AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Michael Qian

Milk is the first source of nutrition for all mammalian neonates. The complex matrix that constitutes milk has evolved to provide optimal nutrition for the newborn, for each species, respectively. Milk fat, originating in mammary epithelial, forms lipid droplets which are stabilized in the aqueous environment by bioactive polar lipids and proteins called the milk fat globular membrane (MFGM). The fluid phase of the membrane is made of amphiphilic polar lipids, largely glycerophospholipids and sphingolipids. The MFGM acts to compartmentalize the milk fat in the aqueous phase of milk and likewise, stabilize the oil in water emulsion.

Polar lipids, specifically phospholipids, make up the cellular membranes of all living things. Infants require milk polar lipids for growth and development, but also have specific benefits in neurological/neurobehavioral develop, gastrointestinal development, and establishment of the gut microbiome reported in pre-clinical and clinical trials. Adults also benefit from the consumption of milk polar lipids, with positive effects in lipid metabolism, reduced inflammation, and reduced cardiovascular disease risk. Polar lipids also play a role in the neurological health of aging adults and are associated with reduced risk of colon cancer in pre-clinical trials.

In the dairy industry, MFGM can be concentrated into several different products, including cream, beta serum, buttermilk, whey cream and MFGM enriched whey. The fat dominant fractions, such as cream, buttermilk, and whey cream,

previously were the focus of a large body of research using new technologies to recover milk polar lipids. MFGM enriched whey is available commercially as whey protein phospholipid concentrate (WPPC), a co-product of the production of high purity whey protein by membrane filtration technologies. However, there are no standards for phospholipid content in WPPC. Determination of phospholipid content of WPPC would give a better understanding of value this product has as a source of milk polar lipids, compared to other MFGM enriched products.

Polar lipids are also valuable functional ingredients, acting as emulsifiers, commonly used throughout the food industry. These polar lipids are usually sourced from plants, such as soy and sunflower, called lecithins. Plant based lecithins are regularly used in dairy products because of their emulsifying properties. Milk and dairy products are a potential source of polar lipids; however, no common commercial processes exist to extract phospholipids at concentrations high enough to serve as dairy-based lecithins.

During this project, a validated method for quantitating milk phospholipids was developed using hydrophilic interaction high performance liquid chromatography coupled to evaporative light scattering detection (HILIC HPLC-ELSD). This method was developed in collaboration with the dairy industry to be used as a standard method for future quality assurance testing and production of new MFGM and milk polar lipid products. Lipids are extracted using the Folch method, modified to reduce the required volumes of halogenated solvents. A final sample preparation step using solid phase extraction is used to remove neutral lipids and concentrate polar lipids. This HPLC method utilizes a quaternary solvent system which results in baseline resolution of phospholipid classes allowing for confident, reproducible quantitation of major milk polar lipids. The method was validated using spike-recovery, injection stability, and intermediate precision tests. The sample preparation steps also successfully isolated phospholipids for accurate quantitation when challenged with high protein, lactose, and fat content dairy products and plant based lecithins.

Next, the use of a food-grade solvent, ethanol, as an extraction solvent for the recovery of polar lipids from WPPC was investigated. The ethanol extraction procedures developed allowed for the recovery of a total lipid residue, and avoided

specialized equipment reported by other methods in the literature. Using absolute ethanol and the boiling point of the reaction mixture as an indicator of temperature, a total of 20 mass equivalents of solvent was used to recovery lipids over four extraction cycles. Additionally, the potential for polar lipids to be fractionated from neutral lipids using ethanol was also investigated. Total lipids dissolved in ethanol extraction solvent were concentrated slightly and then partitioned using fractional crystallization to produce a polar lipid-enriched fraction. Neutral lipids are crystallized away from polar lipids when incubated at 15°C due to temperature-dependent solubility in high ethanol concentration solutions. The resulting polar lipid fraction was analyzed by HPLC and determined to contain 38% total phospholipids by mass. Sphingomyelin was observed to concentrate into the polar lipid fraction during ethanol fractionation.

Finally, mass spectrometry-based lipidomics methods were applied to the ethanol extracted lipids from WPPC to profile polar lipids present in the commercially available product. Detailed structural information can be obtained from mass spectrometry, allowing for the identification of fatty acids attached to individual lipids species and the identification of the sphingoid backbone of sphingolipids. A total of 33 sphingomyelin species were detected and represented more than 27% of the total polar lipids detected in the total lipid extract. In the previous experiment some phospholipid species decreased in concentration after fractional crystallization, this observation was confirmed by the LC-MS analysis. Phosphatidylserine, phosphatidylinositol, and several glycosylated ceramide species were observed to co-crystallize with neutral lipids during fractionation. Correlation diagrams and principal component analysis (PCA) support the claim that phospholipids, specifically sphingomyelin and phosphatidylcholine, are recovered in a polar lipid-enriched residue after ethanol extraction and fractionation.

The Extraction and Analytical Profiling of Bioactive Phospho- and Sphingolipids
from Commercial Whey Protein Phospholipid Concentrate

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

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Joe Hale was involved with the design and writing of the first manuscript chapter.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1: Introduction—Literature Review

Genesis of Milk and the Milk Fat Globular Membrane

Milk is a complex biological matrix evolved to provide optimal nutrition to mammalian infants. The lipid content of milk is largely triglycerides (TG) found in milk fat globules (MFG) surrounded by a biological membrane of proteins and polar lipids, referred to as the milk fat globular membrane (MFGM) (Heid & Keenan, 2005). MFGM is a tri-layer system acquired from secretion events from the endoplasmic reticulum (ER) and the cell's apical plasma membrane (Heid & Keenan, 2005), shown in Fig. 1.1 and 1.2.

TGs are first synthesized in the ER, accumulate and are released into the cytoplasm called microlipid droplets (MLD) (Deeney et al., 1985). Electron microscopy studies have shown MLDs gain a membrane layer of proteins and polar lipids from the ER when released into the cytoplasm (Mather & Keenan, 1998). These initial droplets have diameters less than 0.5 μm and are found in proximity to the ER. Lipid droplets coalesce in the cytosol, growing in volume, to form cytoplasmic lipid droplets (CLD) (Heid & Keenan, 2005) and range in diameter between 0.5 μm and 4 μm (Deeney et al., 1985; Dylewski et al., 1984). This progression of lipid droplet development is supported by the similarity of droplet surface composition and single layer structure (Mather & Keenan, 1998).

Transport of CLDs from the basal region of the cell to the apical surface is not well understood but is believed to involve elements of the cytoskeleton (Heid & Keenan, 2005; McManaman & Neville, 2003). Evidence to support this claim is inconsistent across studies of alkaloid disruption of microtubule assembly and the effects on serum phase, protein and lipid secretion (Mather & Keenan, 1998). Several studies have reported numerous motility and motor

proteins associated with the MFGM and are likely involved in transporting CLDs along the cytoskeleton (Wu et al., 2000).

In the traditional explanation of secretion, CLDs transit the apical membrane through a budding process and are enclosed by the cellular bilayer membrane of the mammary epithelia until the droplet pinches off from the cell into the alveolar lumen (Heid & Keenan, 2005). The distribution of droplet size in milk suggests that MLDs can also directly migrate to the apical membrane and be secreted. However, other evidence suggests that the number and size of MFGs would exceed the surface area of the apical membrane available, and that a different mechanism must be at play (Honvo-Houéto et al., 2016). Another theory has been proposed for the secretion of lipid droplets into milk in which droplets accumulate at the apical membrane and fuse with secretory vesicles originating at the Golgi (Wooding, 1973), and the direct contribution of vesicle membranes to the release of lipid globules has been reported (Metka & Nada, 1992). Proteomic studies have confirmed the involvement of the ER in lipid secretion (Wu et al., 2000), and strongly imply the involvement of the ER, secretory vesicles, and plasma membrane material to form the final MFGM (Honvo-Houéto et al., 2016). Casein micelles have also been observed integrated into the MFGM, visualized by cryo-scanning electron microscopy (Luo et al., 2014), and could additionally support the involvement of vesicle transport of lipid globules, detailed images shown in Figure 1.4.

Polar lipids of the milk fat globular membrane (MFGM)

The major polar lipids of the MFGM are glycerophospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), and sphingolipids, including sphingomyelin, ceramides, glucosylceramide, and lactosylceramide (Rombaut et al., 2005). Polar lipids are amphiphilic,

meaning they contain both hydrophilic and hydrophobic regions within the molecule, chemical structures are shown in Figure 1.5. This unique nature of polar lipids gives them emulsifying properties, acting as the intermediate between fat droplets and the aqueous phase of milk (Contarini et al., 2013). The general structure of polar lipids is largely conserved and are classified into two major groups, glycerophospholipids and sphingolipids.

Glycerophospholipids, or phospholipids (PL), contain two fatty acids (FA) ester linked to the sn-1 and sn-2 positions of a glycerol molecule, with the sn-3 position occupied by a phosphate head group. A free acidic phosphate group is referred to as phosphatidic acid (PA), and a subsequent addition of another glycerol group results in phosphatidylglycerol (PG). More common moieties are inositol (PI), ethanolamine (PE), serine (PS), and choline (PC). This group of lipids, more specifically PC, are referred to as lecithins from the Greek word "lekithos" for egg yolk, and their structure was first published by Theodore Nicolas Gobley in 1847 (Zeisel, 2012). The removal a FA from PC via phospholipase A$_2$ (PLA$_2$) or by lecithin-cholesterol acyltransferase (LCAT) results in lysophosphatidylcholine (lysoPC) (Law et al., 2019).

Sphingolipids (SL) are a group of polar lipids identified by their sphingoid-base backbone, a long chain alkyl group containing an amino group and between one to three hydroxy groups, examples of chemical structures are shown in Figure 1.6 (Bielawski et al., 2006). The common structural moieties of the sphingoid base are sphingosine (Sph, (2S, 3R, 4E)-2-amino-1,3-dihydroxyalkenes), dihydrosphingosine (dhSph, (2S, 3R)-2-amino-1,3 dihydroxyalkanes), and phytosphingosine (phytoSph, (2S, 3S, 4R)-2-amino-1,3,4 trihydroxyalkanes). From these sphingoid-bases other additions to the primary hydroxyl group, and N-acyl group can create various SL species. The addition of a FA as an N-acyl group generates a ceramide. The most common FA chains in SLs are 14 to 22 carbons and vary in saturation and branched methyl

groups. Glycosphingolipids are ceramide species attached to a mono- or disaccharides. Gangliosides are ceramides with oligosaccharides attached and at least one sialic acid linked on the carbohydrate chain. The addition of a phosphoryl choline group to the primary hydroxy group of a ceramide creates a sphingomyelin (SM). The addition of a phosphate group to a sphingosine or ceramide molecule results in sphingosine 1-phosphate (Sph 1-P, or S 1-P) and ceramide 1-phosphate (Cer 1-P), respectively, which are import cell signaling molecules (Bielawski et al., 2010).

Phospholipid composition of bovine and human milk

Bovine milk consists of 3.5 to 5% fat depending on the breed and 0.5 to 1% phospholipids of the total fat (Jensen et al., 1991). Bitman & Wood (1990) also report a phospholipid concentration of 0.5% of total lipids. Rombaut et al. (2005) report raw milk to contain 0.029% PL by weight (0.23% db) and 0.7% PL of the total fat. Glycosphingolipids were quantitated in addition to PLs with glucosylceramide and lactosylceramide reported at 2.7 and 6.6% of PLs, respectively. Other PLs were reported approximately as PE 42%, PI 4.8%, PS 6.7%, PC 19.2%, and SM 17.9% of total PLs (Rombaut et al., 2005). Concentrations of polar lipids in bovine milk have been reported using modern chromatographic and mass spectrometry techniques, approximately PI 0.6 mg/L, PS 5.34 mg/L, PE 33.37 mg/L, PC 3.69 mg/L, SM 3.23 mg/L (Donato et al., 2011). Pimentel et al. (2016) review the phospholipid concentration of numerous dairy products (Tables 1.1 and 1.2) and report bovine milk to contain about 29 to 40 mg PL/100g fluid milk and 0.7 to 1% PL of total fat which agrees with Jensen et al. (1991).

The FA composition of PLs in bovine milk is also observed to change over the lactation period, however, the overall content of PLs was stable (Bitman & Wood, 1990). It is also well understood that the FA profile of PLs change with the diets of cows (DePeters et al., 2001;

Lopez et al., 2014). Interestingly, the total fat content of milk was observed to decrease in spring when cows are fed fresh pasture (approximately 40 g/kg in spring vs 42 g/kg in winter), however, the concentration of polar lipids increased (approximately 138 mg/kg milk in spring vs 112 mg/kg milk in winter). Likewise, the SM content increased significantly from 32 mg/kg milk in spring vs 25 mg/kg milk in winter, and the FA profile of PLs shifted to higher levels of unsaturated FAs (Lopez et al., 2014).

Comparing human milk polar lipid concentrations with bovine milk-derived ingredients is important for infant nutrition (Claumarchirant et al., 2016). Human milk fat accounts for 50% of the total energetic value available to infants (Jansson et al., 1981). The TG content of human milk is 98%, and the PLs represent 0.2-2% of the total fat (Bitman et al., 1984), slightly higher PL content compared to bovine milk. The higher PL content of human milk agrees with Garcia et al. (2012) reporting of 0.324 mM for human milk and 0.265 mM in cow milk. The three major classes of PL in human milk are PE, PC, and SM (62-80%), and minor components include PI and PS (12-15%) (Cilla et al., 2016). Concentrations of SM in human milk were reported to range from 24.5-174 mg/L (Fontecha et al., 2020). The major sphingoid base of human milk, representing 60% of the population, was reported as C_{18}-sphingosine with long chain fatty acids C20:0, C22:0, C24:0 and C24:1, determined by gas chromatography and direct-inlet mass spectrometry techniques (Bouhours & Bouhours, 1981).

A comprehensive analysis of mother's milk investigated phospholipid concentrations during seven different stages of lactation, including colostrum, transition, 1 month, 3 months, 6 months, 9 months, and 12 months, across three geographical regions in Spain (Claumarchirant et al., 2016). The most abundant PL present in human milk were SM and PE, 28-36% and 22-33%, respectively. Quantitation of individual PL concentrations were determined by high performance

liquid chromatography coupled to evaporative light scattering detection (HPLC-ELSD); approximately SM (8.0-16.5 mg/100mL), PE (6.5-16.0 mg/100mL), PC (3.6-8.0 mg/100mL), PS (3.3-6.7 mg/100mL), and PI (3.0-6.3 mg/100mL) including all lactation stages. PL levels started low in colostrum, were observed the highest in transition milk, and then decreased over the duration of lactation stages (Claumarchirant et al., 2016).

Importance of Polar Lipids in Nutrition and Related Health Effects

Infant nutrition and development: the role of polar lipids

Milk is the first source of nutrients for neonates. Polar lipids, including phospholipids and sphingolipids, like those present in the MFGM, make up the bilayer membrane of all cells. It is obvious that polar lipids are a required nutrient for cell growth and division. Formula feed infants receive significantly lower amounts of MFGM than breastfed infants, due to the common addition of vegetable oils in place of milk fats (Timby et al., 2017). However, SM and PS cannot be sourced from plant-based lecithins (Fontecha et al., 2020). The importance of MFGM and milk polar lipids in infant nutrition and development is well reviewed in the literature (Abrahamse et al., 2012; Fontecha et al., 2020; Lee et al., 2018). MFGM and milk polar lipids are involved in neurological development, establishing the early immune system, intestinal and gut microbiome development. Many pre-clinical and clinical trials which aim to investigate the effects of MFGM do not control for the protein or lipid components, thus it is impossible to directly attribute the outcomes directly to milk polar lipids. Such studies include tests of neurological development, gastro-intestinal development, and formation of the gut microbiome. Trials of MFGM on infant development are discussed in this section, and the polar lipid-specific variables tested are denoted when applicable.

Neurological development

Animal studies have revealed evidence that the addition of MFGM to infant formula can improve reflex development and changes brain lipid composition and metabolism. Rat pups fed formula supplemented with MFGM better resembled the mother-reared pups. In contrast, pups fed a standard formula differed significantly in brain PL composition compared to mother-reared animals. MFGM supplementation improved ear and eyelid twitch, negative geotaxis and cliff avoidance reflexes (Moukarzel et al., 2018). Studies in piglets found minor effects in neurodevelopment and lipid metabolism for animals fed whey-derived extracellular vesicles and PLs over 19 days. Piglets fed whey polar lipids had increased hippocampal maturation and lipolysis activities compared to animals fed soy lecithin formula (Henriksen et al., 2021).

Several human clinical trials have demonstrated the effects of milk polar lipids on infant neurodevelopment. Infants fed formula supplemented with milk PL and gangliosides (GM3 and GD3) had increased cognitive scores, improved hand-eye coordination and performance IQ scores comparable to breastfed infants (Gurnida et al., 2012). The addition of milk SM to formula improved neurobehavioral scores of pre-mature infants, suggesting SM plays a role in the development of the pre-frontal cortex (Tanaka et al., 2013). Another study reported similar results, that infants fed MFGM formula (70 mg/100mL) had significantly higher cognitive scores than the control group, and comparable to the breastfed group (Timby et al., 2017).

Intestinal development and the gut microbiome

Pre-clinical trials in neonatal mice fed MFGM responded better when challenged with LPS, having lower inflammatory response and lower cytokine levels (Huang et al., 2019). Lipopolysaccharide (LPS) is a bacterial endotoxin, a glucosamine-based phospholipid found on the outer membrane of pathogenic gram-negative bacteria, which causes an inflammatory

response in mammals (Raetz & Whitfield, 2002). Improved gastro-intestinal development characterized by increased enzyme activity and weight gain was observed in neonatal piglets fed MFGM. Piglets who received MFGM also had decreased communities of pathogenic bacteria in their colon and feces. Larger communities of *Clostridium IV*, *Parabacteroides*, *Lutispora*, and *Sutterella* were found in the MFGM treatment group, and these organisms are associated with increased weight gain of infants (Berding et al., 2016). The effects of MFGM ingredient supplementation to formula on gut microbial species, associated with reducing the impact of stress, were studied in rat pups (Thompson et al., 2017). The test group had improved non-rapid eye movement (NREM) and rapid eye movement (REM) sleep patterns when a stressor was applied. This improvement in stress response and sleep was attributed to the higher population of *Lactobacillus rhamnosus* present in pups fed the MFGM ingredient formula (Thompson et al., 2017).

Clinical trials of the addition of ganglioside enriched milk fat (GMF, 1.43 mg/100 kcal) to formulas found lower levels of *E. coli* after seven days of consuming GMF, and higher populations of *Bifidobacteria* after 30 days of consuming the enriched gangliosides in human infants. Malnutrition is considered the ultimate cause of death for millions of children under the age of five every year due to infectious disease and diarrhea. Episodes of bloody diarrhea and GI infection were reduced after the consumption of MFGM in Peruvian infants (Lee et al., 2018; Zavaleta et al., 2011).

Importance of polar lipids in adult health and later life

Neurological health and aging

Polar lipids continue to play a role in neurological health well into adulthood for humans. Specifically, concentrations of phosphatidylserine (PS) in the brain have been shown to affect

ligand binding to receptors (Pepeu et al., 1996). Several human clinical trials investigating the effects of PS treatment on Alzheimer's Disease (AD) and age-associated memory impairment found a slight improvement in neuropsychological tests (Amaducci, 1988; Crook et al., 1991; Crook et al., 1992). Since no side effects were observed across hundreds of patients, the treatment with PS should still be considered a potential therapy (Pepeu et al., 1996). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) concentrations in four regions of the brain of deceased patients diagnosed with dementia and AD was studied by Söderberg et al. (1991). In patients with AD the fatty acid profile of PE changed in all regions of the brain, compared to control deceased patients not suffering from mental disorders.

Polar lipid metabolites may play a role in the mental health of adults. In a clinical trial low blood plasma levels of choline, a metabolite of phosphatidylcholine and sphingomyelin, have been associated with higher levels of anxiety. Furthermore, this human clinical trial (n=5918) found no significant correlation of choline levels to depression symptoms (Bjelland et al., 2009).

Polar lipid effects on the gut and related cardiovascular effects

The inclusion of gangliosides to the diet of an animal model decreased inflammatory markers (prostaglandin E2, LTB4, IL-1β and TNF-α) of gut mucosa after being challenged by LPS injection. Serum levels of LTB4, IL-1β and TNF-α were reduced 41%, 58%, and 55%, respectively (E. J. Park et al., 2007). Another animal study concluded that orally administered PC functions preventively on GI permeability or leakiness, but did not have an effect on inflammatory markers (Dial et al., 2008). Mice feed a diet containing 10% MFGM had lower serum levels of interleukin cytokines and other inflammatory markers when challenged with LPS, compared to mice fed with a fat equivalent corn oil diet (D. R. Snow et al., 2011). The

study shows the potential for dairy ingredients to positively impact gut permeability and inflammation, however, it does not allow for the effects to be attributed to membrane proteins or polar lipids, both bioactive components of MFGM.

MFGMs and several components, including gangliosides GM3 and GD3 and lactosylceramide, are capable of directly binding to enterotoxigenic *E. coli* strains and preventing bacterial hemagglutination (Sánchez-Juanes et al., 2008). Milk SM was shown to decrease serum cholesterol and LPS in mice. Furthermore, the gut microbiota was improved with an observed decrease of fecal Gram-negative bacteria and an increase in *Bifidobacterium* (Norris et al., 2016) Similarly, mice feed milk SM displayed reduced hepatic steatosis and inflammation markers in adipose tissue (Norris et al., 2017).

Western diets are categorized by high intake of saturated fats and sugar, and low intake of fiber (Statovci et al., 2017). Saturated fatty acids are known to cause postprandial, after a meal, inflammation (Margioris, 2009)**.** Demmer et al. (2016) investigated the effect of consuming MFGM with a high saturated fat meal on postprandial inflammation in obese adult humans. The MFGM was sourced from a cream-derived milk lipid fraction powder, which contained 13.2% membrane proteins, 13.7% phospholipids, and 0.63% gangliosides. Analysis of serum after consumption of the meal showed lower levels of insulin, LDL cholesterol, total cholesterol, and intracellular adhesion molecule (sICAM); an increase in the concentration of anti-inflammatory cytokine IL-10 was also observed. IL-10 has atheroprotective effects, while sICAM is involved in early atherosclerosis development. The consumption of MFGM with a high-fat meal was shown to lower cardiovascular disease (CVD) risk and reduce postprandial inflammation (Demmer et al., 2016). However, the study showed the effect of intact MFGM and did not discriminate between bioactive membrane proteins and polar lipids.

Moreover, Vors et al. (2019) reported a similar decrease in cholesterol absorption and markers of CVD in postmenopausal women fed diets supplemented with milk polar lipids. PLs were delivered in cream cheese dosed with PL-rich buttermilk concentrate at 0, 3, and 5 g PL/day over 4 weeks. The concentration of SM was determined to be 0.8% wt and 1.3% wt in 3 and 5 g PL cheese, respectively. The study supports SM's role in decreasing intestinal cholesterol absorption and offers health professional an alternative to pharmaceutical treatments with undesirable side effects (Vors et al., 2019).

Anticancer proliferative effects of dairy polar lipids

Colon cancer is characterized by the abnormal proliferation of colonic mucosa epithelia, and the first detectable change is the formation of aberrant crypt foci (ACF) which can lead to carcinogenesis (Alrawi et al., 2006). *In vitro* studies have tested isolated MFGM from raw milk against HT-29 and Caco-2 colon cancer cells, and reported the inhibition of proliferation (Zanabria et al., 2013, 2014). Another study used *in vivo* rat models reported reduced incidence of cancer and ACF when feed a diet including MFGM and dairy fat at a concentration of 25g/kg over 3 weeks (Snow et al., 2010). It is important to note again, that these studies include intact MFGM and do not discriminate between effects associated with bioactive protein or polar lipids. Later studies controlled for the effects of milk polar lipids and showed that PLs extracted from buttermilk using food-grade solvents completely inhibited the growth of HT29 colon cancer cells below concentrations of 250 μL/mL (Castro-Gómez et al., 2016). It was also shown that using food-grade solvents is critical for maintaining the bioactive properties of dairy polar lipids.

Limited investigation of dairy PLs on breast, ovarian, and prostate cancers is inconclusive. The increased consumption of dairy foods may reduce the risks of breast cancer (Dong et al., 2011). Although, buttermilk PLs did not have antiproliferative effects on MCF-7

breast cancer cells, *in vitro*. In the same *in vitro* study, buttermilk concentrated with PLs and sphingolipids showed antiproliferative effects on NCI/ADR-RES ovarian cancer cells (Castro-Gómez et al., 2016). This effect on ovarian cancer cells was attributed to the high concentration of SM (5%) present in the polar lipids (Castro-Gómez et al., 2016). There is no evidence to support that dairy consumption affects the risks of prostate cancer (Huncharek et al., 2008; Parodi, 2009).

Recovery of Polar Lipids from Dairy

Polar lipids are important functional ingredients in the food industry, serving as emulsifiers in many food systems. PL emulsifiers used in food are referred to as lecithins, sourced from soybeans, egg yolks, and oil-seeds (sunflower, rapeseed, and cottonseed) (McClements et al., 2017). The word "lecithin" is regularly used in direct reference to PC which is the dominant species in soybeans. In the dairy industry, it is common practice to use plant-based PLs as emulsifiers added to powdered products to improve dispersion and solubility.

Current commercial whey protein products, e.g., whey protein concentrates (WPC 34 and WPC80) and whey protein isolates (WPI), are used as ingredients in many sectors of the food industry. These products can be used as binders in processed meat products, fat replacers lipids in "reduced-fat" products such as salad dressings and mayonnaise. They improve structure in baked goods in place of eggs, enhance browning in baked goods due to the presence of lactose, and form the base for fitness nutrition in protein powders and bars (Królczyk et al., 2016).

One whey product recently defined by the American Dairy Products Institute, whey protein phospholipid concentrate (WPPC), is a MFGM enriched co-product from the manufacture of WPI. WPPC is defined to have a minimum protein content of 50% and a

minimum fat content of 12%. However, there is no standard for PL content. The only the requirement is that it is manufactured using processes that concentrate MFGM and polar lipids.

To manufacture MFGM-enriched products commercially, the processes must destabilize the emulsion created by the milk fat globule (Huang et al., 2020). This can be achieved mechanically by centrifugation for cream separation and churning, or under pressure in membrane filtration systems. Other technologies of supercritical fluid extraction (Astaire et al., 2003; Spence et al., 2009a) and pressurized liquid extraction (also known as accelerated solvent extraction, ASE) (Castro-Gómez et al., 2014; Richardson, 2001) have been applied to dairy products for the concentration of MFGM. Studies have also investigated chemical methods of separation to recovery polar lipids, including enzyme hydrolysis (Barry et al., 2017a), food-grade solvent extraction (Price et al., 2018), and metal ion precipitation (Damodaran, 2010). Many production methods of PL-enriched or isolated ingredients are reviewed by Huang et al. (2020).

Membrane filtration

Membrane filtration technologies have revolutionized the dairy industry, allowing for the efficient recovery of whey solids. They have created an entirely new product category, powdered whey protein concentrates and other co-products. "Membrane filtration technologies" refers to the use of semi-permeable membrane filters for the fractionation or concentration of liquids. The liquid that passes through the filter is called permeate, and the retained fluid is called retentate (Strathmann et al., 1992).

The most widely used membrane materials are porous ceramics and polymer-based membranes, including polypropylene, polysulfone, polyacrylonitrile, and polyvinylidene fluoride (PVDF) based polymers (Akhtar et al., 1995). Cellulose acetate membranes were first used at an industrial scale, but their applications were limited due to their sensitivities to pH (Pouliot,

2008).   Membranes are loosely categorized into four groups determined by their porosity. Starting from the largest pores, the separations are microfiltration (MF, > 0.1 μm), ultrafiltration (UF, 1-500nm), nanofiltration (NF, 0.1-1nm) and reverse osmosis (RO, <0.1nm) being the smallest.   RO arguably has no pore size, having no engineered pores and allows molecules less than 100 Da to permeate.   As the pore size decreases, higher pressures are required to drive the separation.   The differences in porosity dictate which molecules pass through to the permeate or remain in the retentate.   In general, membrane filtration in dairy processing seeks to concentrate components into the retentate, as water largely constitutes the permeate.

The major challenge in membrane filtration is the prevention of fouling, or the build-up of solids on the membrane surface and pores, specifically during UF operations (Pouliot, 2008). Performance decreases gradually as the membrane accumulates foulants requiring regular cleaning to restore the clean surfaces.   Several techniques can be implemented to reduce fouling, such as controlling transmembrane pressure, use of cross-flow configurations where the feed stream is pumped tangentially to the membrane, and the use of high flow rates (Akhtar et al., 1995; Walstra, 1999).

Membrane filtration is a greener technology for the dairy industry (Kumar et al., 2013) with a lower energy consumption (9 kWh t^{-1} water removed) compared to previous techniques like vacuum evaporation (9-150 kWh) (Daufin et al., 2001).   Furthermore, membrane systems are simple to operate (Kumar et al., 2013).

Membrane separations most commonly consist of consecutive filtration steps using different molecular weight cut-offs to produce the desired product (Daufin et al., 2001). Application of UF in the dairy industry allows for the recovery of whey protein concentrate (WPC, >70% protein) which can be further purified to whey protein isolate (WPI, >90% protein)

using diafiltration, a technique of diluting the retentate with water and further filtering to remove lactose and smaller components (Daufin et al., 2001). Microfiltration, using the largest pore size membranes, allows for the concentration of lipid globules in the retentate as whey proteins pass into the permeate.

Boyd et al. (1999) used a ceramic tubular membrane (0.2 μm porosity) to concentrate MFGM (components >400 kDa) from Cheddar cheese whey. Morin et al. (2006) reported a greater effect on PL concentration in regular buttermilk compared to whey buttermilk using tubular ceramic MF membranes (0.7 m^2 total surface area, 0.45 μm pore size). The researchers investigated two different processes, one using volume reduction to half original volume (2-fold concentration), and a second in which continuous diafiltration was done using twice the original volume with water (2-fold diafiltration). Transmission of PLs through the membrane (loss into permeate) was higher in regular buttermilk using the diafiltration method, while the opposite was true for whey buttermilk where the highest transmission was observed in volumetric concentration (Morin et al., 2006a). Rombaut et al. (2007) investigated the effect of temperature and pH on the retention of polar lipids from acid whey using membrane filtration. The increase of pH from 4.6 to7.5 at 50°C allowed for near-complete recovery of PLs.

Supercritical fluid extraction

Supercritical fluid extraction (SFE) uses a solvent above its critical point to selectively extract molecules in a mixture (Williams, 1981). Supercritical fluids have unique properties of low density and viscosity with a high diffusivity, making them excellent for permeating samples and extracting specific components (Taylor et al., 1997). The process of SFE is completed in several steps: first a pump delivers the fluid to the extraction cell or column, the fluid diffuses

into the sample matrix, the soluble analytes are removed from the sample, then the extract is collected in a fractionation cell, and the solvent is depressurized (Herrero et al., 2006).

A commonly used solvent in SFE is carbon dioxide due to its low critical temperature and pressure, 31.1^oC and 73.8 bar respectively. Another benefit of using carbon dioxide as an extraction solvent is that it can be rapidly removed from the extracted materials simply by releasing the system pressure leaving no residual (Hauthal, 2001). Additionally, the solvent can be captured and recycled in high consumption systems found in industry, making it a green technology (Herrero et al., 2006).

The application of SFE to dairy products for the enrichment of PL acts to remove the unwanted lipid material, leaving the polar lipids behind. Astaire et al. (2003) used SFE (375 bar CO_2, 77^oC) after a microfiltration process resulting in an enriched product containing 19.7 mg PL/g dry powder. A five-fold increase in PL concentration of buttermilk powder was observed by Spence et al. (2009b), and later reported the parameters of 35MPa and 50^oC to resulting in a 70% reduction of total lipids (Spence et al., 2009a). The combination of UF (10-fold conc.), diafiltration (5-fold) on a 10 kDa membrane, and subsequent SFE (350 bar CO_2, 50^oC) yielded a final product with 73% protein and 21% total fat, where PL represented 61% of the total fat (Costa et al., 2010).

The addition of polar co-solvents, such as alcohols, can change the solvation power during SFE, allowing for more efficient extraction of polar analytes (Herrero et al., 2006). Sprick et al. (2019) utilize a two-stage SFE method in which neutral lipids are removed using traditional carbon dioxide methods, followed by a second stage using the addition of ethanol as a co-solvent for the extraction of PL. The yield of PLs was greatly affected by the concentration

of ethanol, and lesser so by temperature and pressure changes. A final PL residue contained approximately 26% polar lipids by optimized parameters of 35 MPa, 40°C and 15% ethanol.

Pressurized liquid extraction

Pressurized liquid extraction (PLE) is a technology similar to SFE and uses solvents under pressure and elevated temperatures, but below their critical point, to extract components from a solid matrix. Increased temperature allows for better penetration of solvent into solid particles and diffusion of analytes into the solvent phase. Applying pressure to the system maintains the solvent as a liquid allowing for solvents to be used above their boiling points (Carabias-Martínez et al., 2005).

PLE technology was initially developed for testing environmental samples but has been applied to various food stuffs for analysis of a wide variety of molecular classes. Pressurized liquid extraction has been applied in dairy products to detect carbamate insecticides, sulfonamide antimicrobials, penicillins (amoxicillin and ampicillin), and aminoglycoside antibiotics in milk (Bogialli, Capitolino, et al., 2004; Bogialli, Curini, et al., 2004; Bogialli et al., 2003, 2005), endocrine disruptor compounds such as BPA in powdered milk and infant formula (Ferrer et al., 2011), pesticides in milk-based infant formula (Mezcua et al., 2007), and UV ink photoinitiators from packaging of milk and yogurt (Morlock & Schwack, 2006).

PLE could be a potential tool for polar lipid recovery. It has been used in various dairy matrices to determine total lipid content (Richardson, 2001). Operating procedures were optimized for some dairy products, including powdered products, cheese, fluid milk, and butter. The solvent systems used for powdered products were composed of hexane, dichloromethane, and methanol at differing concentrations. These solvents yielded lipid recoveries similar to reference methods of tested powders (Richardson, 2001). Castro-Gómez et al. (2014) use the

same solvent system and report PLE equivalent to Roese-Gottlieb and IR determination methods. These studies amass evidence that PLE can be used for lipid extraction from dairy products. PLE using food-grade ethanol yielded higher recoveries of PLs than other organic solvent methods, from buttermilk (Castro-Gómez et al., 2016).

Enzyme hydrolysis

Research investigating the use of proteolytic enzymes for the recovery of milk PLs often involved the combination of techniques mentioned above. The hypothesis is that enzymes could be used to lyse the protein components of MFGM which act in stabilizing the membrane, and then the free PL fragments can be recovered through an additional process. Barry et al. (2017) use enzyme hydrolysis in combination with UF techniques. The use of a 50 kDa membrane causes the permeation of hydrolyzed peptides to create a PL enriched fraction. Total PL content was observed to increase 7.8-fold, approximately 6.2% total PL from an initial concentration of 0.8% PL in buttermilk powder. Total lipid content also significantly increased, 43.4% (db) vs 6.8% (db) initially (Barry et al., 2017a). Later, they incorporated SFE with ethanol as a co-solvent after UF to yield a final lipid extract containing and impressive 56% PL (db) (Barry et al., 2017b).

Food-grade ethanol solvent extraction and fractionation

Recently, it has been shown that a food-grade solvent, ethanol, can be used to extract total lipids from reconstituted whey protein phospholipid concentrate (WPPC) (Price et al., 2018). The principles of extraction were originally developed for the recovery of PL from egg yolk (Wang et al., 2017). Reconstituted WPPC is pumped through small diameter tubing into a bath of heated ethanol. Both temperature and ethanol concentration variables were investigated. Immediately, the proteins precipitate into thin strings, and the lipids are dissolved into the

ethanol solution. The solution is filtered away and, the solids are reconstituted in water and re-extracted. The final solution is evaporated to recover a total lipid extract (Price et al., 2018).

It was concluded that a 5-cycle extraction using 70% ethanol at 70°C were the optimal parameters, allowing for the recovery of a total lipid extract with a PL content between 40.7 to 58.1%. The use of temperatures above 70°C showed diminishing returns in the recovery of both total lipids and total PL. However, the increase in ethanol concentration (70%, 80%, 90%) resulted in higher recoveries of total lipids and PLs. The use of 90% ethanol results in near-complete recovery of lipid material, including PL content, but causes the relative concentration of PLs to decrease in the final lipid residue (Price et al., 2018).

The pilot-scale application using ethanol extraction of PLs has recently been reported in the literature (Lee et al., 2020). This pilot study used 10kg of dairy product (WPC and butter serum powders) extracted with 60L of ethanol solution (85%, 90% and 95%) in a single 5h cycle at 60°C. Precipitated solids were filtered, and the filtrate was concentrated by forced thin-film evaporation. Fractionation of the concentrated lipids was completed by leaving the mixture at 40°C for 30 min, resulting in a polar lipid enriched phase and triglyceride phase. The final PL content of the polar lipid enriched phases were 30.5-31.0% for WPC samples and 36.7-45.1% for butter serum samples.

The idea of fractionating lipids in ethanol could allow for the complete recovery of total PLs from dairy products and later isolation of a polar lipid fraction from other triglyceride content. The principle of fractionating mixtures based on temperature-dependent solubility is referred to as fractional crystallization. Such processes are common in the food industry for the production of low-temperature stable oils frequently used in salad dressing oils; this process is called winterization when in reference to food production (Puri, 1980). Little evidence can be

found in literature about the properties of dairy lipids in ethanol, however, the fractionation of dairy lipids in acetone solutions has been investigated (Chen & deMan, 1966). Additionally, several industrial processes have been patented which use ethanol/alcohols in a combination of other organic solvents such as acetone, hexane, and heptane (Bruecher et al., 2017; Nyberg & Burling, 1997; Shulman et al., 2009; Suzuki et al., 2018).

Metal ion precipitation

Glycerophospholipids and SM contain a negatively charged phosphate group which can participate in ion-ion interactions. In milk, these lipids could interact with Ca^{2+} present in casein micelles. This interaction also explains the potential of casein micelles to be incorporated into the MFGM discussed previously (Luo et al., 2014). Moreover, PLs would be expected to interact with other metal ions and potentially participate in chelation complexes.

Damodaran (2010) investigated the use of metal chloride and acetate salts, $CaCl_2$, $MgCl_2$ and $Zn(Ac)_2$, to precipitate MFGM and recover a fat-free whey protein isolate (WPI). The study revealed that zinc ions present at concentrations above 20 mmol/kg resulted in the complete precipitation of MFGM. It was proposed that the zinc ions complexed with the phosphate groups of PL in a tetrahedral geometry (Damodaran, 2010). These methods could theoretically be used to recover the MFGM. However, because the goal of the study focused on the creation of high purity protein discussion of potential safety risks associated with zinc present in the MFGM concentrate and methods to remove the metal ions was not included.

Another application of zinc and calcium ions to precipitate PL in MFGM from beta stream, a by-product of anhydrous milk fat, found that significantly higher amounts of Ca^{2+} are required compared to Zn^{2+}. Optimal conditions were determined to be zinc acetate (25 mM) at pH >6.5 and 30°C, and calcium acetate (>75 mM) at similar pH but a higher temperature of

60ºC. Subsequent extraction of the precipitated MFGM with ethanol permitted the recovery of PL and removal of metal ions (Price et al., 2020).

Quantitation and Analysis of Dairy Lipids

Crude fat analysis

Compositional analysis of food is required for products specifications and nutritional labeling including moisture, protein, fat, ash, and carbohydrate contents. Standard methods of analysis exist for each food component from AOAC and other standard methods. Many methods have been validated for specific food-stuff to accommodate for physical properties. A complete reference of standard methods for the analysis of dairy products can be found by Wehr et al. (2004). The earliest methods developed for fat analysis in dairy use chemical reagents to digest or extract the fat into a separate layer that can be read directly in the graduated sections of specialized glassware. These simple direct measurement methods, including Babcock, Gerber, Mojonnier and Roese-Gottlieb, are used to this day in the dairy industry for crude fat analysis. With advances in technology new electronic scanning devices or "analyzers" have been introduced to the dairy industry and are not limited only to determining fat. However, these devices are always calibrated to one of the standard chemical methods stated previously.

The Babcock and Gerber methods use sulfuric acid to generate heat through exothermic reactions to disrupt the emulsion of milk and dairy products and release free fat to be measured in the graduated neck of specialized glass bottles. The Gerber method includes a small portion of isoamyl alcohol into the reaction and is sometimes preferred for the analysis of fermented dairy products containing high amounts of sugar due to an observed decrease in acid-scorched particles in the final fat layer (Wehr et al., 2004).

The Mojonnier and Roese-Gottlieb methods are also very similar, using ammonia – ethanol solutions to break the emulsion and dissociate milk proteins. Then organic solvent in the form of an ether – petroleum ether mixture is added to dissolve the free fat. The organic layer is decanted away and evaporated to leave dried fat to be measured gravimetrically. The Mojonnier method AOAC 989.05 (AOAC, 2016) is reported to be more precise than the Babcock method and has been adopted by the dairy industry as the gold standard for fat determination (Wehr et al., 2004).

Gas chromatography of lipids

Specific structural information is commonly sought in detailed lipid analysis beyond gravimetric crude fat determinations. Often knowing the overall size and distribution of acylglycerols, or the profile of fatty acids (FA) present in the sample can indicate physical properties and nutritional value of food fats and oils. Lipid profiles of food stuffs can also confirm the authenticity of the product (Medina et al., 2019), identifying adulterated ingredients rejected by consumer standards for ethical and/or environmental reasons and legally defined limits on composition.

A common analytical technique for the regular analysis of lipids in the food industry is gas chromatography (GC). Selection of analytical detector after separation by GC adds further versatility to the method, commonly flame ionization detection (FID) and mass spectrometry (MS) which provides additional structural information. GC techniques allow for the analysis of intact acylglycerols, FA and the long chain bases of sphingolipids (Christie, 1989).

Acylglycerol analysis by GC

Acylglycerols, including mono-, di-and triglycerides, can be analyzed in their native state, without modifications, using high-temperature GC separations. High temperature stable

capillary columns are required for the analysis and often high phenyl concentration (>50%) bonded stationary phases are used. Intact triglycerides (TG), the major component of milk fat, are separated by total carbon number (i.e. total FA chain length) and unsaturation (Castro-Gómez et al., 2017; DePeters et al., 2001).   The application of high temperature GC methods for acylglycerol analysis in foods was reviewed by Ruiz-Samblás et al. (2015).   Fraga et al. identified eighty-one molecular species of TG in milk fat using a combination of silver ion thin layer chromatography and capillary GC (Fraga et al., 1998). GC methods have been used to show that TG composition changes with the diet of dairy cows (DePeters et al., 2001). However, cows feed consistent diets will produce a unique profile of TG. This pattern in the TG profile can be used to authenticate milk which will be distinctly different if adulterated with other food fats or oils. The validation of milk by GC analysis of TG composition is a valuable tool for the protection of high-value cheeses with geographical indications (i.e. DOP, AOP, PDO) (Fontecha et al., 2006)

Fatty acid analysis and synthesis of fatty acid methyl esters (FAME)

For individual FA to be analyzed by GC they must first be converted into more volatile esters, frequently methyl esters, resulting in fatty acids methyl esters (FAME). Several methods for the transesterification (or methanolysis) process are available differing in reagents used to react or catalyze the addition of a methyl group to the carboxylic acid of the FA, including sodium methoxide, boron trifluoride in methanol, acidic methanol solutions using hydrochloric acid or sulfuric acid, and alkali methanol solutions using sodium or potassium hydroxide (Christie & Han, 2003). Standard methods for FAME analysis by GC of food are established by AOAC and ISO, including AOAC 2012.13 for the applications to milk products and infant

formula (AOAC, 2016). It is common practice to report fatty acid profiles on a percentage basis and understood as a representation of the crude total fat content.

The result of each transesterification reaction is the same thus selection of a method can be made to accommodate a laboratory's risk assessment and equipment. Some methods are reported to cause fewer secondary reactions and interesterification products and may be considered when selecting a method (Christie, 1989). It is important to note that the transesterification reactions, in general, will not discriminate between acylglycerols, glycerophospholipids, and sphingolipids present in the sample. However, there is an exception for sphingolipids which are unsusceptible to attack in alkali catalyzed methanolysis due to the amide bonding between FA and sphingosine backbone. This phenomenon can be advantageous in experimental design where acylglycerols and glycerophospholipids are first reacted by alkali methanolysis, and then sphingolipids can be recovered and reacted under acidic conditions.

The most commonly selected GC stationary phases for FAME analysis are wax (bonded polyethylene glycol) and cyanopropyl columns (Zeng et al., 2013). General purpose polar columns of 25-30m can be used for FAME separation, but columns lengths of 60-100 m are not uncommon allowing for separation of long chain FA. The use of higher polarity stationary phases, such as bis-cyanopropyl columns, allows for the resolution of cis/trans isomers (i.e. C18:1c and C18:1t) and long chain FA in the CLA region (Harynuk et al., 2006). Elution order patterns of FAME can be identified in commercially available standard mixtures and are useful for the identification of FA species. In general, FAME elute in order of carbon chain length followed by increasing unsaturation. Early in the elution, saturated FA elute in front of the corresponding unsaturated FA, however, after steric acid (C18:0) many polyunsaturated FA (PUFAs) have a complex elution. In addition, while high polarity phases have the advantage of

resolving many of these unsaturated FA, the elution order changes when compared to wax columns (Sidisky et al., 1996).

Ionic liquid stationary phases for FAME analysis

The highest polarity GC stationary phases commercially available are ionic liquid (IL) columns. The first ionic liquid phase for GC was described in 1959 (Barber et al., 1959). The advances of IL phases have been reviewed in the literature (Fanali et al., 2017; Poole & Lenca, 2014; Poole & Poole, 2011). Performance of IL phases is attributed to physical and thermodynamic properties including high thermal stability, low volatility, and good selectivity for a range of compound classes (Fanali et al., 2017). IL phases are formed by pairing an organic cation, usually containing nitrogen or phosphorus, with either an organic or inorganic anion (Ragonese et al., 2009). The successful demonstration of imidazolium (Anderson & Armstrong, 2003) and phosphonium (Patil et al., 2018) cations as IL stationary phases have allowed for the commercial distribution of novel GC columns (Figure 1.9).

Similar to other polar phases, IL columns have unique retention properties resulting in greater FAME resolution when compared to wax and cyanopropyl phases (Zeng et al., 2013). The application of IL phases to FAME is well studied in literature (Ragonese et al., 2009; Talebi et al., 2018; Zeng et al., 2013). These extremely polar phases are also valuable tools in two dimensional GC (2D GC), or GCxGC, in which the selectivity of two GC columns are combined in tandem for higher resolution of peaks in a single separation. Applications of GCxGC in FAME analysis showed selection of a higher polarity first dimension followed by a lower polarity second dimension yielded the best results by increasing peak area occupancy in the second dimension (maximizing the benefit of using a second dimension in general) (Pojjanapornpun et al., 2018). An unique approach to GCxGC utilized a palladium catalyst

between two IL columns causing the unsaturated sites to be fully hydrogenated into the respective saturated FAME (Delmonte et al., 2013). This online hydrogenation resulted in the separation of trans FA from PUFAs and prevents PUFAs from overlapping with different chain lengths.

The importance of fatty acid composition in food stuffs specifically trans FA, PUFAs, and long chain FA has warranted the application of IL phases and advanced techniques like GCxGC. A summary of IL phases and GC methods used for FAME analysis in food, with several applications to dairy, is presented in Table 1.4, recreated from Fanali et al. (2017). Specific applications of IL phases to dairy samples also include the detection of odd and branched chained FA in butter, where several *iso* and *anteiso* branched FAs could be separated from overlapping species (Gómez-Cortés et al., 2017).

Sphingolipid analysis by gas chromatography

While modern analysis of sphingolipids almost exclusively applies liquid chromatography and lipidomic techniques, methods exist for the analysis of sphingolipids by high-temperature GC. These methods were developed before advancements in liquid chromatography platforms and sought to take advantage of a reliable and commonly available analytic technique of GC, albeit a laborious process. Native sphingolipids are non-volatile and must be modified to increase volatility, including removal of FA and derivatization, before GC analysis. Optimized procedures of acidic methanolysis to reduce secondary reaction products were reported by Gaver and Sweeley (1965) and procedures for the formation of trimethylsilyl derivatives of the resulting free sphingoid bases by Carter and Gaver (1967). In the case of sphingomyelin (SM) the phosphocholine head group must also be removed before analysis, this is important in the analysis of dairy matrices where the major sphingolipid class present is SM.

Removal of the phosphate head group is achievable through the enzymatic reaction of phospholipase C sourced from *Clostridium welchii* (Christie, 1989).

Moreover, these methods only allow for the determination of sphingoid bases of ceramide structures and do not allow for the identification of glycosphingolipids such as lactosylceramide and galactosylceramide found in milk. This limitation is largely due to molecular weight and/or hydrolysis of glycoside during FA removal. Likewise, this methodology will also not allow for the distinction between SM and other ceramides after the removal of the polar head group.

Derivatization of ceramides and subsequent analysis by GC-MS using modern commercial reagents and fused-silica wall-coated open tubular (WCOT) columns are reported by Bleton et al. (2001). This method allows for the analysis of free ceramides (sphingoid base + FA) a significant improvement over the old methods which require removal of the FA. In this method, ceramides (100 µg) are reacted with trimethylsilylation reagent (pyridine— hexamethyldisilazane – trimethyl – chlorosilane, 9:3:1 v/v/v) (100 µL) at room temperature for 30 min. After the reaction, the excess reagents are removed by evaporation, and the resulting residue is dissolved in 100µL of hexane for GC-MS analysis (Bleton et al., 2001).

The methodologies discussed above provide alternate means of sphingolipid analysis compared to liquid chromatography platforms, taking advantage of a readily available and less expensive analytic platform of GC-MS than LC-MS. The required structural modifications, to form free ceramides, limits the structural information gained by such analysis of dairy lipids which are comprised of a range of sphingolipid including SM, glycosphingolipids, and ceramides.

Extraction of Polar Lipids and Liquid Chromatography

Analysis of dairy polar lipids represents a very small fraction of all the research on bioactive lipids, and thus many advances in the extraction, purification, and analysis of polar lipids have resulted from the investigations of other biological matrices. These methods are mainly based on unfriendly chemicals in nature, and their developments are influenced by biochemistry laboratory settings taking advantage of reagents and techniques uncommon in dairy industry laboratories. Over time these methods have been applied to dairy products and have proved to be valuable tools for the analysis of dairy polar lipids.

Chemical methods for polar lipid extraction

The most common methods for polar lipid extraction from biological matrices utilize chloroform – methanol mixtures. The Folch method was initially developed for the extraction of polar lipids from fish tissues (Folch et al., 1957). Shortly after, a modification to the Folch method was published adjusting the solvent system to optimize the recovery of polar lipids (Bligh & Dyer, 1959). In brief, the Folch method uses a chloroform—methanol—water ratio of 8:4:3 (v/v), and the Bligh and Dyer method (B&D) uses a solvent system of chloroform – methanol – water 2:2:1.8 (v/v/v).

Both methods are widely used and result in a total lipid extract from biological matrices. Some researchers regard the B&D method as the gold standard for lipid extraction in biological matrices (Bielawski et al., 2010). Efficacy of total lipid determination methods, including Roese-Gottlieb and B&D, have been compared across several different foods where high precision was reported for each method (Manirakiza et al., 2001). A standard method based on B&D for the determination of total fat in foods has been established in AOAC 983.23, and

modifications thereof have been validated by Phillips et al. (1997), tested on dietary food composites.

Chloroform – methanol extraction methods have been applied to a variety of dairy products representing high protein products such as whey protein concentrates (Vaghela & Kilara, 1995) and high fat products such as cream, butter, and buttermilk (Rombaut et al., 2006; Spence et al., 2009b). These methods can be found in the literature for most dairy products with varying modifications to accommodate for protein, fat, lactose and water contents.

Purification of polar lipids by solid-phase extraction (SPE)

Isolation of polar lipids from neutral lipids is optimal for phospholipid (PL) quantitation by high performance liquid chromatography (HPLC). Using a sample preparation method to concentrate PL is necessary for dairy samples with low polar lipid concentrations. Solid phase extraction (SPE) is an efficient solution for polar lipid purification after the extraction of total lipids. SPE technologies take advantage of common liquid chromatography stationary phases and solvents to selectively elute analytes. The diversity of polarities and chemistries commercially available has made SPE a widely used technique.

Several methods have been developed for the recovery of polar lipids from biological matrices using SPE. The most common stationary phases are silica and aminopropyl for the recovery of polar lipids. The selection of polar phases is preferred for polar lipid recovery because neutral lipids present elute first as they do not interact with the stationary phase. Polar lipids can then be eluted with higher polarity solvents. The basic separation of dairy polar lipids from neutral lipid content can be achieved on silica SPE with two elution steps: neutral lipids are first eluted with hexane – diethyl ether (8:2, v/v), then PLs are eluted with methanol followed by chloroform – methanol – water (3:5:2, v/v/v) (Avalli & Contarini, 2005).

The comparison of total lipid extraction methods and SPE methods for PL purification from dairy products was investigated by Gallier et al. (2010). The Mojonnier and Folch methods were compared as total lipid extraction methods in combination with the SPE methods of Bitman et al. (1984), Vaghela & Kilara (1995), and Avalli & Contarini (2005) for the recovery of total PLs from neutral lipids. Several dairy products were used in the study, including raw milk, processed milk, cream, and buttermilk powder. It was concluded that the Folch/Bitman method was optimal for the recovery of PLs when compared to the combinations with the Mojonnier method (Gallier et al., 2010).

Isolation of polar lipid classes by solid phase extraction (SPE)

More advanced SPE methods have been developed for the isolation of lipid classes (Kaluzny et al., 1985), and later individual PL classes (Fauland et al., 2013; Pietsch & Lorenz, 1993). These separations of lipid classes can be achieved on the same aminopropyl cartridges as the basic separation of polar and neutral lipids, with the precise control of solvent polarity, pH, and ionic strength. The use of aminopropyl phases allows for better control of polarity difference between mobile and stationary phases, compared to the highly polar silica gel, and provides for the separation of the PL classes which only differ in polar head group species.

One of the earliest reports of SPE purification of lipid classes is by Kaluzny et al. (1985). A complex solvent system is used to recover seven fractions containing a single lipid class, with all fractions reported at a purity of >95%. Using an aminopropyl column, FAs are eluted with acetic acid (2%) in diethyl ether, total PL with methanol, cholesterol esters with hexane, triglycerides with diethyl ether (1%), dichloromethane (10%) in hexane, cholesterol with ethyl acetate (5%) in hexane, diglycerides with ethyl acetate (15%) in hexane, and monoglycerides with chloroform – methanol (2:1, v/v) (Kaluzny et al., 1985).

Pietsch and Lorenz (1993) separated PLs into four classes (PC, PE, PS, and PI) after first eluting neutral lipids and free fatty acids (FFA) with chloroform – isopropanol (2:1, v/v) and diethyl ether – acetic acid (98:2, v/v). PC was then eluted with acetonitrile – n-propanol (2:1, v/v), PE with methanol, PS with isopropanol – methanolic HCl (4:1, v/v), and finally PI with methanol – methanolic HCl (9:1, v/v) (Pietsch & Lorenz, 1993). Later Banni et al. (2001) modified this method for higher sample capacity and increased solvent elution volumes. Another improvement of this method further increased solvent volumes and altered the final solvent for the recovery of PI using chloroform – methanol – 3N HCl (200:100:1) (Pérez-Palacios et al., 2007).

The separation of PL classes was also achieved using a silica SPE cartridge and an aminopropyl SPE cartridge (Fauland et al., 2013) by eluting several PL classes together (PC, lysoPC, SM, and PE) from the aminopropyl phase directly into a second silica SPE cartridge for further separation. A multi-step solvent system was also developed for selective elution of PL classes, controlling polarity and pH like other methods discussed previously. The complete SPE procedure results in five fractions consisting of (1) non-polar lipids, (2) PG + cardiolipin (58.9%), (3) PA + PS + cardiolipin (38.9%), (4) PE, and (5) PC + lysoPC + SM (Fauland et al., 2013). This method is less effective due to the co-eluting of several PL classes.

Thin-layer chromatography separations of lipid classes

Thin layer chromatography (TLC) was first developed by Izmailov and Shraiber in 1938, who separated plant extracts on a microscope slide coated with adsorbent. In 1958, commercial adsorbents were available, and the technique surged in popularity. TLC is a simple technique for the rapid separation of mixtures using inexpensive materials and apparatus (Perry et al., 1972). The most common adsorbents in TLC are silica gels of varying particle size and porosity.

However, plates can be found coated with most modern liquid chromatography stationary phases. TLC itself is a liquid chromatographic method that separates analytes by polarity and the difference in affinity for the adsorbent (stationary phase) and solvent (mobile phase). Additional compounds can be added to the adsorbent to alter the separation of analytes, such as borate salts.

Polar lipids, and lipids in general, are separated well on the highly polar silica gel adsorbents in which the polarity of the solvent has the greatest effect on separation. Many different methods have been reported in the literature for the separation of polar lipids on TLC. Commonly, these methods rely on chloroform—methanol mixtures similar to extraction solvent systems discussed previously.

The separation of polar lipids has also been done using silica and borate impregnated TLC plates (Bouhours & Guignard, 1979). Both plates were developed with chloroform – methanol 90:10 (v/v). Ceramides were visualized by a 20% ammonium sulfate/4% sulfuric acid spray and charring, and glycolipids were visualized using 2% 1-napthol in ethanol and 50% sulfuric acid (aq) followed by gentle heating. Separation of fractions first on silica allowed for the confirmation of purity, then the separation on borate impregnated silica resolves ceramide subfractions (J. F. Bouhours & Guignard, 1979).

Complex separations of polar lipid fractions were reported by (Bodennec et al., 2000) using two and three-dimensional TLC techniques. Multi-dimensional TLC methods use two or more solvent systems developed in perpendicular directions to increase the resolution of analytes. Multi-dimension TLC separations of polar lipids have been reported in the literature (Parsons & Patton, 1967; Yokoyama et al., 2000). A silica TLC method using chloroform – methanol – water (65:25:4, v/v/v) has also been successfully used to separate milk PL (Astaire et al., 2003; Gallier et al., 2010; Spence et al., 2009b). A solvent system by Bitman et al. (1984)

also provides a good resolution of milk PLs. The solvent mixture consists of chloroform – n-propanol – ethyl acetate – methanol – 0.25% aqueous potassium chloride (25:25:25:15:9, by vol).

Analytes can be visualized using a wide assortment of chemical stains developed for the reaction of and subsequent identification of specific chemical structures in molecules. General-purpose chemical stains include iodine crystals that vaporize and adduct to organic molecules, and sulfuric acid and heat that char organic molecules present. A common stain used for the visualization of polar lipids is Dittmer-Lester reagent, a molybdenum-based reagent that has been improved with the addition of acetic acid (Ryu & MacCoss, 1979). Dittmer-Lester reagent reacts with analytes at predictable molar ratios and allows for the quantitation of the analytes by measuring color intensities with a densitometer (Bodennec et al., 2000). Other methods have been developed to visualize UV-active compounds on fluorescently doped adsorbents. Compounds that are UV-active appear blue against a green background, and non-active molecules appear as dark shadows if local concentrations are high enough.

Liquid chromatography and mass spectrometry of polar lipids

Modern liquid chromatography (LC) is an evolution of TLC separations. Bonded stationary phases are packed into columns and analytes are separated under high pressure mobile phase. Several different chromatographic modes are regularly used in LC metabolomics, predominantly reverse phase (RPLC). However, lipidomics can also take the advantage of hydrophilic interaction liquid chromatography (HILIC) and normal phase (NPLC). NPLC resembles silica-based separations on TLC using a polar stationary phase such as silica or amino propyl phase in combination with organic solvent mobile phases; lipids are separated based on their head group, non-polar eluting first followed by increasing polarity. In contrast, RPLC

utilizes non-polar stationary phases of C18 and C8 and high polarity aqueous solvents in gradient to more organic mobile phases; lipids are separated according to their lipophilicity, the carbon chain length and degree of saturation. HILIC techniques are orthogonal to normal phase using polar stationary phases in combination with organic solvents in gradient to increasing polar solvents as mobile phases; separations are dependent on lipid head groups similar to NPLC (Cajka & Fiehn, 2016).

Several high-performance liquid chromatography (HPLC) techniques have been applied to dairy products for the quantitation of PL classes present. The detection of PL is widely done using evaporative light scattering detection (ELSD), a universal detector in which the sample is nebulized into an aerosol and passed through a laser beam. The molecule particles pass through the laser and scatter light, and the decrease of light reaching the detector generates a signal. Several methods were developed using silica columns and chloroform – methanol – aqueous solution mixtures as mobile phases for the analysis of polar lipids (Rombaut et al., 2005; Spence et al., 2009b; Vaghela & Kilara, 1995). A HILIC-ELSD method has been reported for the quantification of PLs in human milk, with 23.8 mg/100g on average. Concentrations of PLs were reported as PI at 0.6 mg/100g, PE 4.2 mg/100g, PS at 0.4 mg/100g, PC at 2.8 mg/100g, and SM at 4.6 mg/100g (Giuffrida et al., 2013).

Mass spectrometry is the method of choice for lipidomic analysis because it offers higher levels of specificity in the identification of complex structures using accurate molecular mass and fragmentation techniques such as tandem mass spectrometry (MS/MS). The higher sensitivity of LC-MS allows for the detection of compounds at fmol concentrations. Different modes of ionization such as matrix-assisted laser desorption ionization (MALDI), and electrospray (ESI), different mass analyzers including quadrupoles (Q), time-of-flight (TOF), ion

traps (IT), and Fourier-transform ion cyclotron resonance (FT-ICR), are available for lipidomic studies. The most common ionization method applied for the analysis of dairy lipids is ESI followed by MALDI. ESI is a soft ionization technique, meaning molecular ions are formed with little fragmentation in the ion source. Analytes elute from the LC column and infuse into the MS ion source through a charged capillary (1-6 kV) where charged droplets form. As the droplets travel toward the MS inlet, solvent rapidly evaporates, and charges are transferred to the analytes (Merrill et al., 2005). The ionization processes that occur in MALDI are more complex and not completely understood. In brief, the sample is mixed or coated in a chemical matrix which assists in the volatilization and ionization of the analytes when pulsed by a high-powered UV laser. Sample mixtures are applied to a metal target plate as spots which allows for high-capacity sampling. New techniques have been developed allowing for the use of entire histological samples and direct mapping of analyte concentrations on the sample image (Karas & Krüger, 2003).

Tandem mass spectrometry configures two mass spectrometers in sequence so that ions can be selected in the first mass analyzer and fragmented, and the resulting daughter ions can be detected in the second mass analyzer. Fragments of ions are commonly formed through collision-induced dissociation (CID) in which analyte ions collide with neutral gas molecules. The degree of fragmentation can be controlled by altering the velocity of the precursor ions and the mass of neutral gas molecules (Merrill et al., 2005). Two data acquisition modes are used in MS/MS analysis, data-dependent acquisition (DDA) and data-independent acquisition (DIA). During the former, DDA, the mass spectrometer selects the highest intensity ion in the first stage to fragment and then analyze in the second stage. In DIA, for every duty cycle the mass spectrometer acquires MS/MS data for a mass window collecting all fragments of all precursor

ions. The disadvantage of DIA is the loss of information connecting precursors to their fragments, resulting from the presence of several precursor ions contributing to the fragmentation event. DIA methods result in convoluted spectra which must be filtered by software in data post-processing. Regardless of data acquisition mode, identification of precursors and fragments is near entirely dependent on the available databases. Even before database searches it is best practice to filter data and use processing algorithms for feature detection, peak alignment, and data normalization (Cajka & Fiehn, 2016).

Mass spectrometry techniques have been used to identify PLs in milk, sphingolipid species in buttermilk and butter serum, and PLs in infant formulas. Donato et al. (2011) applied HILIC-IT-TOF MS to analyze cow and donkey milk samples and concluded cow's milk contained 20 times more PLs (approx. 46.2 μg/mL) than donkey milk; PE was the most abundant PL (approx. 33.4 μg/mL). Bourlieu et al. (2018) profiled sphingolipid isolates from buttermilk (BM) and butter serum (BS) using positive ionization mode direct flow injection triple-quadrupole mass spectrometry (QTrap MS/MS). Sphingolipids are easily ionized in positive-ion mode with long-chain bases and complex sphingolipids forming $(M + H)^+$ ions. Sphingosine 1-phosphate (S 1-P), SM, and gangliosides can also form $(M - H)^-$ ions in negative mode (Merrill et al., 2005). Three major isoforms of ceramide were found in BM and BS, assuming the dominant sphingoid base as d18:1, the species were tentatively identified as C22:0, C23:0 and C24:0. These three ceramide species represented 56.8% and 65.8% of total ceramides in BM and BS, respectively. The two dairy samples were also determined to contain similar SM species, a total of 32 isoforms were detected at concentrations over 0.1% (Bourlieu et al., 2018). Determination of PL content of infant formulas was successful using a HILIC-MS/MS method.

PL concentrations ranged from PC at 0.63-0.84 mg/g, PE at 0.61-0.75 mg/g, PI at 0.26-0.46 mg/g, PS at 0.13-0.28 mg/g, and SM at 0.31-0.82 mg/g (Fong et al., 2013b).

Conclusions

Milk polar lipids are beneficial to human health, beginning with infant nutrition and then later in life as an adult. Infants require polar lipids for growth and development, playing a critical role in neurological and intestinal development, aid in the early immune system, and help establish the gut microbiome. As adults, humans benefit from milk polar lipids due to their contribution to lipid metabolism and related cardiovascular effects, effects on neurological health, and potential anticarcinogenic effects. In the food industry, polar lipids are useful emulsifiers. To the dairy industry, milk polar lipids are a potential new value-added nutraceutical and functional ingredient. Currently, there is no common commercially available dairy-based lecithin product. Several new technologies, such as SFE and PLE, have been presented for the recovery of polar lipids from dairy products. However, these technologies have not been adopted by the dairy industry. MFGM is concentrated into several different dairy products, including whey protein phospholipid concentrate (WPPC). However, there is no standard for PL content in WPPC, only the requirement that it is produced from processes that concentrate MFGM and PLs. Additionally, there are no standard methods for PL analysis. The composition of polar lipids, specifically sphingolipids, concentrated into WPPC is not well studied in the current literature.

The goal of this project was to 1) develop an analytical method for PL quantitation to be used in the dairy industry for regular quality assurance and development of future PL products, 2) investigate a novel food-grade solvent extraction method for the recovery and fractionation of

lipids from MFGM enriched WPPC, and finally 3) use mass spectrometry-based lipidomic

techniques to profile polar lipids in commercial WPPC, with an emphasis on sphingolipids.
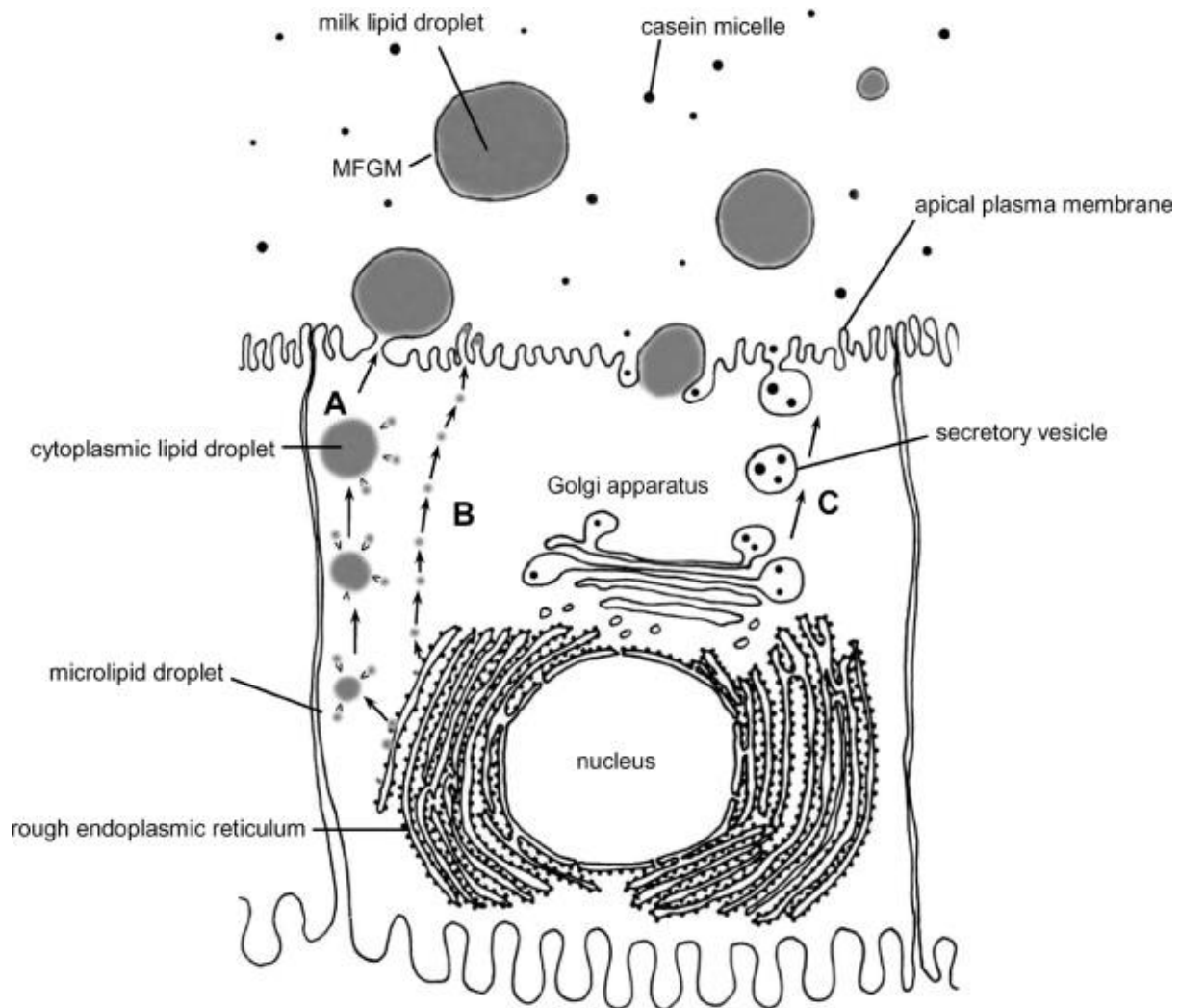
Figure 1.1. Major secretory pathways in mammary epithelial cells during lactation. A = cytoplasmic lipid droplet pathway; B = microlipid droplet pathway; C = secretory pathway for skim milk components. From (Bauman et al., 2006)

Milk fat globule membrane:
a trilayer structure with a lateral organisation of polar lipids
and a heterogeneous distribution of proteins
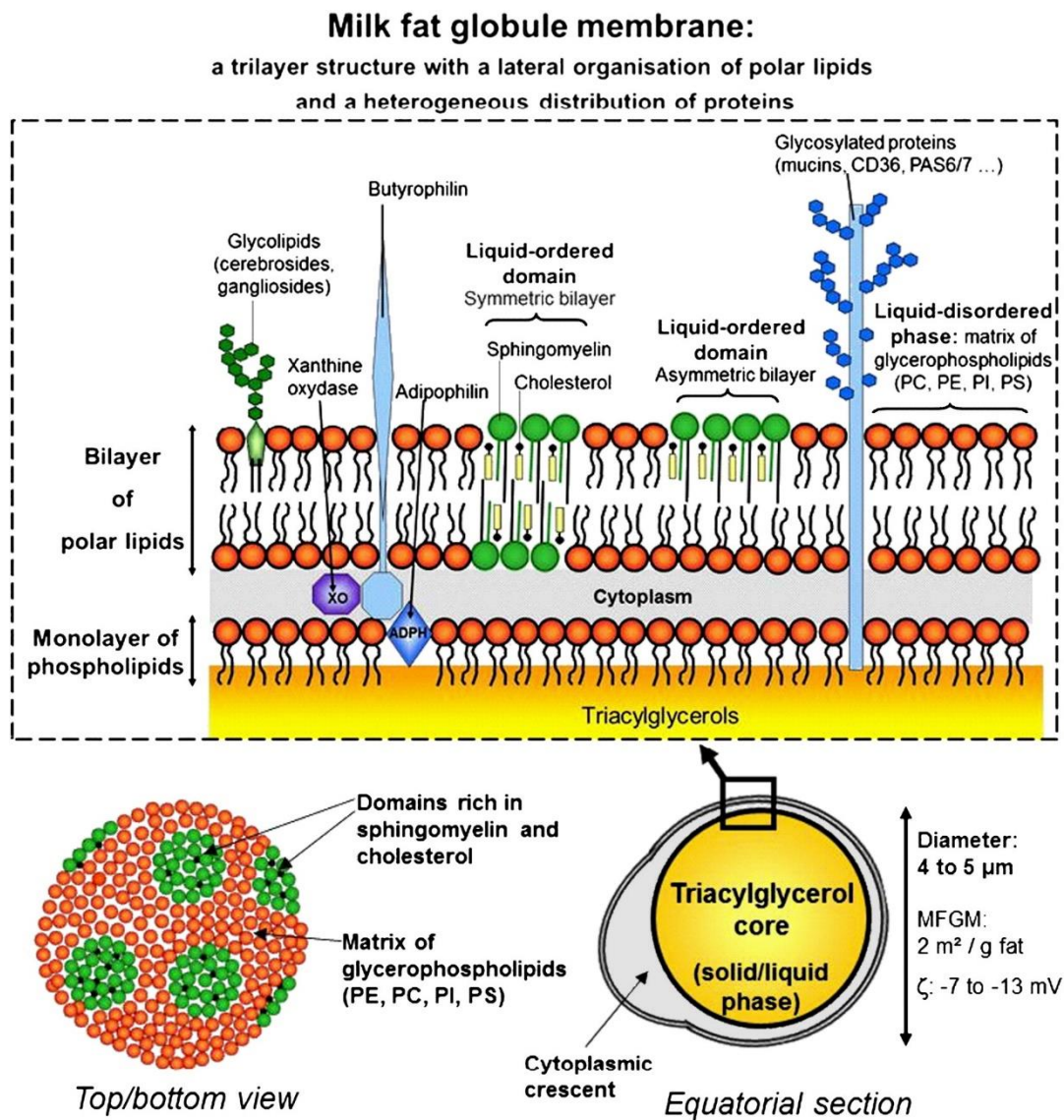
Milk fat globules

Figure 1.2. Supra-structure of milk fat globules with a schematic representation of the milk fat globule membrane (MFGM). The tri-layer of polar lipids is the backbone of the MFGM, with a lateral organization in the plane of the bilayer corresponding to the phase separation of sphingomyelin-rich domains (liquid-ordered phase). The glycerophospholipids are organized in a liquid-disordered (Ld) phase matrix surrounding the SM-rich domains. The MFGM transmembrane and peripheral bound proteins are located along the membrane in the Ld phase of phospholipids. The glycosylated moieties of glycoproteins and glycolipids are distributed over the external membrane surface, in the glycocalyx. From (Lopez, 2011)
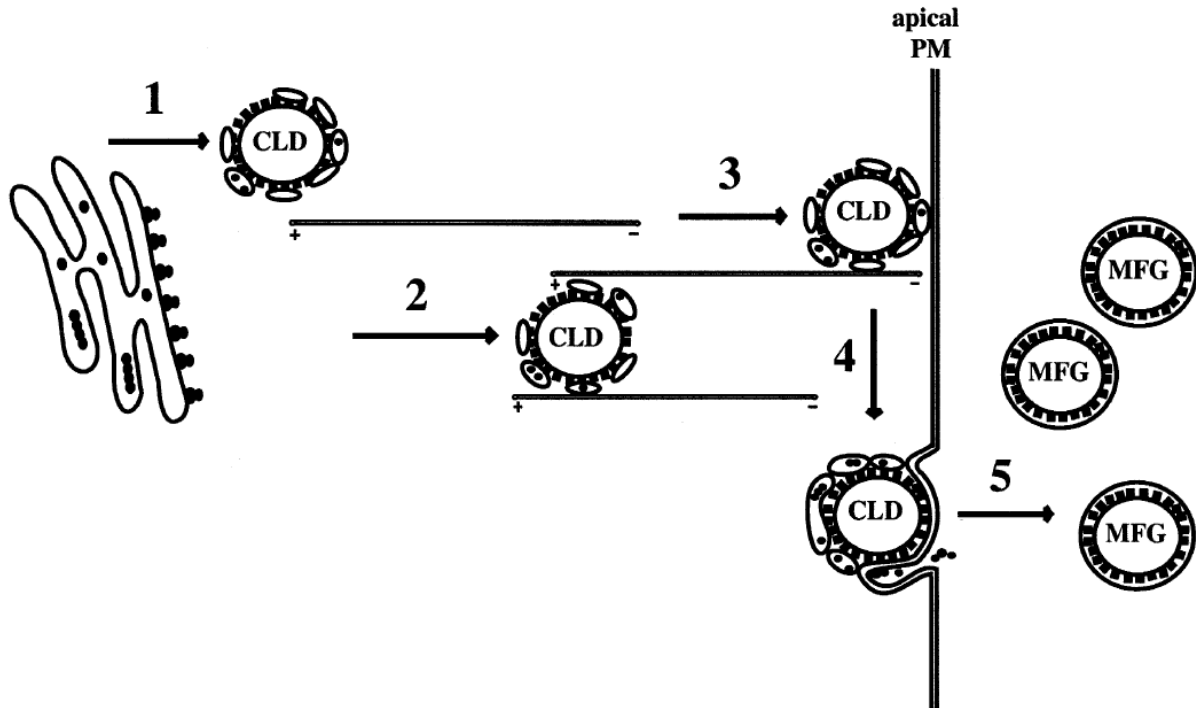
Figure 1.3. Schematic of five proposed steps involved in lipid droplet secretion in the mammary epithelial cell. (1) Assembly of lipid, protein coat, and vesicles onto the CLDs. (2) Attachment of CLDs on to microtubules. (3) Vectorial transport of CLDs to the apical plasma membrane. (4) Fusion events at the apical plasma membrane. (5) Secretion of MFG into the lumen of the alveolus. From (Wu et al., 2000)
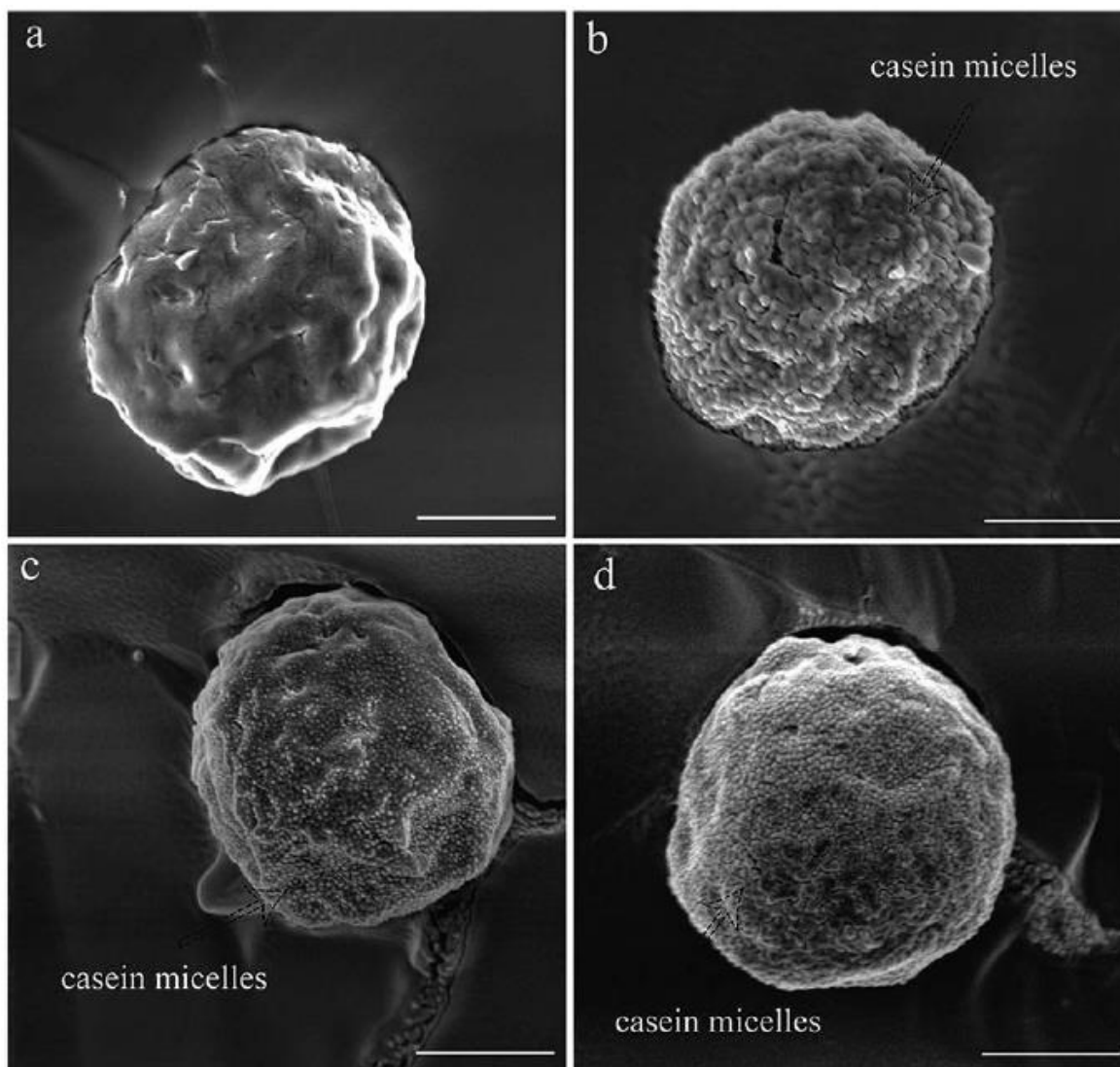
Figure 1.4. Cryo-SEM image of fat globules from milk with different proportions of protein to fat (a) 0.01 g protein per g fat; (b) 0.1 g protein per g fat; (c) 0.5 g protein per g fat; (d) 5 g protein per g fat. The scale bar represents 1 mm. From (Luo et al., 2014)
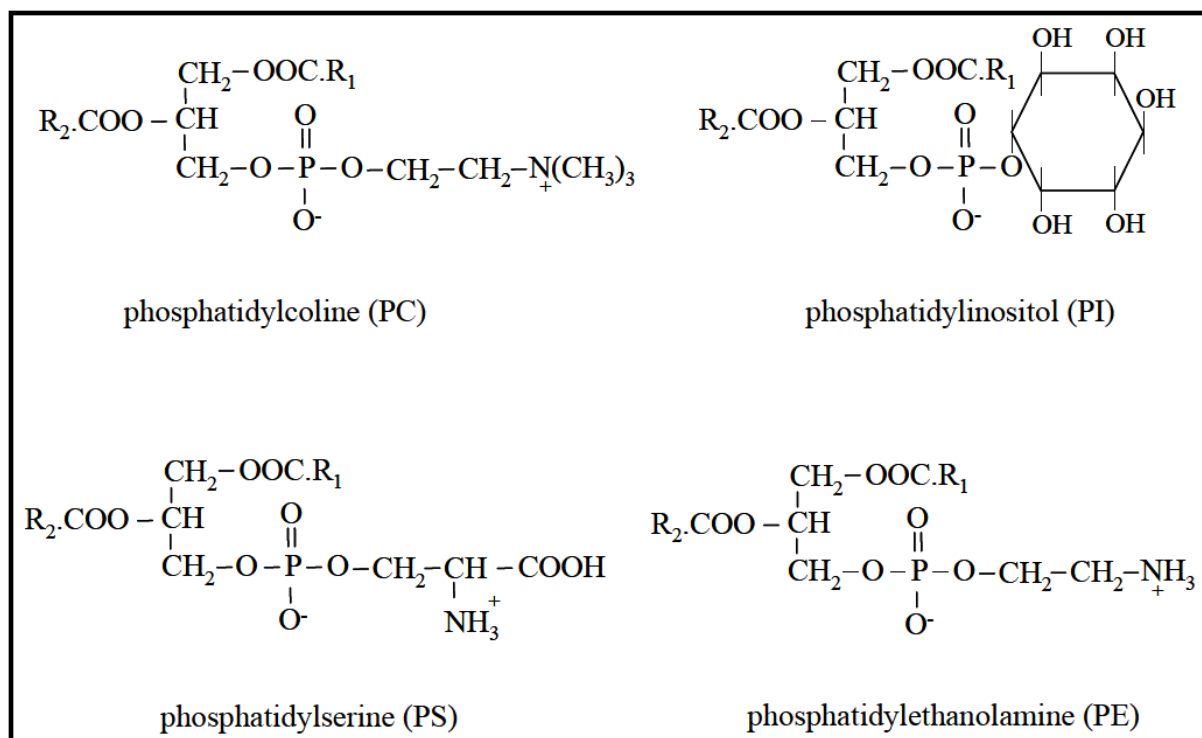
Figure 1.5. Structure of glycerophospholipids common in milk fat. From (Contarini et al., 2013)
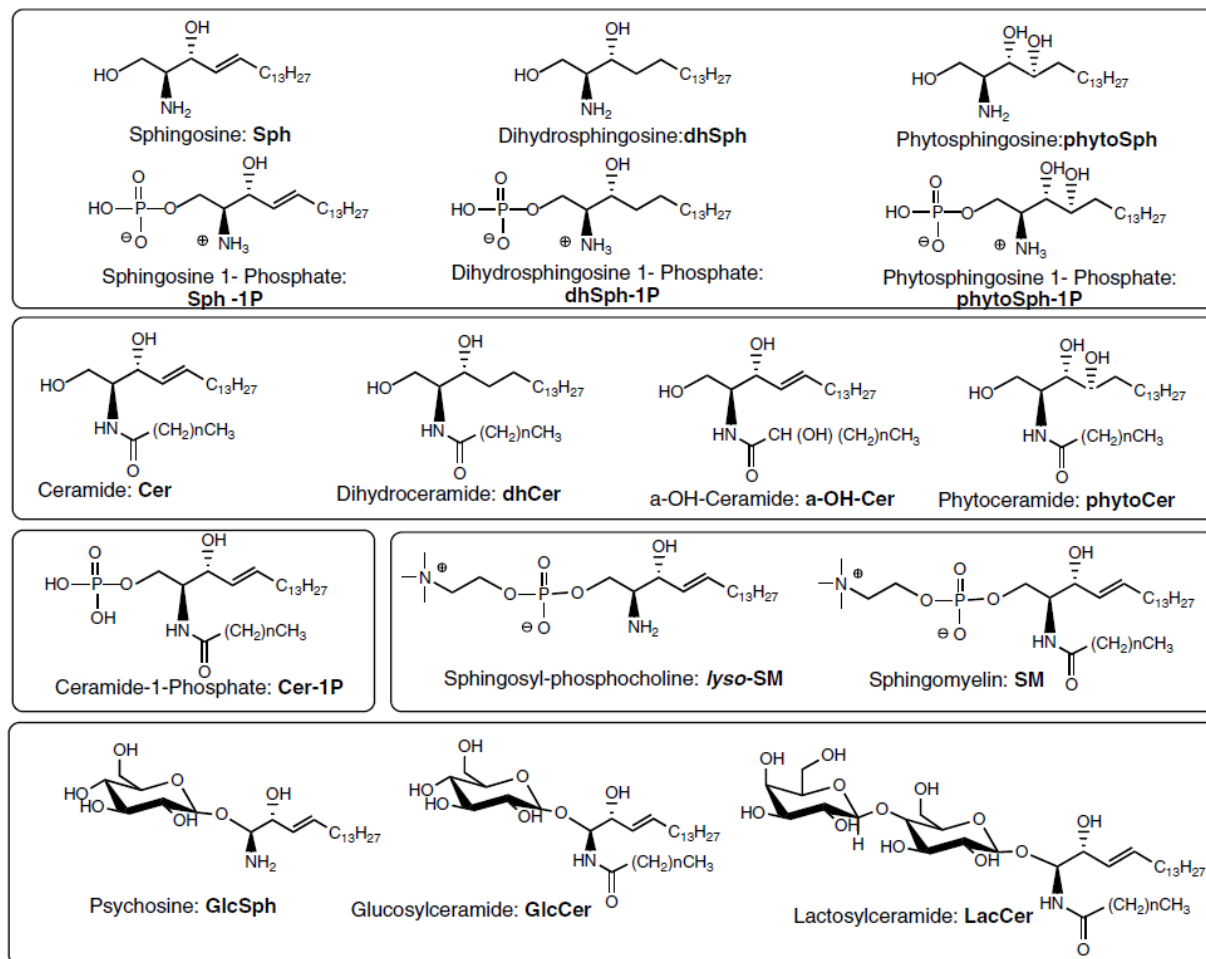
Figure 1.6. General structures, nomenclature, and common abbreviations of sphingolipids. From (Bielawski et al., 2006)
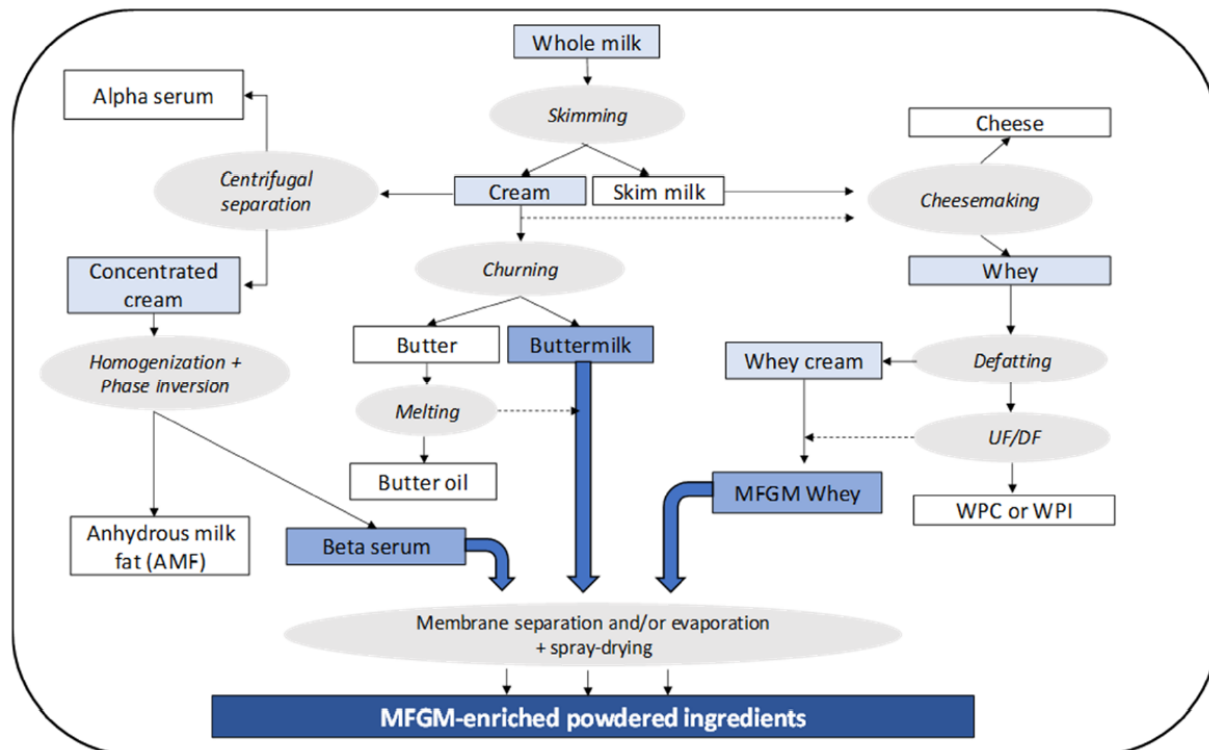
Figure 1.7. Processing alternatives to produce MFGM enriched powdered ingredients. From (Fontecha et al., 2020)
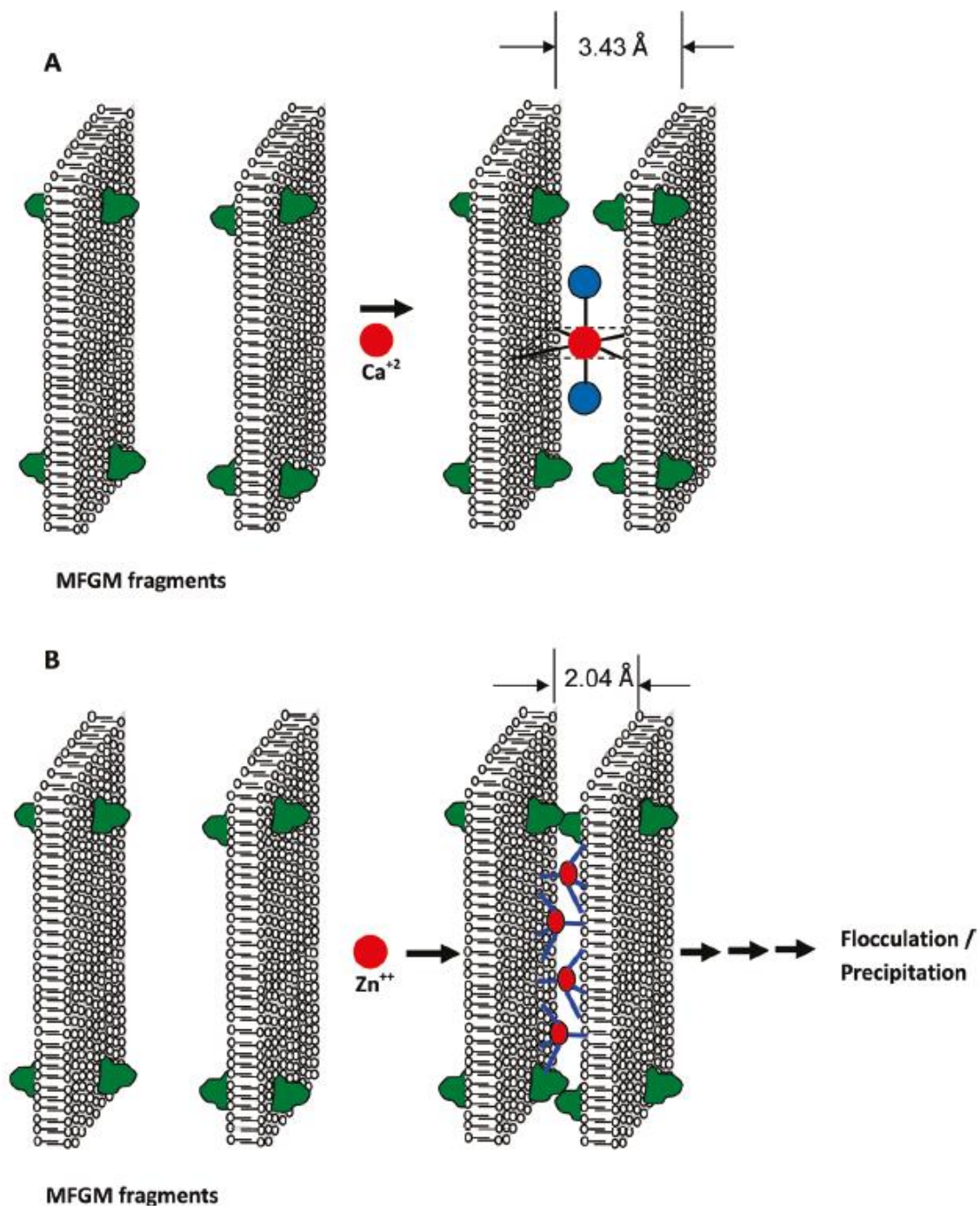
Figure 1.8. Representation of the proposed (A) hexacoordination of Ca^{2+} in a octahedral geometry and (B) tetracoordination of Zn^{2+} in a tetrahedral geometry with MFGM particles. From (Damodaran, 2010)
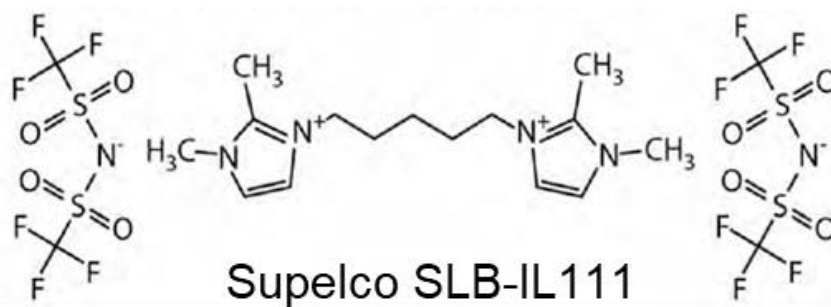
Figure 1.9. Example of imidazolium ionic liquid stationary phase found in commercially available Supelco products (Sigma-Aldrich, St. Louis, MO, USA)

TABLES

Table 1.1 Phospholipid Content of Dairy Products

Product	mg/100 g product	g/100 g Fat	References
Ewe raw milk	29.8	0.390	
Goat raw milk	27.6	0.710	A-F, H, I
Cow raw milk	29.4-40.0	0.700-0.980	
Skimmed milk	20.0-81.9	11.1-19.1	J
pasteurized semiskimmed	18.8	1.30	
UHT full fat	21.2	0.600	
UHT semiskimmed	14.2	0.900	B
UHT skimmed	12.8	10.7	
Sterilized semiskimmed	16.0	1.00	
Infant formula (0 - 6 mo.)	228-304	NR	K
Infant formula (0 - 3 mo.)	607	NR	
Nonsweetened condensed milk	75.1	1.00	B
Cream	139-190	.35-.86	A-C, G
Butter	70.5-230	0.09-0.27	A-C, J
Butteroil	10.0	0.0100	J
Buttermilk	1.12-160	4.49-33.1	A-C, G, L
Buttermilk (skimmed)	110	20.00	M
Buttermilk (acid)	160	33.05	
Buttermilk (reconstituted)	130	21.66	A-C
Buttermilk quark	310	29.06	
Butterserum	660-1250	14.8-48.4	
Butterserum (whole)	97.0	40.0	M
Butterserum (skimmed)	93.0	49.0	
Goat buttermilk	NR	0.19	G
Goat butterserum	NR	1.010	
Fermented buttermilk (sweet)	91.8	21.8	
Fermented buttermilk (sour)	115.5	23.1	
Yogurt skimmed	17.9	5.50	
Kefir semiskimmed	34.0	2.30	
Whey (cheddar)	18.0	5.32	
Whey (emmental)	22.0	45.2	
Fresh cheese	310	29.1	
Ricotta	279	2.70	A-C
Quark skimmed	32.4	24.70	
Quark cream	58.1	0.90	
Cottage cheese	55.8-376	1.30-5.30	
Sphingolipid quark mix	480	10.60	
Fresh cheese	149	0.600	
Mozzarella (buffalo)	115	0.500	
Gouda light (8 weeks)	93.9	0.400	

Table 1.1 cont.	mg/100 g product	g/100 g Fat	References
Gouda (8 weeks)	151	0.500	
Gouda (36 mo.)	147	0.400	
Cheddar	154	0.500	
Camembert	123	0.500	
Emmental	110	0.400	A-C
Parmigiano (24 mo.)	111	0.400	
Mozzarella (whey)	19.1	6.20	
Cheddar (whey)	17.6	5.30	

NR = not reported. Recreated from (Pimentel et al., 2016)
Reference list: **A** (Rombaut et al., 2006), **B** (Rombaut et al., 2007), **C** (Rombaut & Dewettinck, 2006), **D** (Yao et al., 2016), **E** (Castro-Gómez et al., 2014), **F** (Zancada et al., 2013), **G** (Contarini et al., 2013), **H** (Rodríguez-Alcalá & Fontecha, 2010), **I** (Park, 2009), **J** (Barry et al., 2016), **K** (Fong et al., 2013b), **L** (Ferreiro et al., 2016), **M** (Lambert et al., 2016)

Table 1.2 Phospholipid species distribution (g/100 g PL) in dairy products

Products	PE	PC	PS	PI	SM	Reference
Ewe raw milk	26.1-40.0	26.4-27.2	4.96-10.7	4.16-6.40	22.6-29.7	
Goat raw milk	19.9-41.4	27.2-31.9	3.2-14.0	4.00-9.37	16.1-29.2	A-E
Cow raw milk	23.4-46.7	19.1-33.2	2.00-9.07	3.98-8.97	17.8-29.2	
pasteurized semiskimmed	35	20.2	8.9	7.9	17	
UHT full fat	34	20.5	9.1	7.9	19.5	
UHT semiskimmed	33	22	7.9	4.8	22.9	F
UHT skimmed	38.2	19.6	9.9	5.5	16.7	
Sterilized semiskimmed	34.3	24.2	7.7	5.1	17.4	
Buttermilk (powder)	19.8	33.9	20.6	4.93	19.9	D
Buttermilk	33.5-39.5	27.7-35.5	10.3-22.8	2.40-7.20	16.7-18.3	G, H
Butterserum	27.2	29.8	7.2	10.8	24.9	G
Butteroil	20.3	50.7	ND	ND	25.9	I
Goat buttermilk	35.3	24.8	9.9	9.8	20.3	G
Goat butterserum	27.1	26.2	8.2	11.7	26.8	
Yogurt (skimmed)	31.1	19.9	7.9	6.3	24.9	
Fresh cheese	39.9	21.7	8.2	6.5	14.1	
Cheddar	38	20.3	8.5	7.7	16.3	F
Cheese whey (cheddar, mozzarella)	40.6-41.1	19	9.3	3.70-4.60	15.7-16.4	
Cream	42.7	14.6	7.2	6.8	28.6	G
Butter	31	24.7	15.3	11.9	17.1	
Infant formula (0 - 6 mo.)	23.2-29.5	25.9-35.6	5.51-10.1	10.2-19.5	13.1-26.9	J
Infant formula (0 - 3 mo.)	23.6	41.7	4.28	9.88	20.6	

ND = Not Detected. Recreated from (Pimentel et al., 2016).

Reference list: **A** (Yao et al., 2016), **B** (Castro-Gómez et al., 2014), **C** (Zancada et al., 2013), **D** (Rodríguez-Alcalá & Fontecha, 2010), **E** (Park, 2009), **F** (Rombaut et al., 2007), **G** (Contarini et al., 2013), **H** (Ferreiro et al., 2016), **I** (Barry et al., 2016), **J** (Fong et al., 2013b)

Table 1.3 Membrane filtration spectrum used in the separation of milk constituents

Pore size	Operating Pressure (MPa)	Module configuration [a]	Retained species	Dairy Product Use [b]
MF > 0.1 µm	0.01-0.2	T, MC	Somatic cells, bacteria, spores, MFG, Casein micelles	Micellar casein, Native whey proteins
UF 1-500 nm	0.1-1.0	T, HF, SW, PF	Soluble proteins, Caseinomacropeptide	WPC, WPI, MPC, β-LG, α-La
NF 0.1-1 nm	1.5-3.0	T, HF, SW, PF	Peptides, Salts (divalent cations)	Whey protein hydrolysates, Glycomacropeptide
RO <0.1 nm	3.0-5.0	SW, PF	Lactose, Salts (monovalent cations)	Deproteinized whey, Delactosed permeate

[a] T, open tubular; MC, multichannel; HF, hollow fiber; SW, spiral wound; PF, plate and frame.
[b] WPC, whey protein concentrate; WPI, whey protein isolate; MPC, milk protein concentrate; β-LG, beta-lactoglobulin; α-La, alpha-lactalbumin.
Adapted from (Pouliot, 2008)

Table 1.4 Application of ionic liquid columns for the analysis of FAMEs in food products

Column	Conditions	Detection	Sample	Ref.
SLB-IL111 (100 m x 0.25mm id, 0.20 μm df)	(a) Gradient: 150 °C (hold 60 min) to 210 °C at 10 °C min^{-1} (hold 15 min) (b) Gradient: 160 °C (hold 60 min) to 210 °C at 10 °C min^{-1} (hold 15 min) (c) Gradient: 170 °C (hold 60 min) to 210 °C at 10 °C min^{-1} (hold 15 min) (d) Gradient: 180 °C (hold 60 min) to 210 °C at 10 °C min^{-1} (hold 15 min)	FID	Milk	A
AA-IL (10 m × 0.25 mm id)	Gradient: 80 °C (hold 3 min) to 105 °C (hold 5 min) at 40 °C min^{-1}, then up to 150 °C (hold 5 min) at 5 °C min^{-1} and then up to 200 °C at 5 °C min^{-1} (hold 2 min)	FID	Milk	B
SLB-IL111 (200m× 0.25 mm id, 0.20 μm df)	Gradient: 170 °C (hold 50 min) to 185 °C at 6 °C min^{-1} (hold 35 min)	FID	Butter	C
SLB-IL59, SLB-IL60, SLB-IL65, SLB-IL111 (30 m × 0.25 mm id, 0.20 μm df)	(a) Isotherm: 220 and 180 °C (b) Gradient: 140 °C (hold 12 min) to 170 °C at 5 °C min^{-1}, then up to 240 °C at 30 °C min^{-1} (hold 10 min) (c) Gradient: 140 °C (hold 12 min) to 170 °C at 5 °C min−1, then up to 185 °C at 30 °C min^{-1} (hold 30 min)	FID, MS, VUV	Fish oil, flaxseed oil	D
SLB-IL111 (75 m × 0.18 mm id, 0.18 μm df)	Isotherm: 171 °C	FID	Margarine, olive oil, rapeseed oil, salad oil, sesame oil	E
SLB-IL111 (1D column) (30 m × 0.25 mm id, 0.20 μm df), SLB-IL59 (2D column) (1 m × 0.10 mm id, 0.08 μm df)	Gradient: 100 °C (hold 1 min) to 230 °C at 8 °C min^{-1}	FID, MS-Q-TOF	Safflower oil, linseed oil, fish oil, butter	F
SLB-IL111, SLB-IL100, SLB-IL76 (2D long column) (30 m × 0.25 mm id, 0.20 μm df)	(a) Gradient: 100 °C (hold 1 min) to 220 °C at 3 °C min^{-1} (SLB-IL100) (b) Gradient: 100 °C (hold 1 min) to 260 °C at 3 °C min^{-1} (SLB-IL111, SLB-IL76)	FID	Fish oil, butter	G

Adapted from (Fanali et al., 2017).
Reference list: **A** (Gómez-Cortés et al., 2017), **B** (Mendoza et al., 2015), **C** (Delmonte et al., 2012), **D** (Weatherly et al., 2016), **E** (Inagaki & Numata, 2015), **F** (Nosheen et al., 2013), **G** (Zeng et al., 2013)

Chapter 2: Phospholipid analysis in whey protein products using HILIC high performance liquid chromatography-evaporative light scatter detection in industry setting

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Abstract

The main objective of this work was to develop an analytical method that can be used in a dairy manufacturing facility for the quantitation of phospholipids in dairy products. Total lipids from a dairy matrix were obtained first by Folch extraction. The total lipid extract was then applied to a silica gel based solid-phase extraction column, triglycerides and other non-polar lipids were separated from the phospholipids and sphingolipids. Quantitation was performed by hydrophilic interaction high performance liquid chromatography (HILIC) coupled to evaporative light scattering detection (ELSD) using a quaternary separation method. The method was validated using a commercial whey protein phospholipid concentrate and was used to analyze phospholipid and sphingolipid composition in buttermilk, whey protein concentrate (WPC), whey protein phospholipid concentrate (WPPC) and several other dairy ingredients. This method was sensitive, reproducible, and can be used in the dairy industry as a research tool to

develop new value-added dairy phospholipid products then later as a standard protocol for quality assurance analysis of current and future products.

Introduction

Milk fat globule membrane (MFGM) is used to compartmentalize triglycerides (TG) in the secretory cells of mammary glands. This membrane consists of three layers of polar lipids (PLs) and membrane associated proteins. Fat droplets originate in the endoplasmic reticulum of the cell where they gain the first layer of polar lipids. Then once the lipid vesicles reach the apical end of the cell to be secreted as part of milk, a true phospholipid bilayer with membrane associated proteins is added to the surface as the droplet 'buds' through the cells membrane into the alveolar lumen (Rombaut & Dewettinck, 2006; Holzmüller & Kulozik, 2016).

The MFGM polar lipids include two major groups: phospholipids and sphingolipids (Dewettinck et al., 2008). The major PLs of importance in the dairy industry are glucosylceramide, lactosylceramide, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) (Rombaut et al., 2005). The nature of these polar lipids is amphiphilic which allows them to act as emulsifiers between the fat droplets and aqueous serum phases of milk (Contarini & Povolo, 2013). It is because of this functional property that PLs have been of interest to the dairy industry as a dairy based emulsifier. In addition to PL functional properties, the food industry also has great interest in associated nutritional properties and health benefits of polar lipids (Dewettinck et al., 2008; Spence et al., 2009a).

During the processing of milk, PLs are concentrated into the cream and fat dominant fractions, thus a large portion of research on dairy PL has been focused on cream, or buttermilk and buttermilk products (Astaire et al., 2003; Fong et al., 2007; Spence et al., 2009; Gallier et al.,

2010; Le et al., 2011; Barry et al., 2017; Bourlieu et al., 2018). During the cheese making process, lipid droplets in milk are disrupted, which results in MFGM fragmentation and the transfer of PL into whey. The PL species in whey products were investigated by (M. N. Vaghela & Kilara, 1995), Boyd et al. (1999), and Levin et al. (2016).

The dairy industry has made great efforts to isolate and concentrate PL from various dairy processing streams, including cream washing and centrifugation (Holzmüller and Kulozik, 2016), membrane filtration, supercritical fluid extraction (Astaire et al., 2003; Spence et al., 2009b) enzymatic hydrolysis of proteins and combinations of the formers (Barry et al., 2017b). Research and development efforts are continuing to develop commercial processes for PL enriched ingredients and products. In all of these endeavors, sensitive and reliable analytical methods are needed for PL determination.

Many analytical methods for the quantitation of PL have been developed over the years including TLC-densitometry (Bitman and Wood, 1990), high performance liquid chromatography (HPLC) (Rombaut et al., 2005; Russo et al., 2013), mass spectrometry (Donato et al., 2011; B. Fong et al., 2013b), and ^{31}P nuclear magnetic resonance (NMR) (Donato et al., 2011; B. Fong et al., 2013b). TLC-densitometry is a semi-quantitative method at most, and it cannot be used for quality control purposes. Use of ^{31}P NMR for detection of PL is an excellent research tool, however, it is a specialized technique not viable in a dairy industry setting due to high maintenance cost and required expertise. Likewise, mass spectrometry is expensive, high maintenance and requires expertise to operate.

The dairy industry needs an analytical procedure for PL detection using common techniques already present in the day-to-day routine testing laboratory. The technique of HPLC is already implemented in the dairy industry, specifically among whey processors, for the

analysis of proteins and milk sugars. Many published HPLC methods in literature are difficult to recreate and apply directly as plug-and-play solutions for beginning analysis of PL in an industry setting. These methods normally requiring some degree of modification for acceptable results on the specific system being used. Additionally, methods such as those used by Spence et al. (2009a) involve the heavy use of chloroform which is uncommon in dairy industry laboratories, poses new hazards in the laboratory environment, and requires additional chemical waste disposal.

In this study, an analytical method was developed and validated for the analysis of PL in multiple dairy products applicable to an industry setting, whilst minimizing the requirement for halogenated solvents. Chloroform extraction of lipids is a valuable tool providing quantitative recovery that we chose not to exclude from the study, however modification to reduce solvent demand was a necessary achievement. The methods presented here include solvent extraction of PL from several dairy matrices, sample purification, and analysis by high performance liquid chromatography coupled with evaporative light scattering detection (HPLC-ELSD) including intermediate precision conducted among several industry scientists. This method can be easily implemented in the dairy industry for product development and quality control purposes.

Materials and Methods

Chemicals and Reagents

Chloroform, 2-propanol, methanol, n-hexane, and diethyl-ether were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and were of HPLC grade. Phospholipids PG, PI, PE, PS, PC, and SM were purchased from Sigma-Aldrich Chemical Company. Ammonium formate, and acetonitrile were of chromatographic grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Whey protein concentrate 34 (WPC34) and Whey protein phospholipid concentrate (WPPC) samples were supplied by Agropur Dairy Cooperative (Appleton, WI) and meet American Dairy Product Institute (ADPI) standards. Extra grade buttermilk powder was purchased from Dairy America (Fresno, CA), liquid refined soy lecithin and de-oiled soy lecithin powder were purchased from The Solae Company (St. Louis, MO), de-oiled sunflower lecithin powder was purchased from Sternchemie Lipid Technology Company (Hamburg, Germany).

Lipid Extraction

Lipids were extracted from samples using the Folch method (Folch et al., 1957), modified for a smaller volume. Samples were weighed to load 0.1 g of fat in the extraction based on total fat content determined by the Mojonnier method (AOAC 989.05) or material composition data provided by manufacturer for commercial samples. A 60 µL aliquot of 25 mg/mL phosphatidyl glycerol (PG) in chloroform – methanol (2:1, vol/vol) was added to the sample as a surrogate standard. An aliquot (1.8 ml) of 125 mM sodium chloride solution was added and mixed on a laboratory vortex until uniform. A 12 mL volume of chloroform – methanol (2:1, vol/vol) was added to the mixture and vigorously mixed. The suspension was centrifuged at 2000 x g for 10 min at room temperature. The bottom layer (chloroform layer) was removed and reserved. Two subsequent extractions were performed by adding 8 mL of chloroform to the top aqueous layer, removing the bottom organic layer after each extraction and pooling with other organic phases. Pooled extracts were evaporated using a vacuum centrifuge and the residues were re-dissolved in 4.5 g of chloroform – methanol (2:1, vol/vol), noting the use of solvent mass to compensate for the high density and potential pipetting errors between scientists in an industry setting.

Separation of Lipids with Solid Phase Extraction (SPE)

SPE clean-up of polar lipids was performed using the method of Avalli & Contarini (2005) to remove non-polar lipids. A silica SPE cartridge with 1 g bed weight and 6 mL capacity was used in this study (Supelclean LC-SI, Sigma-Aldrich). The SPE cartridge was conditioned with 4 mL hexane. Raw-lipid extract (0.5 mL) was loaded on the cartridge at a concentration of 30 mg/mL. Non-polar lipids were eluted with 3mL of hexane – diethyl ether (8:2, vol/vol) followed by 3mL hexane – diethyl ether (1:1, vol/vol). Phospholipids were eluted with 2 mL methanol followed by 2 mL of chloroform – methanol – water (3:5:2, vol/vol/vol). Collected phospholipid solution was evaporated under a stream of nitrogen. Phospholipids were re-dissolved in 0.5 mL chloroform – methanol (2:1, vol/vol) before injection on HPLC.

HILIC HPLC-ELSD Analysis of Phospholipids

Extracted phospholipids were analyzed using an Acquity UPLC H-Class Plus system equipped with a quaternary pump, CH-A column heater, FTN Sample Manager, and an Acquity Evaporative Light Scatter Detector (Waters, Milford, MA). The analytical column was an Ascentis Express Silica HILIC LC column with dimensions of 150 mm x 2.1 mm, 2.7 µm bead, and 90 Å pore size purchased from Sigma-Aldrich. The method was run at 0.3 mL/min column flow with a column temperature of 55°C and injection volume of 6 µL. Samples were maintained at 20°C in the auto-sampler. Separation of phospholipids was performed using a quaternary, multi-step gradient in Table 2.1. Solvent A consisted of 180 mM ammonium formate, solvent B was isopropanol, solvent C was methanol, and solvent D was acetonitrile. Evaporative light scattering detector conditions were set at 70°C drift tube temperature, 40 psi nitrogen nebulizer pressure, signal gain setting of 50, and 12°C nebulizer temperature.

Mixed calibration standards were prepared in chloroform – methanol (2:1 vol/vol) by diluting from individual phospholipid standard stock solutions (25 mg/mL PG, PE, PS, PC, SM, and 20 mg/mL PI). PG was selected as a reference for the method and was prepared the same as the other chemical standards. Quantitation was performed by a quadratic regression curve of ELSD response vs. PL concentration. Calibration standards were prepared daily from stocks stored at -20°C.

Method Validation Procedures

Method validation including analyte spiking and recovery, injection stability, and intermediate precision were performed. Phospholipid standards (PI, PE, PS, PC and SM) in chloroform – methanol (2:1, vol/vol) were spiked into WPPC before lipid extraction. In addition, recovery of the SPE process was analyzed by spiking pure phospholipid standards (2.83 mg/g PI, 3.52 mg/g PE, 3.60 mg/g PS, 3.63 mg/g, and 2.92 mg/g at sample matrix concentration) into the lipid extract of WPPC in chloroform – methanol (2:1, vol/vol). Due to the volatility of solvents used in preparation of phospholipids, injection stability was analyzed using statistical control charts (I chart, MR chart) according to guidelines set by the ASTM (ASTM International, 2016). A mid-level mixed phospholipid standard with 0.39 mg/g PI, 1.04 mg/g PE, 1.25 mg/g PS, 0.51 mg/g PC and 0.51 mg/g SM in chloroform – methanol (2:1, vol/vol) was injected on the HPLC consecutively for 6 hours and 40 minutes equaling a total of eighteen repeated injections. Intermediate precision analysis was performed to include multiple calibration curves (n=2) and sample preparations (n=3) of WPPC by three analysts each day, over two days.

Results and Discussion

HPLC Calibration and Limits

A chromatogram of a typical phospholipid calibration standard injection is shown in Figure 2.1. Quantitation of phospholipids was performed using a quadratic regression curve ($R^2 > 0.999$) of detector response (peak area) vs. concentration over the quantitation range used in this study. All analytes of interest are baseline resolved and elute within 13 min. The additional time in the method (gradient program t=12.60 to t=25.00) shown in the chromatogram as baseline signal after the elution of SM is required to equilibrate the silica column for reproducible immediate subsequent injections.

The upper limit of quantitation (ULOQ) is arbitrarily set to 125% of the highest phospholipid calibration standard concentration for each standard. Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined by the signal-to-noise (S/N) ratio method (Snyder, 1997) using the S/N of phospholipid chromatographic peak regions in a blank injection. Thresholds of three-times and ten-times the S/N were used to determine LLOD and LLOQ respectively for each PL, these results are presented in Table 2.2.

Spiking and Recovery

The potential loss of PL during sample preparation was monitored at the two major extraction steps by quantifying the PL recovered from the Folch extraction and the SPE clean-up. Table 2.2 reports the recoveries of each PL species from the two extraction steps. The lowest recovery was seen in PS during the SPE clean-up (89.3%). All other values exceed 90% recovery and we conclude that the Folch and SPE procedures are suitable for the purification of PL from dairy matrices. The SPE method presented here is useful for the removal of non-polar lipids and thus a concentrating step for the PL, however, it was not a necessary step for

successful PL quantitation. Combinations of Mojonnier or Folch extractions with SPE methods including the Avalli method used in this study have been compared in the literature (Gallier et al., 2010). It was concluded that the cold operating conditions of Folch are preferred to Mojonnier, which uses a strong base and direct heat, for the quantitation of PL (Gallier et al., 2010). The Folch method used in this study was modified to minimize the required halogenated solvents, while still delivering quantitative lipid recovery from samples of varying protein and fat contents.

Intermediate Precision

Intermediate precision is the measure of precision within a laboratory using defined methods/procedures. For this study intermediate precision was conducted using the methods above among three industry scientists, exact procedures were prepared in protocol format and distributed to the scientists in advance of conducting the experiment. Measurements were made in triplicate across two days using the same sample of WPPC. Acceptable limits were set at 10% RSD for detection of each PL analyte among the scientists. Reproducibility within the target RSD was met for the detection of all analytes. The resulting RSD for the PL analytes were: 8.4% for PI, 4.7% for PE, 3.6% for PS, 5.4% for PC, and 10.2% for SM.

Phospholipid Sample Stability

To assess the ability of the method to report results that are stable throughout an analysis by HPLC individual value (I) and moving range (MR) control charts were used to monitor the stability of injections of a single phospholipid calibration standard mixture over 6 hours and 40 minutes (data not shown). Control limits were set using the guidelines of ASTM E2587 (ASTM International, 2016). Across the total data collection period signal response for each analyte was monitored to determine the point at which response moved outside of control limits. This would suggest a loss of stability due to factors other than random chance. The prepared sample was

observed to remain stable for 5h 35min including sample preparation time (estimated as 30min). It is recommended that samples prepared using this method be prepared quickly and analyzed within the observed stability time of 5h 35min. This allows for analysis of five to six samples after calibration standards before loss of PL stability. If analysis cannot be completed within the recommended time samples should be stored at -20°C or lower until analysis.

Phospholipid Quantitation of Production and Commercial Products

To demonstrate the capability of the method for determining the PL content of varying dairy products for new product development and quality assurance, the analytic method was applied to buttermilk powder, WPC34 and WPPC. Buttermilk powders were used to challenge the method with a dairy-based high fat matrix, and WPC34 was selected for its high lactose content. The WPPC product was selected to challenge the method with a high protein content matrix, while also being enriched in PL.

Plant based lecithin products were also tested to challenge the method with another high lipid matrix, specifically high in PL content. The ability of measuring PL content of plant-based lecithin and dairy PL enriched streams using the same procedures is of great interest to the dairy industry for future applications in production of a dairy-based lecithin equivalent. Plant lecithin products are used widely in the dairy industry for a variety of products including instantized dairy powders and ice cream. Being able to source PL from under-utilized dairy process streams would allow the dairy industry to become more independent of plant-based lecithin and have a cleaner label by removing an allergen.

As expected, the sampled dairy products had higher levels of SM, widely understood to be more prevalent in animal lipids, compared to the plant lecithin products. In general, the PL profiles for the dairy products (Table 2.3) have similar percent composition among PL species.

Buttermilk powder was found to have 1.71±0.03% PL by mass which agrees with the total PL value reported by Barry et al. of 1.30% w/w (Barry et al., 2017a). Spence et al. report a higher value of 2.2% PL for regular cream buttermilk (Spence et al., 2009b).

The analysis of PL in WPC products is less common in literature, however, Vaghela and Kilara (1996) studied the effects of production variables on PL composition in several WPCs. The control production (non-variable/untreated sample) of WPC 35 and 75 were reported to contain 13.49±0.47% and 13.35±0.40% PL of total lipid mass respectively (Vaghela and Kilara, 1996). These reported values differ greatly from the value reported in this study for WPC34 (1.93±0.12% of total lipid mass, Table 2.3). The exact reason for this disagreement was not investigated in this study but is hypothesized to be caused by major differences in production between the samples of WPC, specifically lower protein content concentrates. WPC34 (or WPC35) can be made differently between processors, and in some cases low protein concentrate products are often back blended from effluent of other processes to meet the minimum required composition of the product identity.

The largest concentration of PL in a dairy matrix in this study was seen in WPPC (8.5±0.5%, Table 2.3). This matrix is the most promising source of PL from dairy and has been investigated by Price et al. (2018) and Levin et al. (2016). Price et al. report a PL content of 29.1±0.7% PL of the total fat content of WPPC (wt/wt), total fat reported as 5.5% by Folch extraction (Price et al., 2018). Levin et al. report a range of PL contents from four different WPPC from four suppliers of 0.57-0.74% PL of total fat content (wt/wt) (Levin et al., 2016). This large variance of PL content in WPPC products again is most likely due to differences in production. PL content is not a requirement of any whey product standard of identity established by ADPI. However, the differences in PL composition seen in literature support the need for an

industry applicable method for PL analysis to better understand the additional value products have from PL content.

Conclusion

The method presented in this study allows for sensitive, reproducible detection of PL in a variety of dairy matrices. The combination of HILIC separation and small scale Folch extraction was successful in reducing the halogenated solvent requirements for analysis. Acceptable reproducibility was seen in the intermediate precision experiment and is expected to improve as the method continues to be used. These procedures are suitable for the dairy industry and are currently being used to research PL composition throughout a cheese-whey process flow. This method will allow for new insights into dairy product composition and the production of new value-added ingredients.

Acknowledgements

Table 2.1. HPLC-ELSD gradient program for the analysis of polar lipids.

Time (min)	Step Detail	% Solvent A	% Solvent B	% Solvent C	% Solvent D
0.00	Initial	5.0	19.0	3.0	73.0
1.00	Hold	5.0	19.0	3.0	73.0
1.50	Gradient	5.0	30.0	4.0	61.0
3.00	Gradient	5.0	34.0	4.5	56.5
5.00	Gradient	8.0	37.0	4.5	50.5
8.50	Gradient	15.0	39.0	4.5	41.5
10.00	Gradient	15.0	50.0	4.5	30.5
12.50	Hold	15.0	50.0	4.5	30.5
12.60	Equilibrate	5.0	19.0	3.0	73.0
25.00	End	5.0	19.0	3.0	73.0

Liquid chromatography solvent gradient program used for the analysis of PL on a silica column (150mm x 2.1mm, 2.7µm). Column flow of 0.3 mL/min, column temperature of 55°C, and auto sampler temperature of 20°C.

Solvent A: 180 mM ammonium formate, Solvent B: isopropanol, Solvent C: methanol, Solvent D: acetonitrile

Table 2.2. HPLC-ELSD method validation data

Phospholipid	Mean[1]	RSD[2]	LLOD[3]	LLOQ[3]	ULOQ[3]	Recovery 1[4]	Recovery 2[5]
PI	3.5	8.4%	0.11	0.35	1.48	104.1%	100.5%
PE	30.2	4.7%	0.26	0.87	3.90	109.8%	96.7%
PS	9.3	3.6%	0.22	0.73	4.68	94.4%	89.3%
PC	24.0	5.4%	0.11	0.37	1.89	95.5%	108.5%
SM	18.3	10.2%	0.06	0.20	1.90	101.1%	111.0%

[1]Mean in units of mg/g in raw WPPC. (N=18)

[2]RSD relative standard deviation from method intermediate precision of WPPC. (N=18)

[3]Limits reported at test concentration in units of mg/g.

[4]Recovery 1: PL recovery through the Folch lipid extraction sample preparation process. (N=3)

[5]Recovery 2: PL recovery through the SPE only sample preparation process for lipids extracted from WPPC. (N=3)

Table 2.3. Phospholipid content (±SD) of commercial dairy products and plant-based lecithin products.

Product	Total PL %	% PI	%PE	%PS	%PC	%SM
BMP	1.71±0.03	7.9±0.6	25.3±1.1	17.2±0.8	34±3	15.1±1.3
WPC 34	1.93±0.12	5.53±0.06	23.0±0.7	15.8±1.9	35.8±1.6	20±2
WPPC[1]	8.5±0.5	4.1±0.2	35.4±0.6	11.0±0.7	28.2±0.7	21.4±1.2
LSL	36.2±0.8	18±2	35.5±1.0	3.0±0.3	41±3	1.94±0.17
PSL	48.4±1.6	22±2	16.1±0.7	3.5±0.5	55±3	2.7±0.4
SunL	56±2	18.7±1.9	25.4±1.9	2.3±0.5	51±4	2.5±0.3

Total PL % reported as %PL of total sample mass, PL species reported as % of total PL content.

(N=3)

[1]WPPC results are from the larger intermediate precision study. (N=18)

BMP buttermilk powder, WPC 34 whey protein concentrate 34, WPPC whey protein

phospholipid concentrate, LSL liquid soy lecithin, PSL powder soy lecithin, SunL powder

sunflower lecithin.
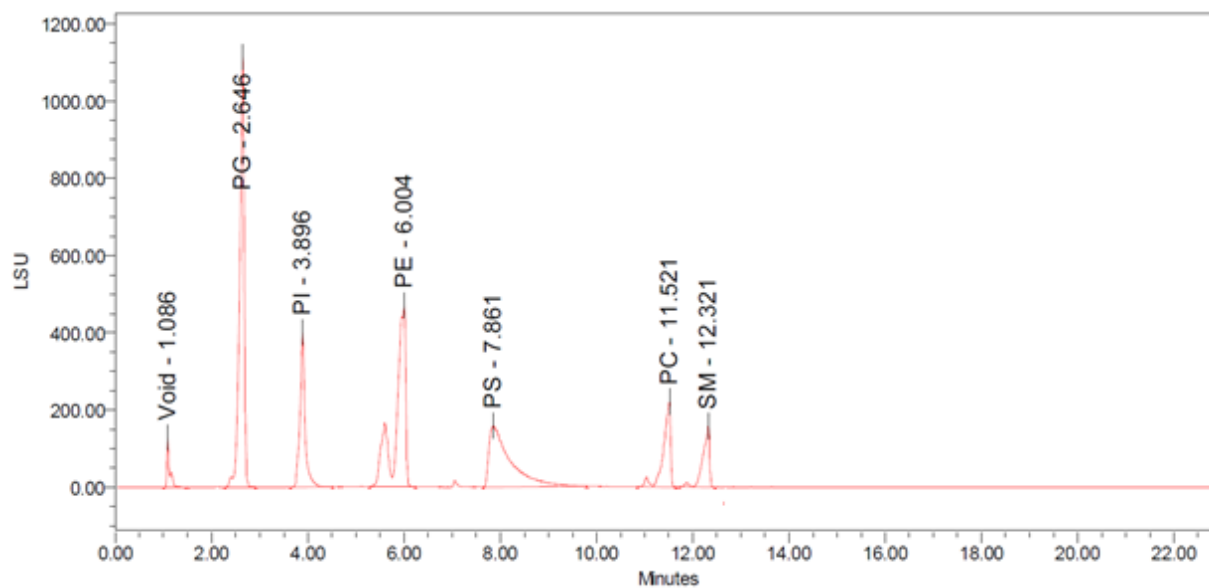
Figure 2.1. Typical chromatogram of PL separation using HILIC quaternary solvent gradient.

PG phosphatidylglycerol, PI phosphatidylinositol, PE phosphatidylethanolamine, PS

phosphatidylserine, PC phosphatidylcholine, SM sphingomyelin.

Chapter 3: Direct ethanolic extraction of polar lipids and fractional crystallization from Whey Protein Phospholipid Concentrate

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Abstract

Polar lipids (PL), including sphingomyelin (SM) and other sphingolipids, have nutritional implications in many important metabolic pathways. Milk and whey are a valuable source of these bioactive lipids differing in SM content compared to other plant-based lecithins in the food industry. Dairy lipids were extracted from whey protein phospholipid concentrate (WPPC) powder by direct exposure to food-grade solvent, ethanol. A total of 20 mass equivalents of solvent were required to completely recover all lipid material of the original WPPC sample. Using a minimal apparatus, absolute ethanol, and heating to a boiling point a final total lipid extract composed of 37.7% PL by mass was achieved. The method was developed with potential pilot-scale and industry application in mind. Investigations into fractional crystallization showed limited success at purifying PL from triglyceride content, <1% increase in % PL by mass. However, the relative percentage of SM was increased in the fractionated sample compared to the total lipid extract. Fatty acid analysis of the PL fraction reported a composition of 33.3% monounsaturated fatty acids (MUFAs) and 9.8% polyunsaturated fatty acids (PUFAs). Additional work is needed to investigate different conditions for lipid recovery, PL recovery, and PL purification by fractional crystallization in ethanol.

Introduction

Polar lipids are a valuable nutraceutical and serve a critical role in the food industry as a functional ingredient. Polar lipids, including phospholipids (PL) and sphingolipids (SL), are

found in dairy as organized membrane structures surrounding fat globules (Lopez et al., 2014; Singh & Gallier, 2017). The milk fat globular membrane (MFGM), containing the polar lipids and bioactive proteins, has evolved to benefit infant development. Polar lipids are required for many aspects of infant development, including organ growth and cellular replication (Cilla et al., 2016) and neurobehavioral development (Tanaka et al., 2013). Additional studies have shown the health benefits of milk polar lipids in adults, including significantly reducing lipid cardiovascular markers with reducing cholesterol absorption (Vors et al., 2019).

Attempts at industry scale isolation of MFGM components, including cream washing, ultracentrifugation, and membrane filtration, have been reviewed by Holzmüller & Kulozik (2016). Many of these methods focus on protein components. Several processes have been used in the attempt to recover or enrich PL from MFGM: butter-milk cream churning (Haddadian et al., 2018), ultrafiltration and supercritical fluid extraction (Costa et al., 2010; Spence et al., 2009b). Polar lipids in MFGM have been recovered with the application of chelating zinc salts (Damodaran, 2010; Price, Fei, et al., 2020). The use of food-grade solvent, specifically ethanol, for the extraction of PL from dairy products has also shown potential industry application (P. Castro-Gómez et al., 2016; Price et al., 2018). Price et al. (2018) pumped reconstituted whey protein phospholipid concentrate (WPPC) into hot ethanol, causing lipids to dissolve into the ethanol phase and recovered after solvent evaporation.

The potential of ethanol as a food-safe solvent for the extraction of polar lipids may offer the dairy industry a new technology requiring limited or no specialty equipment. The fractional crystallization process allows for the separation of mixtures based on solubility and could potentially isolate polar lipids from TAGs with different solubilities in ethanol. Presented in this communication is a novel method for ethanol extraction of polar lipids from WPPC by direct

extraction of the powdered product that is commercially available from the dairy industry. Initial investigations into fractional crystallization of milk fat as a potential method for polar lipid purification from ethanol extracts are also described.

Methods

Ethanolic extraction was conducted in a reflux apparatus directly mixing WPPC (Hilmar 7500, Hilmar Ingredients, Hilmar CA) with absolute ethanol. Preliminary trials for this study were used to determine a ratio of dairy powder to extraction solvent that yielded satisfactory results. The amount of solvent for extraction will be referred to in mass equivalents (mass eq.) of the solid sample used. A final ratio of 1:20 sample-solvent (w/w), or 20 mass eq., was used in this study with 100g WPPC powder for each replicate.

Extraction was completed in 4 cycles, the first cycle used 8 mass eq. of absolute ethanol and the next three subsequent cycles used 4 mass eq. of solvent each. The additional solvent used in this first cycle acts to compensate for the volume of solvent required to wet the surface of the solids and allow for a reasonable supernatant layer to form. The extraction mixture was heated under reflux with magnetic stirring just until the first sight of boiling was achieved in ≤10 min. At which point the flask was removed from heat, boiling allowed to subside, and solids precipitated (approx. 3 min).

While still hot, the ethanol solution was decanted away from solids and vacuum filtered through Whatman 1PS silicone treated filter paper. The flask was returned to the apparatus and refreshed with solvent (4 mass eq.). This cycle of heating, gravity precipitation, decanting/new solvent addition was repeated for 4 cycles, and a total of 20 mass eq. of solvent were used. At the end of the final cycle, the entire reaction mixture is transferred to the filter apparatus, and vacuum applied until solids were dry. The recovered solvent decreased in color intensity as

extraction cycles proceeded, this change in color was used as a qualitative indicator that extraction was complete.

The collected filtrate was concentrated by rotary evaporation under a high vacuum until completely dry. The final mass of total lipid extract was measured gravimetrically, and percent lipids recovered was calculated using the manufacturer provided compositional data. Recovered lipids were transferred to vials for storage and frozen at -20°C until further analysis.

The potential for PL purification and removal of TG content using fractional crystallization was also investigated. Fresh extraction replicates were prepared as described previously until the drying step, where the solution was concentrated to a final mass of 400g for crystallization. At this final mass, the resulting solution was at a concentration of approximately 4.75% lipid w/w, using the average total lipid extract mass from the previous experiment. Fractional crystallization of the final 4.75% w/w solution was done in a circulating water bath at 15°C for 16h.

The crystallization mixture was removed from the water bath, filtered, and rinsed with cold absolute ethanol. The filtrate was dried by rotary evaporation as described previously, and the filtered crystal solids were left to dry on the bench top before both lipid residues were transferred to vials and stored at -20°C. The crystallized solids were observed to be white and fluffy with smaller yellow waxy precipitated solids. The expected PL concentrate residue recovered from the filtrate was an orange/dark yellow viscous liquid at room temperature compared to the total lipid extract which presented as a solid of similar color (Figure 3.1).

Phospholipid composition of the total lipid extract and fractionated polar lipids was determined by HILIC HPLC-Evaporative Light Scatter Detector (ELSD) (Table 3.1) using the method published previously (Ferraris et al., 2020). Residues were assumed to be 100% lipid

material; therefore, no further extraction or sample preparation was performed before dilution for analysis. An external standard calibration curve was used for quantitation using polar lipid standards, including phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM) purchased from Sigma Aldrich (St. Louis, MO).

Fatty acid profiles of all extracts were determined by FAME preparation using methanolic sulfuric acid (1% v/v) according to Christie & Han (2003). GC-FID/MS was used for analysis with an IL-111i (60m x 0.25mm, 0.20 μm) ionic liquid stationary phase capillary column (Sigma Aldrich, Inc.). An oven program of 100°C for 5min, 8°C/min to 180°C, 5°C/min to 260°C hold 5min was used with H_2 carrier at a flow rate of 1.5mL/min. FAME standards were purchased from NuChek Prep (Elysian, MN). The identifications of FAME standards made by Delmonte et al. (2013) using an IL-111i column (200m) in 2D GC-MS was used for reference in determining the elution order of fatty acids in the CLA region in combination with mass spectral data (Table 3.2).

Results and Discussion

The WPPC used in this study has a reported total fat content of 16.24% from the manufacturer. The total lipid extract residue recovered represented 19.03±0.13% of the original sample mass. The assumption was made in the study that the TLE residue was entirely lipid material, which compared to the manufacturer certificate of analysis yields an extraction recovery of 117%. This discrepancy has not yet been formally investigated here but is hypothesized to be the co-extraction of other ethanol-soluble components such as water, lactose, and annatto pigments.

Price et al. (2018) have already shown that higher concentrations of ethanol allow for better separation of lipids from proteins resulting in higher yields and marginal change lipid recovery over temperatures of 70°C. The highest recoveries of total PL were reported using 90% ethanol and 70°C. This information was used in our experimental design, selecting absolute ethanol as an available pre-standardized solvent and ethanol's boiling point as a visual indicator of temperature.

The major PLs detected in the ethanol extracts were PE, PC, and SM respectively. This result agrees with the WPPC lipid composition extracted by the Folch method. The fractionated PL residues showed a small increase (<1%) in total PL content by mass compared to the total lipid extract. This effect of polar lipid isolation during the fractional crystallization process was not as significant as was hypothesized under the conditions used. Interestingly, PI and PS show a substantial decrease in concentration in fractionated PL residue after the fractional crystallization process suggesting co-crystallization with TG into the crystallized lipid residue. The relative percentages of PE and SM were increased substantially. The result indicated the fractionation process could be used to enrich SM.

Milk fat solubility in ethanol and fractional crystallization in organic solvents are not well reported in the literature. However, the fractional crystallization of milk fat in acetone has been reported using butter-fat at a concentration of 10% w/v and temperatures beginning at 15°C (Chen & deMan, 1966). Other research using acetone/ethanol mixtures were applied to pili nut oil to create differing melting point fractions at 10% w/v and freezing temperatures (Kakuda et al., 2000). Higher lipid concentrations were not used in this study due to crystal formation observed during rotary evaporation. Further research is needed to determine the effects of

concentration and temperature on the crystallization of milk fat in ethanol to assess the utility of fractional crystallization for PL purification.

Fatty acid composition analysis of the ethanol extracts reported a saturated—unsaturated ratio of 1.9, 1.6, 2.8 for total lipid extract, fractionated PL, and crystallized lipid residues, respectively (Table 3.2). A higher percentage of saturated FA were found in total lipid extract and crystallized lipid samples (60.8% and 69.2% respectively). In comparison, the fractionated PL residue contained higher levels of monounsaturated fatty acids (MUFA, 33.3%) and polyunsaturated fatty acids (PUFA, 9.8%). Two fatty acids appear to fractionate between the PL residue and crystallized lipids, being detected in one fraction and not the other, C14:1 and C15:0 respectively.

Conclusion

Overall, the ethanol extraction method of polar lipids directly from powdered WPPC was successful while avoiding specialized equipment and considerable dilution of other food-grade solvent extraction methods. Further investigations into the presence of other co-extracted whey components would allow for increased yield accuracies. While the conditions used for fractional crystallization of the total lipid extract have yet to support the current utility of crystal purification of polar lipids in ethanol (<1% increase in total PL), the research proved the concept of ethanol extraction and fractional crystallization for PL extraction and SM enrichment.

Acknowledgements

Table 3.1. Phospholipid composition of total lipid extract and fractionated PL residues by HPLC-ELSD

	WPPC[1]		Total lipid extract[2]		Fractionated PL[2]	
	% PL by mass[3]	% of total PL	% PL by mass[4]	% of total PL	% PL by mass[4]	% of total PL
PI	0.5	5.4	1.6	4.3	0.3	0.8
PE	3.5	39.9	12.8	33.9	14.5	38.5
PS	1.1	12.2	3.1	8.4	1.1	2.8
PC	2.2	25.7	11.8	31.2	12.5	33.1
SM	1.5	16.7	8.4	22.2	10.2	27.1
Total % PL by mass	8.7	N/A	37.7	N/A	38.6	N/A

[1]WPPC lipid extracted using the Folch method as described in (Ferraris et al., 2020) (n = 1). Crude fat of product was 16.24% from manufacturer certificate of analysis.
[2]Total lipid extract (n = 3) and Fractionated PL (n = 3) extracts.
[3]Reported as % PL by mass of WPPC powder
[4]Reported as % PL by of total lipid extract and fractionated PL residues respectively

Table 3.2. Fatty acid composition of Ethanol extracts[1]

Fatty Acid[2]	Total lipid extract	Fractionated PL	Crystallized lipid
C4:0	0.6	0.6	0.2
C6:0	0.5	0.6	0.1
C8:0	0.4	0.5	0.2
C10:0	1.5	1.6	1.0
C12:0	2.2	2.2	1.8
C14:0	8.1	8.2	7.8
C15:0	0.4	N.D.	1.0
C14:1	0.8	1.6	N.D.
C16:0	28.8	28.5	29.9
C16:1	0.7	1.0	0.5
C17:0	0.9	0.7	0.9
C18:0	15.5	11.7	25.1
C18:1	27.5	29.7	21.5
C18:2	7.4	7.9	5.9
C20:1	0.4	0.4	0.5
C18:3 (CLA)	0.3	0.4	0.2
C20:2	0.2	0.4	0.2
C22:0	1.1	1.4	1.0
C22:1	0.3	0.4	0.7
C20:3+C20:4[3]	0.8	0.8	0.7
C24:0	0.7	0.9	0.4
C24:1	0.1	0.2	0.1
C20:5	0.1	0.1	0.1
C22:4	0.2	0.2	0.3
C22:5n3	0.2	0.2	0.3
Total Sat. (%)	60.8	56.9	69.2
Total Unsat. (%)	31.8	35.2	24.9
Sat/Unsat ratio	1.9	1.6	2.8
MUFA (%)	29.9	33.3	23.2
PUFA (%)	9.3	9.8	7.6

[1]Individual fatty acid values reported on wt% basis of total FAs.
[2]Fatty acids listed in chromatographic elution order on IL111i
[3]Fatty acids C20:3 and C20:4 could not be resolved on 60m column.
N.D., not detected

Figure 3.1. Ethanol extraction at several step of process
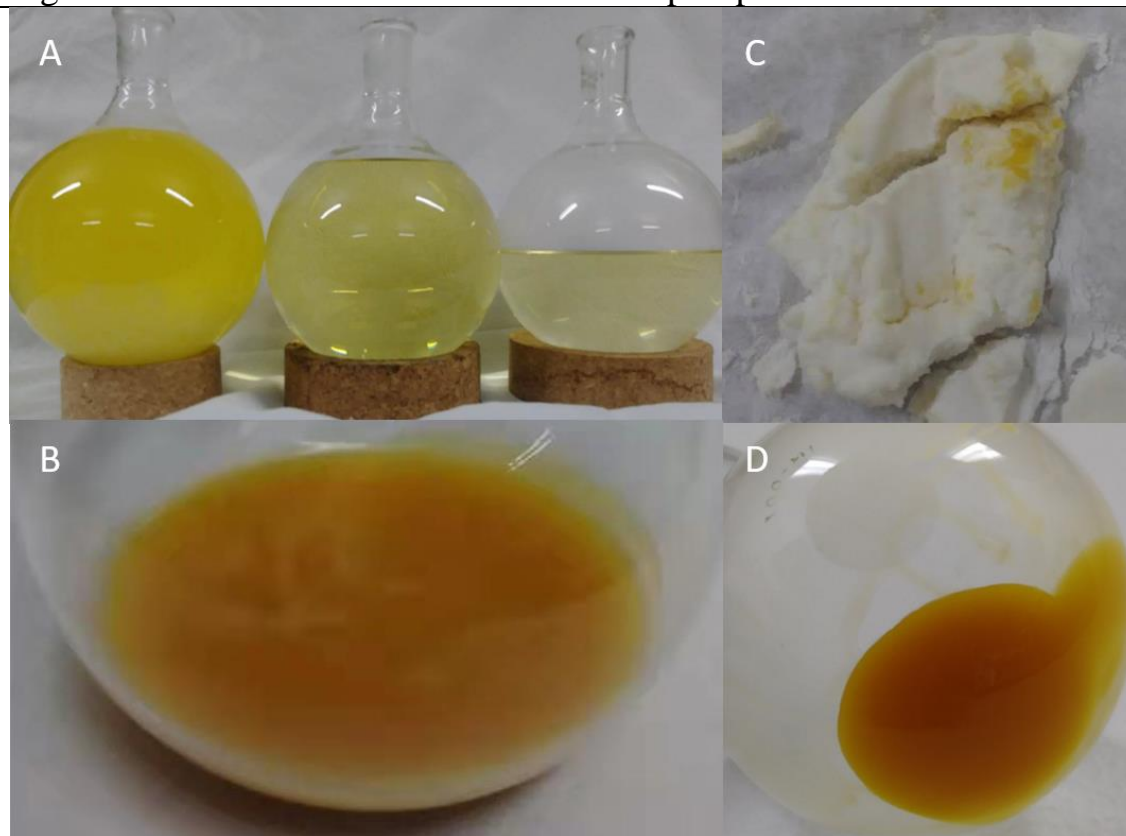
A: Total volume of ethanol solution recovered during total lipid extraction using 20 mass eq.
B: Resulting total lipid extract residue, appears as a solid at room temperature.
C: Recovered crystallized lipids from fractional crystallization process at 15ºC for 16h, yellow waxy precipitated solids present in white crystalized lipid solids.
D: Final fractionated PL residue, appears as a viscous liquid at room temperature.

Chapter 4: Profiling polar lipids in Whey Protein Phospholipid Concentrate by LC-HRMS/MS

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Abstract

Bioactive polar lipids present in the milk fat globular membrane can be recovered and enriched into a co-product of whey protein processing called whey protein phospholipid concentrate (WPPC). A food-grade solvent successfully extracted polar lipids from powdered dairy products, and lipids can be fractionated under temperature induced crystallization. This study investigates the specific lipid species present in ethanol extracted lipid residues from commercially available WPPC using a UPLC-Q-TOF-MS/MS lipidomics method. In general, sphingomyelins and phosphatidylcholines were retained in the polar lipid enriched fraction. Sphingomyelin was found to be a rich source of long chain fatty acids.   Several glycosphingolipids, glucosyl-, galactosyl-, lactosyl-, and galabiosylceramide, were also detected in WPPC; these species were observed to crystallize away from other polar lipids during fractionation. Correlation analysis supported the claim that majority of polar lipids recovered in a total lipid extract using ethanol were retained in a polar lipid enriched residue after fractional crystallization.

Introduction

Milk is the first source of nutrition for mammalian neonates, a biological matrix evolved to provide critical nutrients including protein, fats, and carbohydrates as well as initial immune response antibodies. The majority of milk fat consists of acylglycerols, largely triglycerides found in lipid droplets suspended in the milk. The triglycerides are in the center of a globular

structure resulting from the secretion of the lipids from mammary epithelia (Roeland Rombaut & Dewettinck, 2006). This milk fat globule is enclosed by a tri-layer membrane made of bioactive proteins and polar lipids (PL), referred to as the milk fat globular membrane (MFGM) (Lopez et al., 2014). In addition, the proteins and phospholipids of the MFGM stabilize the emulsion of the lipid core into the aqueous phase of milk (Singh & Gallier, 2017). Because of these emulsifying properties and the specialized nutrition attributed to the bioactive proteins and PL, MFGM has been a research target.

As a functional ingredient, polar lipids are used widely in the food industry as emulsifiers characterized by their amphiphilic structure of a polar head group and a nonpolar tail. The phospholipid emulsifiers common to the food industry are sourced from soybeans, oil seeds (rapeseed, canola, cottonseed, and sunflower), egg yolk, and milk and referred to as lecithins (McClements et al., 2017). It is a regular practice within the dairy industry to add soy and sunflower-based lecithins to powdered milk and whey products to improve solubility forming a commercial instantized product.

During dairy processing, MFGM is concentrated in the fat fraction, thus fat fractions have been selected for MFGM extraction. A large part of the literature focuses on co-products of butter manufacturing including butter serum, buttermilk, and buttermilk powder (Astaire et al., 2003; Barry et al., 2017a; Spence et al., 2009a). After the cheesemaking process, the resulting whey fluid can be mechanically separated into whey cream and whey buttermilk fractions which can also be a source of MFGM and polar lipids (Costa et al., 2010; Morin et al., 2006b; Spence et al., 2009b). The processing of whey was revolutionized with the introduction of membrane filtration technologies allowing for the production of whey protein powders, including whey

protein phospholipid concentrate (WPPC), which is itself a co-product from the production of the whey protein isolate.

While most whey products developed by the industry focus on the recovery of protein content with membrane filtration technologies, the recovery and enrichment of MFGM material on membrane platforms to form WPPC has also shown potential as a source of dairy PLs (Ferraris et al., 2020; Price et al., 2018; Sprick et al., 2019). Attempts to isolate dairy PLs expand beyond membrane filtration techniques to include supercritical fluid extraction (Costa et al., 2010; Sánchez-Macías et al., 2013; Spence et al., 2009a), enzyme hydrolysis (Barry et al., 2017a), metal ion-induced precipitation (Damodaran, 2010; Price, Fei, et al., 2020), pressurized liquid extraction (PLE) (Castro-Gómez et al., 2014) and food-grade solvent extraction using ethanol (Ferraris & Qian, 2021; K. Lee et al., 2020; Price, Wan, et al., 2020).

Beyond use as a functional ingredient, dairy PLs have value as nutraceuticals. The PLs found in milk play critical roles in infant development (Javier Fontecha et al., 2020; H. Lee, Padhi, et al., 2018), including gut microbiome and intestinal growth (Abrahamse et al., 2012; Bhinder et al., 2017), the brain and nervous system (Henriksen et al., 2021; Moukarzel et al., 2018), and neurobehavioral development (Tanaka et al., 2013). The implications of PLs in infant diets have demanded the detailed analysis of human milk and its comparison to bovine sourced ingredients for optimal infant nutrition (Cilla et al., 2016; Claumarchirant et al., 2016; Gallier et al., 2015).

Later in life, PLs have been associated with varying aspects of adult nutrition. Bioactive sphingolipids and phospholipids reduce and regulate inflammatory responses (Demmer et al., 2016; El Alwani et al., 2006; Norris & Blesso, 2017). Dietary sphingomyelin enhances the gut microbiome and lipid metabolism (Norris et al., 2016) with the effect of reducing cholesterol

uptake and improving cardiovascular health (Vors et al., 2019). Lipids found in the MFGM have been associated with anticancer effects (Castro-Gómez et al., 2016; Rodríguez-Alcalá et al., 2017).

Detailed lipidomic analysis using high-resolution mass spectrometry platforms of dairy products and production streams allow for a better understanding of the potential bioactive PL present to inform future nutritional applications and testing. Such analyses have been done on milk powders (Ali et al., 2017), buttermilk and butter serum (Bourlieu et al., 2018; P. Castro-Gómez et al., 2017), and infant formula (B. Fong et al., 2013a). WPPC has been shown to be a potential source of sphingomyelin and other PLs but is not well profiled in literature. It should not be assumed that the PL species present in WPPC are directly comparable to other dairy products due to the intense processing that occurs from raw fluid milk through the hierarchy of co-products finally resulting in WPPC. This study aims to use lipidomic methods by UPLC-Q-TOF-MS/MS to profile the PL species present in extracted lipid residues from WPPC to better understand the potential of this whey product for future nutritional application and research.

Materials and Methods

Chemicals

Formic acid ACS reagent was purchased from Fisher Chemicals (Pittsburg, PA, USA). Methanol, acetonitrile, isopropanol, and water were HPLC-MS grade, and absolute ethanol, were all purchased from EMD Millipore (St. Louis, MO, USA).

Ethanol extraction and fractionation of lipids from WPPC

The food-grade solvent extraction of lipids and subsequent fractionation using fractional crystallization techniques was done using the methods previously presented in the literature (Ferraris & Qian, 2021). Briefly, WPPC was extracted with 20 mass equivalents of absolute

ethanol in a reflux apparatus over four cycles. The extracted lipids were recovered from the ethanol solution through rotary evaporation, resulting in a total lipid extract residue (TLE) (n = 3). Replicate ethanol extractions were prepared following the same procedure as described previously.

Fractional crystallization was used to isolate polar lipids from triglycerides. Lipid extracts were concentrated to approximately 5% w/w. The lipid solution was then placed in a 15°C water bath and allowed to crystallize over 16h. Two fractions were recovered after gravity filtration, the crystallized lipids (F1) (n = 2) and the ethanol-soluble filtrate was recovered after rotary evaporation (F2) (n =2). The F1 fraction contained a yellow waxy precipitate co-crystallized in the surrounding, white-colored solid lipids. This waxy residue (wax) (n = 2) was separated from F1 and subjected to analysis separately.

UPLC-HRMS/MS lipidomics analysis

UPLC-HRMS/MS analysis was conducted in DDA mode (data-dependent acquisition) using a Shimadzu Nexera UPLC system coupled to an AB SCIEX TripleTOF® 5600 mass spectrometer (AB SCIEX, Concord, Canada). UPLC separation was performed according to Cajka et al. (Cajka & Fiehn, 2016) with some modifications. Briefly, data were recorded in negative (ESI-) and positive (ESI+) electrospray ionization modes. Analytes were separated using an Acquity CSH C18 column (2.1 x 150 mm, 1.7 µm, Waters Co). A linear gradient with two mobile phases was used. For the data acquisition in positive ion mode; phase A consisted of acetonitrile:water (60:40) containing 0.1% formic acid, and 10 mM ammonium formate; phase B was isopropanol:acetonitrile (90:10) containing 10 mM ammonium acetate and 0.1% formic acid. The elution gradient was as follows: 0 min, 15% B; 2 min, 30% B; 2.5 min, 48% B; 11 min, 80% B; 11.5 min, 100% B; 12 min, 100% B; and 12-15 min, 15% B. The

autosampler was maintained at 4 ∘C. The flow rate was 0.6 mL/min. For ESI-, the same parameters were used but without formic acid in both mobile phases.

For ESI-, voltage was set at -4500 V; source temperature was set at 550 ∘C; collision energy 35V with collision energy spread (CES) of 15 V; period cycle time was 700 ms; accumulation time 0.1 s; m/z scan range 100–1700; and. For ESI+, voltage was set at 5500 V, other parameters were the same as ESI-. Mass calibration was automatically performed after every fifth LC run. Triplicates were injected for each of the lipid fractions. QCs and blanks were injected after every six analytical runs. All other samples were randomized before injection.

Spectral data were processed using, Progenesis QI^{TM} (NonLinear Dynamics v2.4, United Kingdom). Peak deconvolution for $[M+H]^+$, $[M+Na]^+$, and $[M+NH_4]^+$ adducts in ESI+, and $[M-H]^-$, $[M+FA-H]^-$, and $[M-H_2O-H]^-$ in ESI- and was performed in Progenesis QI^{TM}. For peak annotation, deconvoluted features were screened against METLIN, HMDB, and LipidBlast databases. Tentative lipid annotations were achieved as previously reported by Magana et al. (2020). In brief, annotation confidence was established according to reporting criteria for chemical analysis suggested by the Metabolomics Standards Initiative (Sumner et al., 2007; Viant et al., 2017). Lipid annotation was considered a confident level 2 if the Progenesis score was 50 or higher. A score higher than 50 was reached when good matching against databases is achieved. A confident annotation was considered if isotope pattern similarity is greater than 70%, MS/MS match is > 50% and accurate mass is ten ppm or better (Housley et al., 2018; Magana, Reed, et al., 2020). If a molecular feature was detected in both ion modes, the one with a smaller CV in the QC samples was chosen. Relative levels of the lipid species were obtained by computing their peak area (Magana, Wright, et al., 2020). Relative percent composition of

positively identified species was determined for each replicate injection, using percentage to normalize the data with respect to the total abundance of identified PLs, per injection.

Results and Discussion

The ethanol extraction and fractionation methods resulted in four lipid samples used in LC-MS analysis: TLE, F1, F2, and wax. A total of 3439 targets were detected by UPLC-TOF MS (Fig. 4.1). Relative percent composition of PLs is reported for each unique lipid species identified for both positive and negative ion mode (Table 4.1 – 4.4). Previously, it was observed that the ethanol fractionation process caused phospholipids to crystallize with neutral lipids (Ferraris & Qian, 2021). The use of high-resolution mass spectrometry-based lipidomic methods allowed for a better understanding of this phenomena in addition to profiling bioactive PLs available in a WPPC commercial product.

In general, sphingolipids appeared to ionize in positive and negative ion modes, resulting in many identified species. Several ceramide species were detected and a larger number of glycosphingolipids, including glucosyl-, galactosyl-, lactosyl-, and galabiosylceramide species (Table 4.1). Total ceramide species detected constituted 4.8% and 5.2% relative abundance of the PLs identified in the TLE and F2 fractions, respectively, in positive ion mode. Many glycosylated ceramides identified co-crystallize with triglycerides (F1) during the ethanolic fractionation process, the loss of these bioactive lipids should be considered in the future when using this method. A single species, glucosylceramide (18:1/12:0), represented 3.8% of the PLs found in the crystallized lipid sample. The fatty acids identified in glycosphingolipids were all saturated fatty acids and the saturated fatty acids may have contributed to the crystallization of these lipids.

A total of 33 sphingomyelin (SM) species were detected and represented over 27% of the PLs identified in the TLE by positive mode ionization (Table 4.2). Several different sphingosine backbones structures were identified with chain-length ranging from 14 to 18 carbons, and many monounsaturated fatty acid species were present in SM. Studies have reported SM species to contain fatty acids larger than stearic acid (C18:0) and are a reservoir of long-chain fatty acids (Bourlieu et al., 2018; Castro-Gómez et al., 2017). A large population of SM species with eicosanoic acid (C20:0), docosanoic acid (C22:0), nervonic acid (C24:1) and hexacosenoic acid (C26:1) were present in WPPC. The SM species identified largely remain in the PL phase during ethanol fractionation.

Glycerophospholipids, including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA) (Table 4.3) and phosphatidylcholine (Table 4.4) make up the majority of the identified phospholipids. Modified phospholipids species were also detected, including PE plasmalogens, dimethyl-PE, and lysoPC. Only two branched-chain fatty acid PL species were identified, both present in PA species.

Correlation heat map diagrams were used to visualize the relationships between unique PL species and lipid fractions. Positive or high correlations were displayed as a red gradient in all the graphs with negative displayed as a blue gradient. Strong positive correlations were observed between sphingolipids and PC for positive mode data (Fig. 4.A.1). Similar positive correlations are observed in negative ion mode data (Fig. 4.A.2) for PE, sphingolipids, and PC. Negative correlations were consistent between PS and PI versus SM and PC. Several species of PE and glycosylated ceramides were negatively correlated to SM and PC (blue gradient, Fig. 4.A.1).

Relationships between lipid fractions of WPPC are shown in Figure 4.2. A higher correlation between TLE and F2 in positive ion mode was observed. These two samples also appeared mutually exclusive from F1 and wax samples. The combination of relationships between identified species (Fig. 4.A.1 and 4.A.2) and the relations between lipid fractions (Fig. 4.2) support the claim that the polar lipids found in TLE and F2 were very similar and agreed with trends seen in the tabulated data.

Principle component analysis (PCA) of the PL species determined in the WPPC (Fig. 4.2) resulted in a distinct separation of lipid fractions, and tight clustering of sample replicates as expected from negative ion mode. However, the positive mode data show a near-complete overlap of the TLE and F2 samples. This result agrees with the strong correlation between TLE and F2 using positive ionization mode reported in Fig. 4.2. It can be concluded that, in general, the polar lipids present in WPPC can be enriched with ethanol extraction and fractionation.

Conclusion

The polar lipids in milk can be a valuable functional ingredient essential in growing infants' and aging adults' metabolic pathways. The global dairy industry and consumers can benefit from the recovery of polar lipids from underutilized co-products into a new value-added ingredient. SM, ceramides, glycosphingolipids, and other glycerophospholipids can be extracted from WPPC using food-grade ethanol, and these PL can be further enriched or fractionated from neutral lipids. Correlation analysis between lipid fractions and PL species demonstrated that most PL was recovered after ethanol fractionation. However, several bioactive PL classes including glycosphingolipids, PI and PS remain with neutral lipids during ethanol fractionation.

Acknowledgements

Table 4.1. Identified ceramide species of WPPC lipid extracts

Positive Mode			Rel. % of PL[2]		
m/z[1]	Molecular Species	CN/DB	TLE	F1	F2
520.5075[A]	Cer(d18:1/16:0)	34:1	0.05	0.03	0.05
604.6011[A]	Cer(d18:0/22:1)	40:1	0.19	0.07	0.21
672.6235[B]	Cer(d18:0/24:1)	42:1	0.15	0.06	0.17
713.5669[C]	Galactosylceramide (d18:1/14:0)	32:1	0.60	0.48	0.67
661.5360[D]	Glucosylceramide (18:1/12:0)	30:1	1.55	3.82	1.67
741.5979[C]	Glucosylceramide (18:1/16:0)	34:1	0.34	0.31	0.38
715.5826[D]	Glucosylceramide(d18:2/16:0)	34:2	1.03	1.46	1.15
806.6459[B]	Glucosylceramide (18:1/22:0)	40:1	0.31	0.82	0.31
867.7391[C]	Glucosylceramide (18:1/25:0)	43:1	0.19	0.87	0.21
884.6052[B]	Lactosylceramide (d18:1/16:0)	34:1	0.10	0.65	0.09
968.6995[B]	Lactosylceramide (d18:1/22:0)	40:1	0.17	1.17	0.16
996.7308[B]	Lactosylceramide (d18:1/24:0)	42:1	0.12	0.59	0.11
Negative Mode			Rel. % of PL[2]		
m/z[1]	Molecular Species	CN/DB	TLE	F1	F2
638.5734[E]	Cer(d18:1/20:0)	38:1	0.08	0.00	0.12
666.6052[E]	Cer(d18:1/22:0)	40:1	0.18	0.00	0.28
680.6213[E]	Cer(d18:1/23:0)	41:1	0.23	0.00	0.37
944.7067[F]	Galabiosylceramide (d18:1/22:0)	40:1	2.17	0.59	2.90
744.5625[E]	Glucosylceramide (d18:1/16:0)	34:1	0.85	0.07	1.71
726.5819[F]	Glucosylceramide (d18:1/18:0)	36:1	1.49	0.09	2.02
828.6592[E]	Glucosylceramide (d18:1/22:0)	40:1	2.30	0.21	3.37

[1] The listed m/z represents the most abundant adduct detected.
Adduct identified for each corresponding m/z denoted by superscript letter.
[A] $[M+H-H_2O]^+$, [B] $[M+Na]^+$, [C] $[M+ACN+H]^+$, [D] $[M+NH_4]^+$, [E] $[M+FA-H]^-$, [F] $[M-H]^-$
[2] Relative percent composition of total identified PL species for respective ionization mode. The lowest reporting value is 0.01%, any species detected below this threshold is reported as zero.

Table 4.2. Identified sphingomyelin species of WPPC lipid extracts

Positive Mode			Rel. % of PL[2]		
m/z[1]	Molecular Species	CN/DB	TLE	F1	F2
677.5578[A]	SM(d18:0/14:0)	32:0	0.43	0.20	0.44
675.5420[A]	SM(d14:0/18:1)	32:1	0.95	0.44	1.04
689.5577[A]	SM(d15:0/18:1)	33:1	0.57	0.25	0.58
705.5888[A]	SM(d14:0/20:0)	34:0	0.97	0.60	0.93
703.5730[A]	SM(d18:0/16:1), SM(d18:1/16:0)	34:1	4.83	2.94	4.96
723.5390[B]	SM(d16:1/18:1)	34:2	0.09	0.06	0.10
733.6203[A]	SM(d18:0/18:0)	36:0	0.21	0.09	0.17
731.6046[A]	SM(d14:1/22:0)	36:1	1.22	0.63	1.19
745.6198[A]	SM(d15:1/22:0)	37:1	0.28	0.08	0.26
761.6519[A]	SM(d18:0/20:0)	38:0	0.90	0.58	0.93
759.6362[A]	SM(d14:0/24:1)	38:1	1.99	1.30	2.05
775.6670[A]	SM(d15:0/24:0)	39:0	0.67	0.40	0.69
773.6519[A]	SM(d15:0/24:1)	39:1	2.01	0.34	2.07
771.6354[A]	SM(d15:1/24:1)	39:2	0.14	0.10	0.14
789.6825[A]	SM(d18:0/22:0)	40:0	0.70	0.39	0.72
787.6670[A]	SM(d18:1/22:0)	40:1	4.21	2.42	4.42
785.6515[A]	SM(d18:1/22:1)	40:2	0.49	0.26	0.50
803.6979[A]	SM(d15:0/26:0)	41:0	0.19	0.09	0.21
801.6826[A]	SM(d15:0/26:1)	41:1	3.55	1.97	3.74
821.6493[B]	SM(d15:1/26:1)	41:2	0.16	0.12	0.16
817.7133[A]	SM(d18:0/24:0)	42:0	0.08	0.05	0.09
815.6978[A]	SM(d16:0/26:1)	42:1	2.19	1.19	2.21
813.6819[A]	SM(d18:1/24:1)	42:2	0.29	0.03	0.30
851.6943[B]	SM(d17:0/26:1)	43:1	0.21	0.17	0.21
Negative Mode			Rel. % of PL[2]		
m/z[1]	Molecular Species	CN/DB	TLE	F1	F2
749.5825[C]	SM(d18:0/16:0)	34:0	0.25	0.01	0.47
747.5672[C]	SM(d18:0/16:1)	34:1	2.27	0.12	3.49
775.5981[C]	SM(d18:0/18:1)	36:1	0.26	0.02	0.47
805.6458[C]	SM(d18:0/20:0)	38:0	0.28	0.01	0.44
803.6301[C]	SM(d18:1/20:0)	38:1	2.18	0.14	3.37
785.6561[D]	SM(d18:1/22:0)	40:1	2.46	0.18	3.61
817.6457[E]	SM(d18:0/22:2(OH))	40:2	0.97	0.08	1.55
845.6759[C]	SM(d18:1/23:0)	41:1	3.30	0.44	4.96
859.6923[C]	SM(d18:0/24:1)	42:1	1.11	0.13	1.64

[1] The listed m/z represents the most abundant adduct detected.
Adduct identified for each corresponding m/z denoted by superscript letter.
[A] $[M+H]^+$, [B] $[M+Na]^+$, [C] $[M+FA-H]^-$, [D] $[M-H]^-$, [E] $[M-H2O-H]^-$
[2] Relative percent composition of total identified PL species for respective ionization mode. The lowest reporting value is 0.01%, any species detected below this threshold is reported as zero.

Table 4.3. Glycerophospholipids of WPPC lipid extracts excluding phosphatidylcholine

Positive Mode			Rel. % of PL[2]			*Negative Mode*			Rel. % of PL[2]		
m/z[1]	Molecular Species	CN/DB	TLE	F1	F2	m/z[1]	Molecular Species	CN/DB	TLE	F1	F2
677.5096[A]	PA(20:0/i-14:0)	34:0	0.24	1.41	0.25	701.5135[C]	PA(18:0/18:1)	36:1	0.78	1.61	0.12
733.5722[A]	PA(a-25:0/a-13:0)	38:0	0.19	2.19	0.19	699.4978[C]	PA(18:2/18:0)	36:2	0.79	1.42	0.22
718.537[A]	PE(18:1/16:0)	34:1	1.00	2.70	0.81	750.5308[D]	PE(18:0/15:0)	33:0	1.00	0.08	1.52
716.5210[A]	PE(16:0/18:2)	34:2	1.23	2.84	0.97	716.5249[C]	PE(18:1/16:0)	34:1	3.67	1.47	5.03
748.5833[A]	PE(21:0/15:0)	36:0	0.42	0.11	0.44	714.5094[C]	PE(16:0/18:2)	34:2	3.19	1.01	4.53
746.5681[A]	PE(18:1/18:0)	36:1	0.72	1.44	0.63	778.5621[D]	PE(15:0/20:0)	35:0	1.33	0.13	2.18
744.5529[A]	PE(18:0/18:2)	36:2	4.29	6.13	4.21	744.5568[C]	PE(18:0/18:1)	36:1	6.64	2.57	8.39
764.5185[B]	PE(18:1/18:2)	36:3	0.29	0.57	0.27	742.5404[C]	PE(18:1/18:1)	36:2	15.88	5.74	20.14
788.5161[B]	PE(18:1/20:4)	38:5	0.13	1.20	0.13	738.5094[C]	PE(18:3/18:1)	36:4	1.86	0.20	2.80
700.5263[A]	PE(P-16:0/18:2)	34:2	0.15	0.24	0.14	804.5777[D]	PE(15:0/22:1)	37:1	1.91	0.11	3.47
748.5835[A]	PE-NMe2(16:0/18:0)	34:0	0.48	0.25	0.41	802.5622[D]	PE(15:0/22:2)	37:2	1.02	0.04	1.86
756.5518[A]	PE-NMe2(15:0/20:3)	35:3	0.29	0.04	0.27	770.5711[C]	PE(20:1/18:1)	38:2	1.05	0.04	1.74
774.5992[A]	PE-NMe2(16:0/20:1)	36:1	0.71	0.11	0.73	786.5311[E]	PE(16:0/22:5)	38:5	7.53	14.46	3.25
772.5832[A]	PE-NMe2(18:0/18:2)	36:2	0.35	0.07	0.34	718.5405[F]	PE(P-18:1/16:1)	34:2	1.01	0.08	1.47
887.5606[B]	PI(18:1/18:0)	36:1	0.03	1.26	0.00	726.5463[C]	PE(P-18:0/18:2)	36:2	0.98	0.21	1.27
885.5443[B]	PI(18:0/18:2)	36:2	0.03	1.10	0.00	724.5296[C]	PE(P-16:0/20:3)	36:3	0.37	0.07	0.59
762.5251[A]	PS(14:1/20:0)	34:1	0.07	1.26	0.01	700.5305[C]	PE(P-16:0/18:1)	38:2	0.83	0.26	1.06
814.5576[B]	PS(18:0/18:0)	36:0	0.08	0.93	0.01	744.5564[C]	PE-NMe2(18:1/16:0)	34:1	1.47	0.08	2.40
790.5572[A]	PS(18:1/18:0)	36:1	0.62	12.03	0.15	835.5363[C]	PI(18:1/16:0)	34:1	1.48	3.98	0.32
788.5415[A]	PS(18:2/18:0)	36:2	0.76	13.68	0.17	863.5677[C]	PI(18:0/18:1)	36:1	7.54	20.62	1.13
786.5257[A]	PS(18:2/18:1)	36:3	0.10	1.13	0.03	861.5523[C]	PI(18:2/18:0)	36:2	7.39	18.59	1.30
838.5574[A]	PS(22:5/18:0)	40:5	0.07	1.17	0.01	859.5364[C]	PI(18:2/18:1)	36:3	1.05	2.12	0.30
						887.5671[C]	PI(20:3/18:0)	38:3	2.35	6.24	0.34
						788.5467[C]	PS(18:1/18:0)	36:1	6.65	15.10	1.44
						812.5462[C]	PS(18:0/20:3)	38:3	0.76	1.59	0.31

[1] The listed m/z represents the most abundant adduct detected. [A] [M+H]+, [B] [M+Na]+, [C] [M-H]-, [D] [M+FA-H]-, [E] [M+Na-2H]-, [F] [M-H2O-H]-

[2] Relative percent composition of total identified PL species for respective ionization mode. The lowest reporting value is 0.01%, any species detected below this threshold is reported as zero.

Table 4.4. Phosphatidylcholine species identified in WPPC lipid extracts

m/z[1]	*Positive Mode* Molecular Species	CN/DB	Rel. % of PL[2] TLE	F1	F2
468.3074[A]	LysoPC(14:0)	14:0	0.04	0.01	0.04
496.3389[A]	LysoPC(16:0)	16:0	0.17	0.07	0.19
496.3385[A]	LysoPC(16:0)	16:0	0.03	0.01	0.03
524.3700[A]	LysoPC(18:0)	18:0	0.10	0.04	0.11
522.3542[A]	LysoPC(18:1)	18:1	0.10	0.02	0.10
650.4739[A]	PC(9:0/17:0)	26:0	0.19	0.06	0.21
678.5054[A]	PC(14:0/14:0)	28:0	0.81	0.30	0.89
692.5208[A]	PC(14:0/15:0)	29:0	0.26	0.10	0.26
706.5363[A]	PC(16:0/14:0)	30:0	5.89	2.45	6.28
720.5521[A]	PC(16:0/15:0)	31:0	1.18	0.49	1.08
734.5678[A]	PC(16:0/16:0)	32:0	6.72	3.23	7.11
754.5337[B]	PC(18:1/14:0)	32:1	0.37	0.61	0.41
730.5361[A]	PC(14:0/18:2)	32:2	0.26	0.04	0.27
746.5677[A]	PC(15:0/18:1)	33:1	0.88	0.23	0.80
762.5994[A]	PC(18:0/16:0)	34:0	1.87	0.54	2.01
760.5834[A]	PC(18:1/16:0)	34:1	10.79	4.69	11.39
758.5675[A]	PC(16:0/18:2)	34:2	6.40	2.48	6.47
788.6151[A]	PC(18:0/18:1)	36:1	3.83	1.46	4.06
786.5993[A]	PC(18:0/18:2)	36:2	8.51	3.24	7.94
784.5833[A]	PC(18:1/18:2)	36:3	4.93	1.65	4.60
782.5671[A]	PC(18:1/18:3), PC(16:0/20:4)	36:4	0.93	0.21	0.94
812.6146[A]	PC(18:0/20:3)	38:3	0.32	0.06	0.34
m/z[1]	*Negative Mode* Molecular Species	CN/DB	Rel. % of PL[2] TLE	F1	F2
830.5934[C]	PC(20:1/16:1)	36:2	1.07	0.04	2.00

[1] The listed m/z represents the most abundant adduct detected.
Adduct identified for each corresponding m/z denoted by superscript letter.
[A] [M+H]+, [B] [M+Na]+, [C] [M+FA-H]-
[2] Relative percent composition of total identified PL species for respective ionization mode. The lowest reporting value is 0.01%, any species detected below this threshold is reported as zero.
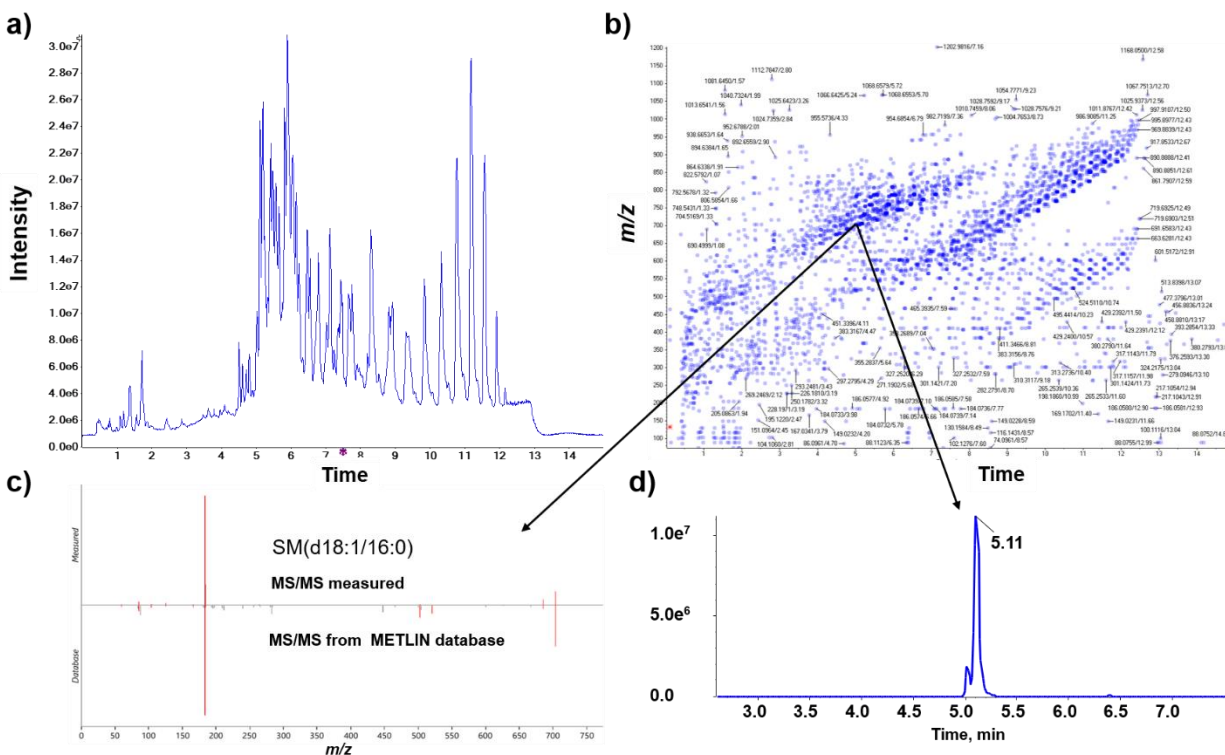
Figure 4.1. Untargeted lipidomics analysis of a pooled sample (QC) using the data-dependent acquisition in positive ionization mode (2 μL injection; 1 mg/mL). a) Total ion chromatogram. b) Distribution map of precursor ions submitted to collision-induced dissociation (CID) during chromatographic elution. The x-axis represents each precursor ion's elution time; the y-axis provides m/z information for the precursor ion. The intensity of the blue color represents the ion abundance of the precursor ion. Each of the 3439 dots contains exact mass, isotopic pattern, retention time and fragmentation spectrum used for lipid annotation. c) Example of library matching for SM(d18:1/16:0). MS/MS spectrum is matched against databases. The red lines correspond to matched fragments among measured ones and the database (e.g. METLIN). d) Precursor ions are integrated, and the area under the curve is used for the semiquantitative analysis.
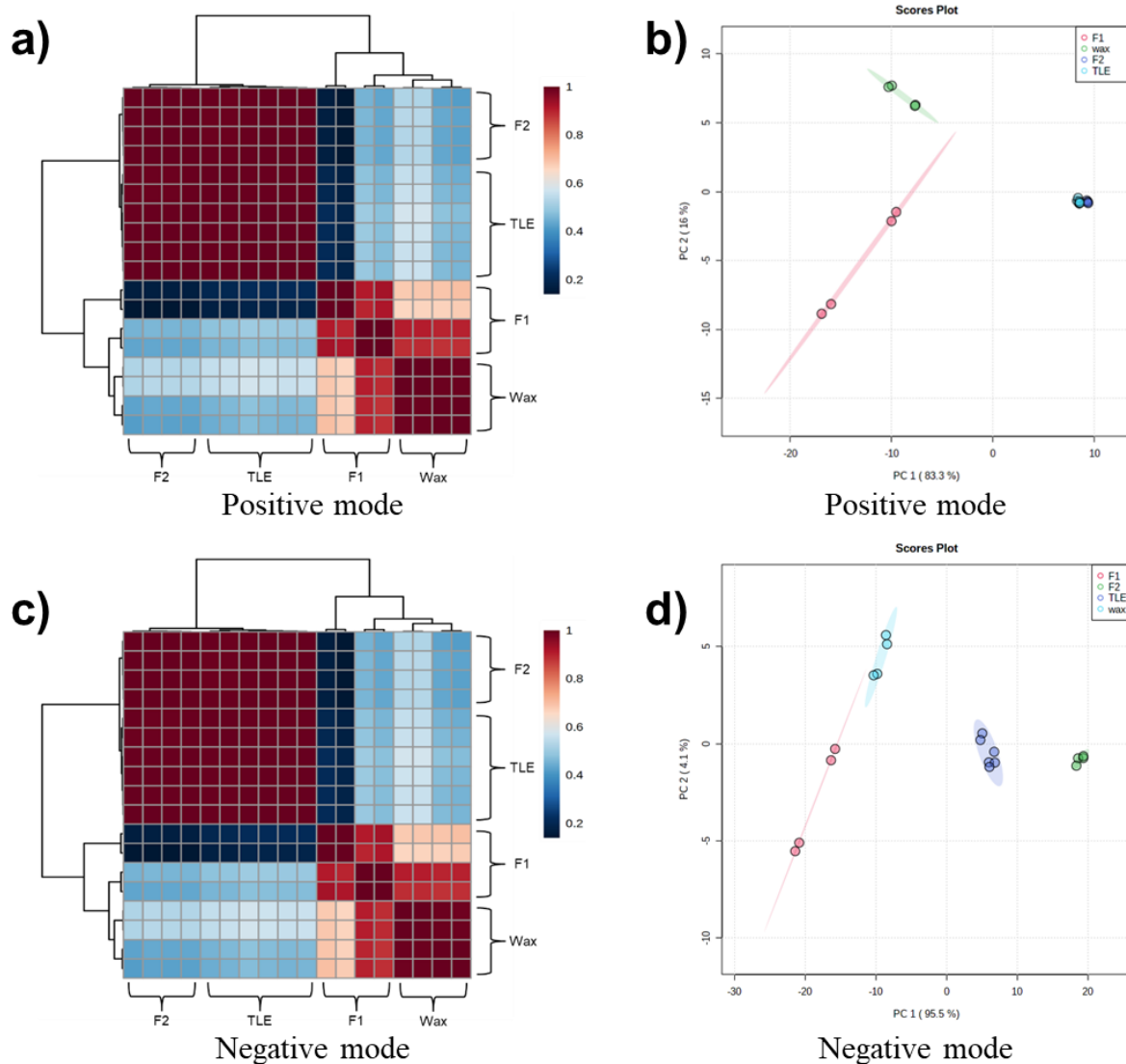
Figure 4.2. Correlation heat map diagrams for WPPC lipid fractions in positive mode (a) and negative mode (c). Higher sample correlations appear red, and lower correlations were represented in blue. Principle component analysis (PCA) of polar lipid composition in WPPC lipid fractions in positive mode (b) and negative mode (d). Total lipid extract (TLE), crystallized lipids (F1), Waxy crystallized lipid (wax), and soluble enriched polar lipids (F2).

Appendix

Figure 4.A.1. Positive mode ionization correlation heat map diagram of identified polar lipid species in WPPC lipid extracts. Lipid species with positive correlation appear red, negative correlations represented in blue.

Figure 4.A.2. Negative mode ionization correlation heat map diagram of identified polar lipids species in WPPC lipid extracts. Lipid species with positive correlation appear red, negative correlations represented in blue.
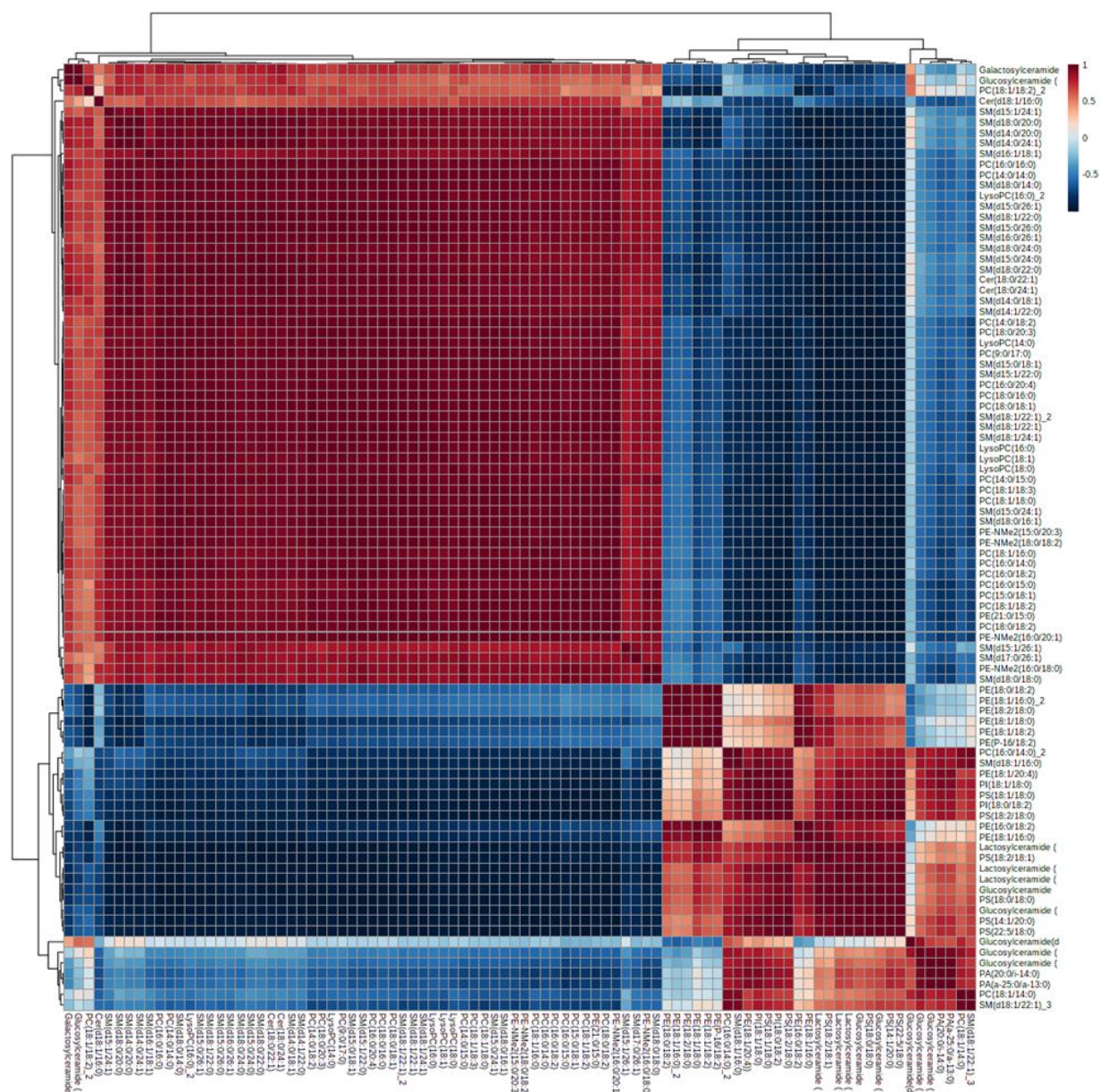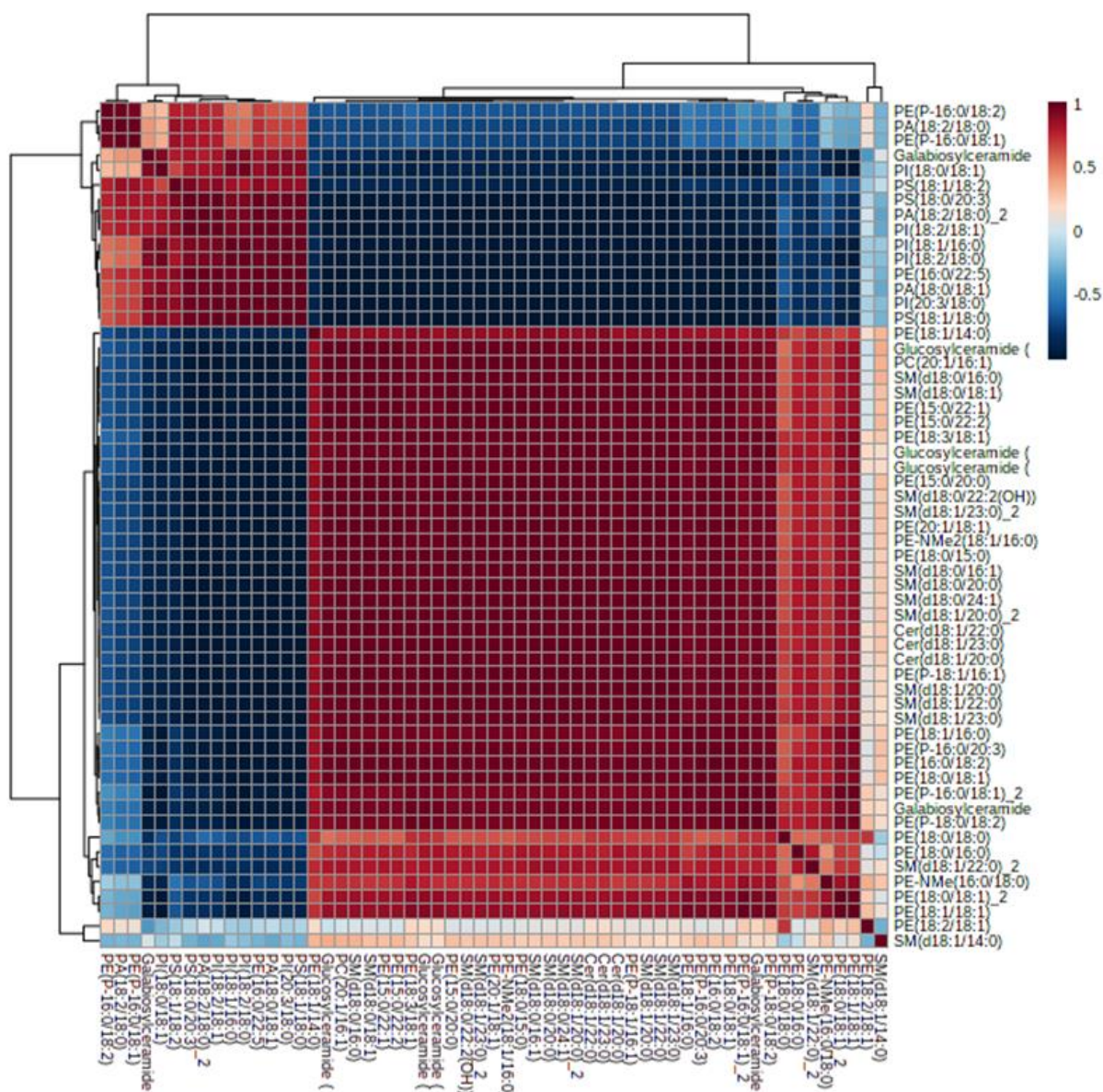
Chapter 5: Project Conclusions

The isolation of polar lipids from dairy is an interesting challenge motivated by the potential nutritional benefits discussed at length previously and the potential for a dairy based lecithin product which could be used in dairy products in place of plant-based lecithins. The use of milk-derived phospholipids in place of soy lecithins in dairy products would also allow for the removal of an allergen creating a cleaner label. The dairy industry largely relies on mechanical separations of fat fractions to yield MFGM-enriched products, furthermore, these common processing techniques cannot be used to create a lecithin equivalent product. This project was in part inspired by the dairy industry's desire for a simple method for the recovery of phospholipids.

Sourcing phospholipids from under-utilized or less-valuable dairy streams and/or co-products was a personal incentive for the project. Experimentation initially began on whey cream, the resulting fat fraction from the centrifugation of sweet whey from cheese production. Whey cream is a good source of milk phospholipids as has been reported in the literature. However, discussion with professionals in the dairy industry revealed that whey cream is not commonly in excess, the whey cream is regularly added back into the next cheese batch and is used to standardize the fat content. Whey cream can also be used as an ingredient in other dairy products. Additionally, whey cream was not readily available or convenient for continued use in this project.

After centrifugation of sweet whey to produce whey cream, fat and MFGM remain in the fluid whey, which is then commonly processed using membrane filtration technologies to produce whey protein concentrates. Whey protein concentrate 34 (WPC 34) is a low protein whey product largely composed of lactose which can also have a fat content up to 5%. WPC 34

is a lower value product, compared to other higher whey protein concentrate products. Composition of phospholipids present in WPC 34 are not well reported in the literature.

The project required an analytical method for the accurate quantitation of milk phospholipids, additionally, the industry partner for the project was seeking a validated standard method for similar analysis to be used in the dairy industry setting. Selection of high-performance liquid chromatography (HPLC) was the obvious choice for a rapid, reproducible, and sensitive analytical method for phospholipids. HPLC methods are widely reported for phospholipid analysis in the literature, as described previously. The recreation of several methods from the literature were attempted but were unsuccessful. These methods were largely normal phase separations relying on chloroform solvent systems. The selection of solvent in combination with the evaporative light scattering detector (ELSD) also influenced the decision to abandon normal phase chromatography and to develop a new method. The use of ELSD and chloroform resulted in large volumes of nebulized halogenated solvent vapors exiting the detector as a waste stream, which had to be vented into a chemical hood. This presented a safety hazard not only in the academic setting but also in the industry setting where halogenated solvents are not used in routine analysis. The large volumes of halogenated solvents that would be required by a chloroform-based HPLC separation, along with the additional hazard of the nebulized solvent exiting the detector, were unfavorable safety risks deemed unacceptable for the project.

The final method developed for the project was a compromise of chloroform-based lipid extraction (Folch method) and SPE purification, where the procedure was scaled down to limit the required volumes of halogenated solvent and exposure risk, in combination with hydrophilic interaction liquid chromatography (HILIC) separation which used solvents already common for

the routine analysis of dairy proteins by HPLC. The final quaternary solvent system developed resulted in improved resolution of major milk phospholipids when compared to other methods in the literature. The baseline resolution of analytes was advantageous in an industry setting, as it allowed for simpler data processing and avoids manual peak splitting by the technician. A commercial reference standard for phospholipids is not readily available, therefore, the method had to be validated to industry standards using a combination of tests to demonstrate consistent, reproducible results during each step of the procedure.

Experimentation on ethanol extraction of dairy polar lipids began using WPC 34. Initially it was observed that at higher concentrations, lipids crystallize in solutions of ethanol cooled to room temperature or below. The high concentrations of lactose present in WPC 34 also resulted in some dissolution of lactose into the ethanol extraction solvent along with the lipid material. A secondary crystal structure was observed in the chilled ethanol solutions on the surface of the glassware, hypothesized to be lactose. The solvation of lactose into heated ethanol presents another challenge in purifying milk polar lipids from WPC34. These challenges and lipid fractionation from WPC34 were not further investigated during the project, however, WPC34 may still be a viable source of milk polar lipids (approximately 2% phospholipids by mass) to generate a new high value polar lipid product.

Ethanol extraction of polar lipids continued with a new powdered whey product, a co-product whey protein phospholipid concentrate, which is specifically generated by processes that concentrate MFGM during the production of high purity whey protein. WPPC is a favorable source of polar lipids because it is a co-product of whey processing and has a higher initial content of phospholipids compared to WPC34. Other research groups have also published ethanol extraction methods for the recovery of milk polar lipids, one method was conducted at a

pilot scale described previously. These methods were used to inform the procedures developed by this project. The apparatus/extraction vessel selected for the method avoided specialty equipment and could be upscaled in an industry setting using commonly available food processing equipment. The direct solvent extraction of whey powder avoids the major dilution effects of other methods in the literature where the whey powder is reconstituted in water and pumped into a solution of heated ethanol. The pumping of an aqueous solution of whey into ethanol causes continuous dilution of the ethanol concentration until the end of the extraction cycle. The specific effects of temperature and the ethanol concentration on polar lipid recovery were not well investigated in this project and should be tested in the future to determine the optimal parameters. Additionally, the percent recovery of phospholipids per extraction cycle was not determined in this project and should be investigated to identify the minimum solvent quantities required to recover the majority of phospholipids from the whey powder.

The crystallization of lipids in ethanol solutions extracted from WPC34, suggested that the lipids could be concentrated and fractionated after extraction. The phenomena of temperature-induced crystallization of lipids is already used in the food industry, called winterization, to create refrigeration stable oils commonly used in salad dressing. Temperature dependent solubility of solutes in a mixture is referred to as fractional crystallization in the fields of chemistry. These principles of winterization and fractional crystallization were applied to the ethanolic lipid extracts from WPPC. Properties of lipids in solutions of ethanol are not well described in the literature, however, a few studies reported lipid fractionation in acetone – ethanol mixtures. For this study ethanol fractionation was briefly investigated in a controlled experiment using a singular crystallization temperature and lipid concentration. Previously during the project, crystallization had been induced at room temperature over prolonged times, or

shorter time periods under refrigeration and/or submerging in an ice bath. The effect of temperature is hypothesized to effect which lipid species crystallize in relation to the degree of saturation of the attached fatty acids. A detailed understanding of the effects of temperature and concentration on lipid crystallization remains incomplete and should be investigated further in the future.

The process of ethanol extraction of polar lipids from dairy products has limitations. The dairy industry is very efficient with limited waste being generated. The majority of milk solids are recovered as a primary product or subsequently as a co-product. For the dairy industry to adapt a new technology or process it needs to meet two major characteristics, 1) the ability to integrate into a continuous fluid production model and 2) the ability to reduce or completely avoid generating a new waste stream.

The technology of supercritical fluid extraction (SFE) has been demonstrated to successfully recover polar lipids from powdered dairy products over ten years ago, and yet the dairy industry has resisted adopting the technology. SFE is a batch process requiring the minor repackaging of raw materials into permeable bags to reduce cleaning of the apparatus, and it requires the addition of flow modulators, such as celite, to increase supercritical fluid penetration. The batch process nature of SFE does not amend easily into the continuous fluid production model of the dairy industry and the addition of flow modulators to the powdered raw material generates an unusable waste product after extraction.

The ethanol extraction procedures presented in this project are also reflective of a batch process, however, the resulting precipitated protein and the other milk solids remain food safe and could be further processed as a separate product. Similar to SFE methods reported in the literature, the ethanol method proposed in this research relies on the raw material existing as a

powder product which requires the input of energy to generate the spray-dried product. Milk proteins are known to denature in high concentration solutions of ethanol, which is expected to limit future utilization of the precipitated protein. The degree of denaturation of the protein with respect to ethanol concentrations during extraction could be measured in future experiments using circular dichroism spectroscopy.

An adjacent industry to the dairy industry could use food-grade ethanol for the recovery of dairy polar lipids with potentially lower initial investment costs for equipment compared to other technologies. The use of food-grade ethanol in the extraction could be supplemented by the fermentation of lactose to yield ethanol. Furthermore, the ethanol can be recycled after every extraction, which would reduce costs of production. The future of ethanol extraction methods remains promising as fluid applications and pilot scale experiments have been reported by other research groups during the time of this project, which demonstrate a potential compromise with continuous fluid production models and the potential for industrial scale-up.

The final part of the project aimed to complete detailed analysis of polar lipid species present in MFGM-enriched WPPC. The use of mass spectrometry lipidomic techniques allowed for the detection of individual polar lipid species. The soft ionization technique results in molecular ions being formed which can then be fragmented to gain detailed structural information for each individual molecular ion detected. The individual fatty acids attached to the polar lipids and the composition of the sphingoid backbone of sphingolipids was determined with high precision. It is understood that these advance techniques are powerful research tools, that would not be found in dairy industry laboratories. However, this project had the opportunity to use these techniques to gain a better understanding of the polar lipids available to the dairy

industry sourced from WPPC. This information was then made available and shared directly with the dairy industry.

The LC-MS analysis confirmed the observation of polar lipid species decreased in concentration during ethanol fractionation and apparent co-crystallization with neutral lipids. Phosphatidylserine, Phosphatidylinositol, and several glycosylated ceramide species were observed to crystallize with neutral lipids. This crystallization dynamic should be investigated in the future using more crystallization temperatures to study how the milk polar lipids act and if they can be recovered along with the other polar lipids. The additional use of mass spectrometry lipidomic methods in a future study could investigate the fatty acid composition of crystallized neutral lipids and co-crystallized polar lipids to determine if there is a correlation of saturation and/or chain length with the lipid species that crystallize in ethanol solutions.

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