8	WPI4.6
SBM4.6	0 0 0 0	11.043 0.95923 7.72244 14.3636	10.727 0.95923 7.40644 14.0476	8.11467 0.95923 4.79411 11.4352
WBM4.6	-11.043 0.95923 -14.364 -7.7224	0 0 0 0	-0.316 0.95923 -3.6366 3.00456	-2.9283 0.95923 -6.2489 0.39222
WBM6.8	-10.727 0.95923 -14.048 -7.4064	0.316 0.95923 -3.0046 3.63656	0 0 0 0	-2.6123 0.95923 -5.9329 0.70822
WPI4.6	-8.1147 0.95923 -11.435 -4.7941	2.92833 0.95923 -0.3922 6.24889	2.61233 0.95923 -0.7082 5.93289	0 0 0 0

Level	Least Sq Mean
SBM4.6 A	11.613667
WPI4.6 B	3.499000
WBM6.8 B	0.886667
WBM4.6 B	0.570667

Levels not connected by same letter are significantly different.

Least Squares Means Table

Level	Least Sq Mean	Std Error	Lower 98.75%	Upper 98.75%
SBM4.6	11.613667	0.64467026	9.527332	13.700002
WBM4.6	0.570667	0.64467026	-1.515668	2.657002
WBM6.8	0.886667	0.64467026	-1.199668	2.973002
WPI4.6	3.499000	0.64467026	1.412665	5.585335

Appendix D: Microfluidization Repeated Measures Data SAS Output

For overall effects				
Effect	Num DF	Den DF	F Value	Pr > F
Trt	3	16	13.48	0.0001
Pass	3	16	23.82	<.0001
Trt*Pass	9	16	6.3	0.0008

Tests for Treatment effects (differences between treatments at each pass)

Effect	Trt	Pass	Num DF	Den DF	F Value	Pr > F
Trt*Pass	SBM4.6	all	3	16	36.76	<.0001
Trt*Pass	WBM4.6	all	3	16	0.9	0.4615
Trt*Pass	WBM6.8	all	3	16	2.11	0.1397
Trt*Pass	WPI4.6	all	3	16	2.95	0.0645

Tests for Pass effects

Effect	Trt	Pass	Num DF	Den DF	F Value	Pr > F
Trt*Pass	all	0	3	16	22.62	<.0001
Trt*Pass	all	1	3	16	2.34	0.1116
Trt*Pass	all	2	3	16	4.04	0.0258
Trt*Pass	all	3	3	16	3.49	0.0404

Estimated Difference by Treatments

SBM 4.6											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj. P
Trt*Pass	SBM 4.6	0	SBM 4.6	1	6.9622	0.7442	16	9.36	<.0001	Tukey-Kramer	<.0001
Trt*Pass	SBM 4.6	0	SBM 4.6	2	7.837	0.8536	16	9.18	<.0001	Tukey-Kramer	<.0001
Trt*Pass	SBM 4.6	0	SBM 4.6	3	8.4344	0.8827	16	9.56	<.0001	Tukey-Kramer	<.0001
Trt*Pass	SBM 4.6	1	SBM 4.6	2	0.8748	0.5382	16	1.63	0.124	Tukey-Kramer	0.94
Trt*Pass	SBM 4.6	1	SBM 4.6	3	1.4722	0.6463	16	2.28	0.037	Tukey-Kramer	0.642
Trt*Pass	SBM 4.6	2	SBM 4.6	3	0.5974	0.2637	16	2.27	0.038	Tukey-Kramer	0.649

WBM 4.6											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	WBM 4.6	0	WBM 4.6	1	0.9246	0.7442	16	1.24	0.232	Tukey-Kramer	0.993
Trt*Pass	WBM 4.6	0	WBM 4.6	2	1.4014	0.8536	16	1.64	0.12	Tukey-Kramer	0.936
Trt*Pass	WBM 4.6	0	WBM 4.6	3	1.411	0.8827	16	1.6	0.13	Tukey-Kramer	0.947
Trt*Pass	WBM 4.6	1	WBM 4.6	2	0.4768	0.5382	16	0.89	0.389	Tukey-Kramer	1
Trt*Pass	WBM 4.6	1	WBM 4.6	3	0.4864	0.6463	16	0.75	0.463	Tukey-Kramer	1
Trt*Pass	WBM 4.6	2	WBM 4.6	3	0.0096	0.2637	16	0.04	0.971	Tukey-Kramer	1

WBM 6.8											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	WBM 6.8	0	WBM 6.8	1	0.9448	0.7442	16	1.27	0.222	Tukey-Kramer	0.992
Trt*Pass	WBM 6.8	0	WBM 6.8	2	1.0542	0.8536	16	1.24	0.235	Tukey-Kramer	0.994
Trt*Pass	WBM 6.8	0	WBM 6.8	3	1.5488	0.8827	16	1.75	0.099	Tukey-Kramer	0.901
Trt*Pass	WBM 6.8	1	WBM 6.8	2	0.1094	0.5382	16	0.2	0.842	Tukey-Kramer	1
Trt*Pass	WBM 6.8	1	WBM 6.8	3	0.604	0.6463	16	0.93	0.364	Tukey-Kramer	1
Trt*Pass	WBM 6.8	2	WBM 6.8	3	0.4946	0.2637	16	1.88	0.079	Tukey-Kramer	0.854
WPI 4.6											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	WPI 4.6	0	WPI 4.6	1	1.9566	0.7442	16	2.63	0.018	Tukey-Kramer	0.441
Trt*Pass	WPI 4.6	0	WPI 4.6	2	2.41	0.8536	16	2.82	0.012	Tukey-Kramer	0.343
Trt*Pass	WPI 4.6	0	WPI 4.6	3	2.4938	0.8827	16	2.83	0.012	Tukey-Kramer	0.342
Trt*Pass	WPI 4.6	1	WPI 4.6	2	0.4534	0.5382	16	0.84	0.412	Tukey-Kramer	1
Trt*Pass	WPI 4.6	1	WPI 4.6	3	0.5372	0.6463	16	0.83	0.418	Tukey-Kramer	1
Trt*Pass	WPI 4.6	2	WPI 4.6	3	0.0838	0.2637	16	0.32	0.755	Tukey-Kramer	1

Estimated Differences by Pass

Pass 0											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	SBM4.6	0	WBM4.6	0	8.2716	1.1604	16	7.13	<.0001	Tukey-Kramer	0.0002
Trt*Pass	SBM4.6	0	WBM6.8	0	8.2266	1.1604	16	7.09	<.0001	Tukey-Kramer	0.0002
Trt*Pass	SBM4.6	0	WPI4.6	0	6.1568	1.1604	16	5.31	<.0001	Tukey-Kramer	0.0045
Trt*Pass	WBM4.6	0	WBM6.8	0	-0.045	1.1604	16	-0.04	0.97	Tukey-Kramer	1
Trt*Pass	WBM4.6	0	WPI4.6	0	-2.115	1.1604	16	-1.82	0.087	Tukey-Kramer	0.876
Trt*Pass	WBM6.8	0	WPI4.6	0	-2.07	1.1604	16	-1.78	0.093	Tukey-Kramer	0.8908
Pass 1											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	SBM4.6	1	WBM4.6	1	2.234	0.9794	16	2.28	0.037	Tukey-Kramer	0.6403
Trt*Pass	SBM4.6	1	WBM6.8	1	2.2092	0.9794	16	2.26	0.039	Tukey-Kramer	0.6551
Trt*Pass	SBM4.6	1	WPI4.6	1	1.1512	0.9794	16	1.18	0.257	Tukey-Kramer	0.9959
Trt*Pass	WBM4.6	1	WBM6.8	1	-0.025	0.9794	16	-0.03	0.98	Tukey-Kramer	1
Trt*Pass	WBM4.6	1	WPI4.6	1	-1.083	0.9794	16	-1.11	0.285	Tukey-Kramer	0.9978
Trt*Pass	WBM6.8	1	WPI4.6	1	-1.058	0.9794	16	-1.08	0.296	Tukey-Kramer	0.9983

Pass 2											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	SBM4.6	2	WBM4.6	2	1.836	0.5703	16	3.22	0.005	Tukey-Kramer	0.191
Trt*Pass	SBM4.6	2	WBM6.8	2	1.4438	0.5703	16	2.53	0.022	Tukey-Kramer	0.4943
Trt*Pass	SBM4.6	2	WPI4.6	2	0.7298	0.5703	16	1.28	0.219	Tukey-Kramer	0.991
Trt*Pass	WBM4.6	2	WBM6.8	2	-0.392	0.5703	16	-0.69	0.502	Tukey-Kramer	1
Trt*Pass	WBM4.6	2	WPI4.6	2	-1.106	0.5703	16	-1.94	0.07	Tukey-Kramer	0.8252
Trt*Pass	WBM6.8	2	WPI4.6	2	-0.714	0.5703	16	-1.25	0.229	Tukey-Kramer	0.9927

Pass 3											
Effect	Trt	Pass	Trt	Pass	Mean	Std Error	DF	t Value	Pr> t	Adjustment	Adj P
Trt*Pass	SBM4.6	3	WBM4.6	3	1.2482	0.5236	16	2.38	0.03	Tukey-Kramer	0.5797
Trt*Pass	SBM4.6	3	WBM6.8	3	1.341	0.5236	16	2.56	0.021	Tukey-Kramer	0.4778
Trt*Pass	SBM4.6	3	WPI4.6	3	0.2162	0.5236	16	0.41	0.685	Tukey-Kramer	1
Trt*Pass	WBM4.6	3	WBM6.8	3	0.0928	0.5236	16	0.18	0.862	Tukey-Kramer	1
Trt*Pass	WBM4.6	3	WPI4.6	3	-1.032	0.5236	16	-1.97	0.066	Tukey-Kramer	0.8102
Trt*Pass	WBM6.8	3	WPI4.6	3	-1.125	0.5236	16	-2.15	0.047	Tukey-Kramer	0.7166

Appendix E: Homogenization and Microfluidization Combined Paired Data JMP

Output and Model Assumptions

Response = Difference in diameter (Before – After)

Factor = Treatment (8 levels: SBM 4.6 Homog, WBM 4.6 Homog, WBM 6.8 Homog, WPI 4.6 Homog, SBM 4.6 MF, WBM 4.6 MF, WBM 6.8 MF, and WPI 4.6 MF)

Blocking Factor = Day (3 levels: 1, 2, and 3)

Estimation Method = Restricted Maximum Likelihood (REML)

Summary of Fit

RSquare	0.885174
RSquare Adj	0.851683
Root Mean Square Error	1.602802
Mean of Response	3.723438
Observations (or Sum Wgts)	32

Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Treatment	7	7	17.71	21.9457	<.0001*

LSMeans Differences Tukey HSD

$\alpha=0.050$

LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	SBM4.6Homog	SBM4.6MF	WBM4.6Homog	WBM4.6MF	WBM6.8Homog	WBM6.8MF	WPI4.6Homog	WPI4.6MF
SBM4.6Homog	0 0 0 0	3.17927 1.2676 -1.0432 7.40175	11.043 1.30868 6.57868 15.5073	10.2027 1.2676 5.98018 14.4252	10.727 1.30868 6.26268 15.1913	10.0649 1.2676 5.84238 14.2874	8.11467 1.30868 3.65034 12.579	9.11987 1.2676 4.89738 13.3424
SBM4.6MF	-3.1793 1.2676 -7.4018 1.04322	0 0 0 0	7.86373 1.2676 3.64125 12.0862	7.0234 1.0137 3.56535 10.4814	7.54773 1.2676 3.32525 11.7702	6.8856 1.0137 3.42755 10.3436	4.9354 1.2676 0.71292 9.15788	5.9406 1.0137 2.48255 9.39865
WBM4.6Homog	-11.043 1.30868 -15.507 -6.5787	-7.8637 1.2676 -12.086 -3.6412	0 0 0 0	-0.8403 1.2676 -5.0628 3.38215	-0.316 1.30868 -4.7803 4.14832	-0.9781 1.2676 -5.2006 3.24435	-2.9283 1.30868 -7.3927 1.53599	-1.9231 1.2676 -6.1456 2.29935
WBM4.6MF	-10.203 1.2676 -14.425 -5.9802	-7.0234 1.0137 -10.481 -3.5654	0.84033 1.2676 -3.3822 5.06282	0 0 0 0	0.52433 1.2676 -3.6982 4.74682	-0.1378 1.0137 -3.5958 3.32025	-2.088 1.2676 -6.3105 2.13448	-1.0828 1.0137 -4.5408 2.37525
WBM6.8Homog	-10.727 1.30868 -15.191 -6.2627	-7.5477 1.2676 -11.77 -3.3252	0.316 1.30868 -4.1483 4.78032	-0.5243 1.2676 -4.7468 3.69815	0 0 0 0	-0.6621 1.2676 -4.8846 3.56035	-2.6123 1.30868 -7.0767 1.85199	-1.6071 1.2676 -5.8296 2.61535
WBM6.8MF	-10.065 1.2676 -14.287 -5.8424	-6.8856 1.0137 -10.344 -3.4276	0.97813 1.2676 -3.2444 5.20062	0.1378 1.0137 -3.3202 3.59585	0.66213 1.2676 -3.5604 4.88462	0 0 0 0	-1.9502 1.2676 -6.1727 2.27228	-0.945 1.0137 -4.403 2.51305
WPI4.6Homog	-8.1147 1.30868 -12.579 -3.6503	-4.9354 1.2676 -9.1579 -0.7129	2.92833 1.30868 -1.536 7.39266	2.088 1.2676 -2.1345 6.31048	2.61233 1.30868 -1.852 7.07666	1.9502 1.2676 -2.2723 6.17268	0 0 0 0	1.0052 1.2676 -3.2173 5.22768
WPI4.6MF	-9.1199 1.2676 -13.342 -4.8974	-5.9406 1.0137 -9.3986 -2.4826	1.92313 1.2676 -2.2994 6.14562	1.0828 1.0137 -2.3752 4.54085	1.60713 1.2676 -2.6154 5.82962	0.945 1.0137 -2.513 4.40305	-1.0052 1.2676 -5.2277 3.21728	0 0 0 0

Level

Least Sq
Mean

SBM4.6Homog	A	11.613667
SBM4.6MF	A	8.434400
WPI4.6Homog	B	3.499000
WPI4.6MF	B	2.493800
WBM6.8MF	B	1.548800
WBM4.6MF	B	1.411000
WBM6.8Homog	B	0.886667
WBM4.6Homog	B	0.570667

Levels not connected by same letter are significantly different.

Least Squares Means Table

Level	Least Sq Mean	Std Error	Lower 99.37%	Upper 99.37%
SBM4.6Homo	11.613667	1.0021242	8.595002	14.632331
SBM4.6MF	8.434400	0.7762421	6.096153	10.772647
WBM4.6Homo	0.570667	1.0021242	-2.447998	3.589331
WBM4.6MF	1.411000	0.7762421	-0.927247	3.749247
WBM6.8Homo	0.886667	1.0021242	-2.131998	3.905331
WBM6.8MF	1.548800	0.7762421	-0.789447	3.887047
WPI4.6Homo	3.499000	1.0021242	0.480336	6.517664
WPI4.6MF	2.493800	0.7762421	0.155553	4.832047

Appendix F: HPLC Calibration Curves

Day 1

Name : PE
 Quantitative Method : External Standard
 Function : $f(x)=9.88028e-008*x-0.016199$
 $Rr1=0.9973523$ $Rr2=0.9947116$
 MeanRF:8.25455e-008 RFSD:8.99119e-009 RFRSD:10.8924
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	739619.2	739619
2	0.067	817008.0	817008
3	0.083	977246.4	977246
4	0.100	1143233.6	1143234
5	0.200	2203246.4	2203246

Name : PI
 Quantitative Method : External Standard
 Function : $f(x)=3.99077e-008*x-0.045818$
 $Rr1=0.9945228$ $Rr2=0.9890756$
 MeanRF:2.60616e-008 RFSD:4.81092e-009 RFRSD:18.4598
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2625636.8	2625637
2	0.067	2608412.8	2608413
3	0.083	3240548.8	3240549
4	0.100	3628913.6	3628914
5	0.200	6164880.0	6164880

Name : PS
 Quantitative Method : External Standard
 Function : $f(x)=6.60424e-008*x-0.0185072$
 Rr1=0.9900322 Rr2=0.9801637
 MeanRF:5.41941e-008 RFSD:7.84539e-009 RFRSD:14.4765
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	1209312.0	1209312
2	0.067	1123177.6	1123178
3	0.083	1593648.0	1593648
4	0.100	1739955.2	1739955
5	0.200	3305958.4	3305958

Name : PC
 Quantitative Method : External Standard
 Function : $f(x)=3.27461e-008*x-0.0208643$
 Rr1=0.9942798 Rr2=0.9885923
 MeanRF:2.62388e-008 RFSD:3.39935e-009 RFRSD:12.9555
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2430507.2	2430507
2	0.067	2443001.6	2443002
3	0.083	3062105.6	3062106
4	0.100	3786339.2	3786339
5	0.200	6732816.0	6732816

Name : SM
 Quantitative Method : External Standard
 Function : $f(x)=3.90976e-008*x-0.0155255$
 Rr1=0.9790287 Rr2=0.9584972
 MeanRF:3.31026e-008 RFSD:5.80398e-009 RFRSD:17.5333
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2188832.0	2188832
2	0.067	1854771.2	1854771
3	0.083	2300633.6	2300634
4	0.100	2928832.0	2928832
5	0.200	5500918.4	5500918

Day 2

Name : PE
 Quantitative Method : External Standard
 Function : $f(x)=8.33219e-008*x-0.000877619$
 Rr1=0.9981752 Rr2=0.9963538
 MeanRF:8.22421e-008 RFSD:4.17881e-009 RFRSD:5.08111
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	638137.6	638138
2	0.067	842817.6	842818
3	0.083	936641.6	936642
4	0.100	1219057.6	1219058
5	0.200	2416830.4	2416830

Name : PI
 Quantitative Method : External Standard
 Function : $f(x)=3.1666e-008*x-0.0279772$
 $Rr1=0.9992758$ $Rr2=0.9985522$
 MeanRF:2.37829e-008 RFSD:2.69507e-009 RFRSD:11.3319
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2430318.4	2430318
2	0.067	2959592.0	2959592
3	0.083	3487073.6	3487074
4	0.100	4167958.4	4167958
5	0.200	7162401.6	7162402

Name : PS
 Quantitative Method : External Standard
 Function : $f(x)=5.38143e-008*x-0.00835658$
 $Rr1=0.9985733$ $Rr2=0.9971487$
 MeanRF:4.8862e-008 RFSD:2.86475e-009 RFRSD:5.86293
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	1125478.4	1125478
2	0.067	1329590.4	1329590
3	0.083	1664590.4	1664590
4	0.100	2091171.2	2091171
5	0.200	3856806.4	3856806

Name : PC
 Quantitative Method : External Standard
 Function : $f(x)=2.81128e-008*x-0.0187485$
 Rr1=0.9975613 Rr2=0.9951286
 MeanRF:2.29993e-008 RFSD:1.8434e-009 RFRSD:8.01503
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2381665.6	2381666
2	0.067	2895388.8	2895389
3	0.083	3714224.0	3714224
4	0.100	4437524.8	4437525
5	0.200	7691233.6	7691234

Name : SM
 Quantitative Method : External Standard
 Function : $f(x)=2.96371e-008*x-0.0107426$
 Rr1=0.9994016 Rr2=0.9988035
 MeanRF:2.61729e-008 RFSD:1.83625e-009 RFRSD:7.01587
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2152448.0	2152448
2	0.067	2577932.8	2577933
3	0.083	3150160.0	3150160
4	0.100	3661456.0	3661456
5	0.200	7141084.8	7141085

Day 3

Name : PE
 Quantitative Method : External Standard
 Function : $f(x)=6.0192e-008*x-0.0131087$
 Rr1=0.9978555 Rr2=0.9957156
 MeanRF:5.20269e-008 RFSD:3.73258e-009 RFRSD:7.17433
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	1069419.2	1069419
2	0.067	1250548.8	1250549
3	0.083	1586465.6	1586466
4	0.100	1975788.8	1975789
5	0.200	3513444.8	3513445

Name : PI
 Quantitative Method : External Standard
 Function : $f(x)=2.41286e-008*x-0.0307957$
 Rr1=0.9989313 Rr2=0.9978637
 MeanRF:1.76888e-008 RFSD:2.27799e-009 RFRSD:12.8781
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	3403600.0	3403600
2	0.067	3970310.4	3970310
3	0.083	4607017.6	4607018
4	0.100	5586214.4	5586214
5	0.200	9536723.2	9536723

Name : PS
 Quantitative Method : External Standard
 Function : $f(x)=4.22573e-008*x-0.0152174$
 Rr1=0.9985826 Rr2=0.9971673
 MeanRF:3.57052e-008 RFSD:2.91958e-009 RFRSD:8.17691
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	1581126.4	1581126
2	0.067	1922307.2	1922307
3	0.083	2226896.0	2226896
4	0.100	2822739.2	2822739
5	0.200	5079750.4	5079750

Name : PC
 Quantitative Method : External Standard
 Function : $f(x)=2.19565e-008*x-0.0186155$
 Rr1=0.9983393 Rr2=0.9966813
 MeanRF:1.7956e-008 RFSD:1.64029e-009 RFRSD:9.13504
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	3205515.2	3205515
2	0.067	3735428.8	3735429
3	0.083	4532177.6	4532178
4	0.100	5630673.6	5630674
5	0.200	9907668.8	9907669

Name : SM
 Quantitative Method : External Standard
 Function : $f(x)=2.17164e-008*x-0.00739199$
 Rr1=0.9995206 Rr2=0.9990414
 MeanRF:1.99059e-008 RFSD:1.00861e-009 RFRSD:5.06689
 FitType : Linear
 ZeroThrough : Not Through
 WeightedRegression : None
 Detector Name : AD1

#	Conc (Ratio)	Area	Area
1	0.050	2742856.0	2742856
2	0.067	3315918.4	3315918
3	0.083	4106772.8	4106773
4	0.100	5010342.4	5010342
5	0.200	9550286.4	9550286