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The isolation and concentration of phospholipids from various dairy by-products

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The isolation and concentration of phospholipids from various dairy by-products

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

Page

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ABSTRACT

There has been great interest in the phospholipids (PLs) found in dairy products because of their health and functional properties. The isolation and concentration of these PLs has been a significant research area in recent years. Dairy by-products contain higher concentrations of PLs compared to raw milk, which make them an economic feedstock for PLs concentration. Technologies that produce isolated lipid fractions with elevated PLs concentrations have yet to be obtained and are a priority for the United States dairy industry. This dissertation summarizes methods used to isolate and concentrate PLs from various dairy by-products. The technologies utilized in these studies provide a means for the dairy industry to utilize dairy by-products more effectively and provide methodologies appropriate for industry scale-up. The end goal of this research is the production of an isolated dairy lecithin with a PLs concentration greater than 50%. The simultaneous texturization and extraction of PLs (STEP) method, modified from the method used for egg yolk, which utilized ethanol, was shown to be an effective and efficient way to produce a dairy PLs concentrate from whey protein phospholipid concentrate. The use of food grade surfactants was shown to be effective in improving the PLs distribution to buttermilk, which is a by-product with potential for PLs utilization, during cream churning. Both zinc acetate and calcium acetate were effective for precipitating the total lipid and PLs found in the beta stream and an additional ethanol extraction has great potential for producing valuable lipid fractions. The use of solvent fractionation was shown to be an effective way to concentrate PLs from the lipid fraction isolated from the beta stream and produced a dairy lecithin. An investigation into the oxidative stability of whey protein phospholipid concentrate lipid fraction suggests that powdered and liquid forms of industrial WPPC were oxidized.

All of these studies provided insight into PLs isolation and concentration methods, along with giving an understanding of the oxidative stability of dairy by-products lipid fractions. The end goal of this research was obtained with an effective means to produce a dairy lecithin that can be utilized in various food and consumer applications.

CHAPTER 1: GENERAL INTRODCTION

In recent years the milk fat globule membrane (MFGM) has been of great interest in the dairy industry due to its potential health benefits and functional properties (Dewettinck et al., 2008; Rombaut & Dewettinck, 2006). The MFGM is the richest source of phospholipids (PLs), glycolipids, gangliosides, and glycoprotein in milk (Jimenez-Flores & Brisson. 2008). The approximate ratio of proteins, lipids, and carbohydrates within the membrane are 4:3:1 (Spence et al., 2009) and a depiction the tri-layer membrane that encompasses the triacylglycerol core can be seen in Figure 1. This unique membrane has been shown to prevent body weight gain and bind to strains of pathogenic bacteria, which can reduce the potential for bacterial infections (Milard et al., 2019). The consumption of MFGM in infants has been associated with lower infection rates and better cognitive performance (Hernell et al., 2016). One component within the MFGM that has drawn the most interest are PLs, which are generally referred to as lecithin in the food industry (Contarini & Povolo, 2013). These lipids are a complex class of polar lipids that are amphiphilic because of a hydrophilic head and hydrophobic fatty acid tail (Contarini & Povolo, 2013; Donato et al., 2011). These properties give PLs excellent emulsification properties and make them important functional ingredients for the food, pharmaceutical, and cosmetic industries. Other than the technological functionalities such as emulsifying and lubricating, PLs also have gained considerable interest because of their nutritional value.

Although dairy PLs represent only 0.5-1.0% of the total milk lipids (Fong et al., 2007; Bourlieu et al., 2018; Conway et al., 2014), they are of particular interest because of their higher content of sphingomyelin (SM, 4.1-29.2% of total PLs), and phosphatidylserine (PS, 2.0-16.1% of total PLs) compared to other lecithin sources such as soybean and egg yolk (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Nejrup et al., 2017). SM is reported to play

important roles in cell regulation and is referred to as a tumor suppressor (Contarini & Povolo, 2013; Dewettinck et al., 2008; Rombaut et al., 2006). Cognitive performance improvement, which is of particular significance to Alzheimer's disease treatment, has been attributed to PS (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Dewettinck et al., 2008; Pepeu et al., 1996; Rombaut & Dewettinck, 2006). Other beneficial biological effects such as reduced cholesterol absorption, antioxidant properties, stress and depression tolerance, and reduced incidence of cardiovascular disease, as well as suppression of multiple sclerosis are also associated with dairy PLs (Contarini & Povolo, 2013; Dewettinck et al., 2008; Rombaut & Dewettinck, 2006). Dairy PLs have greater potential to be utilized in infant formula for mimicking the human breast milk PLs profile (Sala-Vila et al., 2005). The utilization of dairy PLs can improve the overall nutritional quality of infant formula as well as the economics of the dairy by-product utilization.

The low PLs content in milk makes it difficult to extract and concentrate PLs on an industrial scale. If raw milk was utilized for PLs concentration it would take 2,500-3,300 kilograms of raw milk to produce one kilogram of pure PLs. Due to this factor, the selection of better starting materials are required if a dairy lecithin is going to compete with plant-based PLs. Although there are industrial products produced which are enriched in dairy PLs, the exact processing that these products undergo is an industrial trade secret. These products may be produced by technologies such as enzymatic hydrolysis of proteins, microfiltration, ultrafiltration, supercritical fluid extraction, or a combination of these technologies (Spence et al., 2009; Barry et al., 2017; Costa et al., 2010; Astaire et al., 2003; Rombaut et al., 2007). However, these dairy PLs concentrates are not isolated lipid fractions, and this may limit their application as an industrial lecithin. The utilization of dairy by-products which have lower

economic value and higher concentrations of PLs is essential for dairy PLs concentration (Table 1). This dissertation summarizes work we did with various dairy by-products such as whey protein phospholipid concentrate, buttermilk, and beta stream as the materials for PL isolation.

The commercial dairy product with the highest PLs content (Phospholipid Concentrate 700) is manufactured by Fonterra Co-operative Group Ltd. (Auckland, New Zealand), which contains up to 60% w/w PLs (Fong et al., 2013). This product contains a substantially higher amount of PLs than any other product reported in the literature. There has yet to be product with high PLs concentrations produced in the United States. Previously, the highest PLs content dairy product reported was 23.7% on a dry basis, concentrated from butter serum using microfiltration (Le et al., 2010). The major limiting factor with the use of microfiltration on buttermilk and butter serum is the retention of casein, which limits how concentrated the PLs can become. Moreover, certain feedstock and technologies utilized for producing the PLs concentrates are not always cost-effective and this leads to low feasibility for commercial scale production. Therefore, there is a need to develop a more cost-effective, readily scalable extraction method to produce a high purity dairy PLs fraction from an economical source such as dairy by-products.

Developing processing technologies that can be utilized to produce PLs-rich products is a top priority for the United States dairy industry. By applying technologies that were developed for other industries, such as egg and corn processing, and optimizing promising dairy-appropriate technologies, the isolation and concentration of dairy PLs can be achieved. A method that can be utilized for producing an isolated dairy lipid fraction is the simultaneous texturization and extraction of phospholipids (STEP) technique, which was originally developed to extract PLs from egg yolk using ethanol (Wang et al., 2017). This method utilizes a “green” and renewable solvent to create an isolated dairy lipid fraction by effectively denaturing proteins while

solubilizing lipids. Food grade surfactants destabilize the cream emulsion, so an aqueous buttermilk with elevated PLs concentration can be obtained. This buttermilk with elevated PLs concentrations can then be utilized for further PLs isolation and concentration. A similar concept was used in the recovery of corn oil from thick stillage to increase lipid recovery (Fang et al., 2015). These two novel technologies were not initially developed for dairy products but could be modified to apply for dairy PLs concentration. The improvement of dairy PLs concentration technologies is required to produce a dairy lecithin. By modifying and optimizing these technologies an economically scale-able method for creating a dairy lecithin can be obtained.

The thermocalcic aggregation technique was invented to remove lipid interference during the manufacturing of whey protein concentrate. This technique uses a combination of mild heat treatment along with pH adjustment and salt addition to precipitate MFGM components from liquid whey (Rombaut & Dewettinck, 2007). The application of this technique to other dairy products and the addition of an ethanol extraction was performed to obtain isolated lipid fraction.

Another technology that can be optimized is solvent fractionation, which has commonly been used in the dairy industry to fractionate milk fat but has not been used to concentrate PLs. By applying solvent fractionation to the total lipid fraction of a dairy by-products, the production of a dairy lecithin is achievable. Along with this fractionation, the concentration of branched chain fatty acids, which are another unique and beneficial dairy lipid, is obtainable. The manipulation of the fractionation solvent and temperature may lead to the concentration of these valuable dairy components.

With the production of a dairy lecithin there is a need to gain an understanding of the oxidative stability of these lipids. The use of dairy by-products is a concern due to the extensive processing they undergo during manufacturing. Lipid oxidation and the generation of secondary

oxidation products within foods systems are of serious concern because of reduced shelf life and issues related to consumer health (Wang & Wang, 2008). By understanding the oxidative stability of a dairy by-product lipid fraction the quality of the lipid can be understood as well as used to predict how these lipids will affect the quality of the foods when they are used as an ingredient.

All these technologies were utilized in the research summarized in this dissertation to gain an understanding of PLs concentration technologies. The aim of the work described in this dissertation was to develop novel technologies to isolate and concentrate dairy PLs, with the end goal of producing a dairy lecithin. Additionally, gaining an understanding of the oxidative stability of the dairy by-products lipid fraction is vital for its utilization. The concentration of dairy PLs is a complex process and more research is required in this area to bridge the gap between industry and academia. A requirement of these technologies is they must be readily scale-able for the adaption to an industry scale. With this in mind we used technologies from the dairy industry and other industries that have shown promise for PLs isolation. These technologies may be used on low value dairy by-products for the production of a dairy lecithin in the years to come.

Dissertation Organization

Following this overview of why dairy PLs have gained interest throughout the food industry are five research papers and a general conclusion. The format of the five research papers are corresponding to the requirement of the *Journal of Dairy Science*.

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Figures and Tables

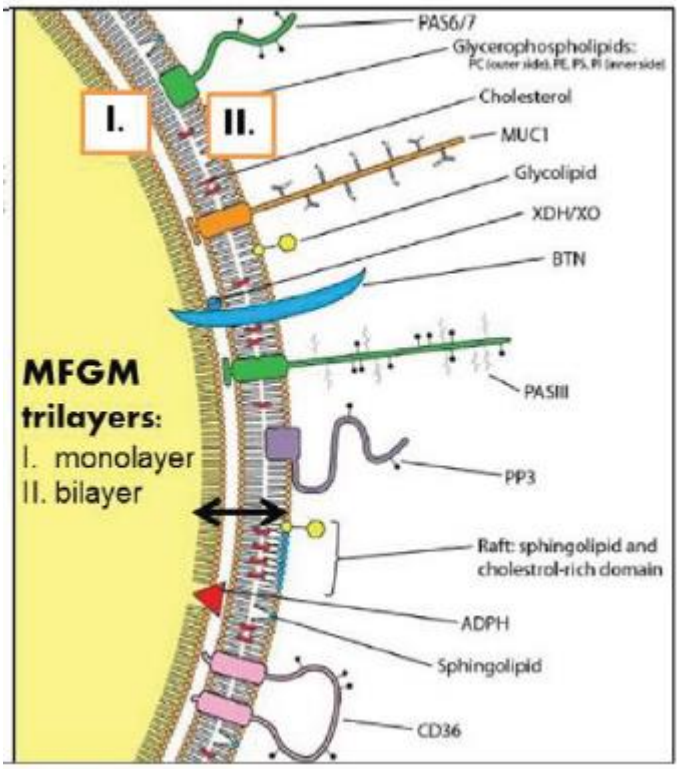

Figure 1. Depiction of the MFGM trilayer membrane (Conway et al., 2014).

Table 1. The PLs content of various dairy products (Rombaut et al., 2006).

Dairy Product	PLs % on Product	PLs % of Total Lipid
Raw Milk	0.04	0.98
Skim Milk	0.02	19.06
Cream	0.19	0.45
Butter	0.23	0.27
Buttermilk	0.16	33.05
Fresh Cheese	0.31	29.06
Acid Whey	0.10	23.66
Whey protein phospholipid concentrate	2.72	29.10
Beta Stream	6.34	48.39

CHAPTER 2: EXTRACTION OF PHOSPHOLIPIDS FROM A DAIRY BY-PRODUCT (WHEY PROTEIN PHOSPHOLIPID CONCENTRATE) USING ETHANOL

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Interpretive Summary

Dairy phospholipids (PLs) are of particular interest because of the higher content of sphingomyelin (SM, 4.1-29.2% of total PLs), and phosphatidylserine (PS, 2.0-16.1% of total PLs) compared to other lecithin sources such as soybean and egg yolk. These PL classes have many health benefits. The current methods that are utilized to concentrate dairy PLs typically produce PL enriched products but not isolated lipid fractions, which may limit their applications. We describe a cost-effective, readily scalable extraction method to produce a valuable dairy lipid extract with high PL purity from a low-value source, whey protein phospholipid concentrate (WPPC).

Abstract

There has been great interest in the phospholipids (PLs) found in dairy products because of their health and functional properties. In this study, a technology that was originally developed for egg yolk PL extraction was applied to whey protein phospholipid concentrate (WPPC). This method successfully precipitated the proteins present in WPPC and extracted the lipids with a renewable alcoholic solvent, ethanol. The effect of ethanol concentration, extraction temperature, and extraction number on the recovery of total lipid, total PLs, and individual PL class was evaluated. The optimum processing conditions for a combined 5-stage sequential extraction for producing a PL-enriched lipid fraction were determined to be 70% ethanol at 70°C, and the total lipid recovery, total PL recovery and PL content achieved were 40.7%, 58.1% and 45.8%, respectively. A lipid fraction with high nutritional value (high content of sphingomyelin or phosphatidylserine) can also be obtained by adjusting extraction conditions and collecting specific fractions, although the yield may decrease. Overall, producing a PL-rich lipid fraction from WPPC using ethanol extraction is feasible and scalable, and different processing conditions can be used depending on the type of lipid product desired.

Key words: dairy lipids, ^{31}P NMR, sphingomyelin, lecithin

Introduction

Phospholipids (PLs), generally referred to as lecithin in the food industry, are a complex class of polar lipids. PLs are amphiphilic because of a hydrophilic head and hydrophobic fatty acid tail (Contarini & Povolo, 2013; Donato et al., 2011), which give PLs excellent emulsification properties and make them important functional ingredients for the food, pharmaceutical, and cosmetic industries. Other than the technological functionalities such as emulsifying and lubricating, PLs also have gained considerable interest because of their nutritional value. Although dairy PLs represent only 0.5-1.0% of total milk lipids (Fong et al., 2007), they are of particular interest because of their higher content of sphingomyelin (SM, 4.1-29.2% of total PLs), and phosphatidylserine (PS, 2.0-16.1% of total PLs) compared to other lecithin sources such as soybean and egg yolk (Burling & Graverholt, 2008; Contarini & Povolo, 2013). SM is reported to play important roles in cell regulation and is referred to as a tumor suppressor (Contarini & Povolo, 2013; Dewettinck et al., 2008; Rombaut et al., 2006). Cognitive performance improvement, which is of particular significance to Alzheimer's disease treatment, has been attributed to PS (Burling et al., 2008; Contarini & Povolo, 2013; Dewettinck et al., 2008; Pepeu et al., 1996; Rombaut & Dewettinck, 2006). Other beneficial biological effects such as reduced cholesterol absorption, antioxidant properties, stress and depression tolerance, and reduced incidence of cardiovascular disease, as well as suppression of multiple sclerosis are also associated with dairy PLs (Contarini et al., 2013; Dewettinck et al., 2008; Rombaut & Dewettinck, 2006). Unlike soy lecithin, which lacks SM (Nejrup et al., 2017), dairy PLs have greater potential to be utilized in infant formula for mimicking human breast milk, which contains SM and PS (Sala-Vila et al., 2005). Utilization of dairy PLs can improve the overall nutritional quality of infant formula as well as the economics of the dairy processing industry.

The majority of the current commercial lecithins are made from soybean and egg yolk. The low PL content in dairy products makes it difficult to extract and concentrate PLs on an industrial scale. Commercial dairy PL concentrates are available that are produced by using technologies such as enzymatic hydrolysis of proteins, microfiltration, ultrafiltration, supercritical fluid extraction, or a combination of these technologies (Spence et al., 2009; Barry et al., 2017; Costa et al., 2010; Astaire et al., 2003; Rombaut et al., 2007). However, these dairy PL concentrates are not isolated lipid fractions, and this may limit their application as an industrial lecithin. The commercial dairy product with the highest PL content (Phospholipid Concentrate 700) is manufactured by Fonterra Co-operative Group Ltd. (Auckland, New Zealand), which contains up to 60% w/w PLs (Fong et al., 2013). The processing steps that this product undergoes are an industrial trade secret. This product contains a substantially higher amount of PL than any other product reported in the literature. Previously, the highest PL content dairy product reported was 23.7% on a dry basis, concentrated from butter serum using microfiltration (Le et al., 2010). The major limiting factor with microfiltration of buttermilk and butter serum is the retention of casein, which limits how concentrated the PLs can become. Developing processing technologies that can be utilized to produce PL-rich products is a top priority for the dairy industry. Moreover, certain feedstock and technologies utilized for producing the PL concentrates are not always cost-effective and this leads to low feasibility for commercial scale production. Therefore, there is a need to develop a more cost-effective, readily scalable extraction method to produce a high purity dairy PL fraction from an economical source other than buttermilk and butter serum.

Whey protein phospholipid concentrate (WPPC) is a by-product that is microfiltered from cheese whey during the production of whey protein isolate (WPI). WPPC contains 63-72%

whey protein, 12-20% fat and approximately 20% of the total lipid content is PLs (Li et al., 2016). It is a highly under-utilized product and would be a great feedstock for dairy PL concentration. A method that can be utilized for producing WPPC lipid with high PL content is the simultaneous texturization and extraction of phospholipids (STEP), which was originally developed to extract PL from liquid egg yolk using ethanol (Wang et al., 2017). The principles in the STEP method may be applied to WPPC for PL concentration, but modifications are needed, as the composition of egg yolk and WPPC are very different.

The use of ethanol was investigated to extract PLs from WPPC. We hypothesized that 1) lower ethanol concentration can lead to a higher PL content in the final product, while higher ethanol concentration can lead to higher recovery yield of total lipids and PLs due to the different affinities of lipids to solvent with different polarity; 2) higher extraction temperatures can result in higher PL content as well as higher recovery of total lipids and PLs due to a more complete denaturation of protein and better lipid solubilization in ethanol. To test these hypotheses, different ethanol concentrations and temperature conditions were used for the extraction, and total lipid and PL recovered from WPPC were quantified. Each individual PL class composition was also quantified to evaluate the effect of ethanol concentration and temperature on recovery of each specific class of PLs.

Materials and Methods

The WPPC used in this study was provided by Bongards' Creameries (Perham, MN). The WPPC was frozen (-20°C) before treatment to guarantee a consistent product quality for each replicate extraction. The extracted lipid was then quantified for PL using ^{31}P NMR (Wang et al., 2014). Reagent-grade solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Extraction of Phospholipids from WPPC

The extraction procedure, which has similar extraction principle as the STEP method (Wang et al. 2017; Wang et al; 2016), was performed using WPPC as a feedstock. A laboratory extraction system (Figure 1) designed for the STEP method on egg yolk was adapted to extract total lipid and PLs from WPPC. Aqueous ethanol, with final concentrations of 70, 80 and 90%, was used and the temperature of the solvent in the solvent reservoir and extraction cylinder was maintained at 60, 70, and 80°C by using a hot water jacket heating system (Haake DC 10, Thermo HaakeD-76227 Karlsruhe-Germany). Liquid WPPC, in the form of a thin stream, was injected using an 18G1$_{1/2}$ PrecisionGlide Needle (Becton-Dickinson & Co.; Franklin Lakes, NJ) into the hot solvent and the protein in WPPC was denatured and solidified into short strings upon contact with ethanol. The liquid WPPC was heated to 60°C prior to injection to make the product more flowable and it was metered using a peristaltic flow pump (UL 3101-1, Wheaton Science Products) at a speed of 0.67 g/sec through the syringe needle. The needle was manually rotated in a circular motion below the surface of the solvent to obtain uniform protein denaturation and to produce a thin diameter WPPC string. Liquid WPPC (100 g) was spun into various amounts of ethanol (depending on ethanol concentration; 187, 320, 720 mL 100% ethanol were used for 70, 80, and 90% ethanol concentration treatment). After protein denaturation, WPPC was immersed in the solvent for 6 min before drainage of the miscella for 1 min. The first extraction was considered complete after the miscella was collected, and then four subsequent extractions were carried out on the partially delipidated WPPC using the same conditions, except only 100 mL of the ethanol solvent was used. For the first extraction, the ethanol solvent concentration was 100% so that the concentration of the mixture would be 70, 80 or 90% after WPPC was injected (taking the moisture content of WPPC into consideration). For

all subsequent extractions, pre-prepared solvent solutions with 70, 80 and 90% ethanol were used. Overall, five samples of miscella were produced and collected. The solvent in each miscella was removed by a rotary evaporator and the lipids were further dried in a vacuum oven at 40°C overnight before weighing.

Total lipid in WPPC extracted by using the Folch method (Rodriguez-Alcala & Fontecha, 2010; Folch and Stanley, 1957) was used as a control. The lipid was extracted by using 2:1 v/v chloroform-methanol, and PL composition of the lipid was analyzed using the method reported in the following section.

Quantification of Phospholipids

The PL content and the class composition were determined by using ^{31}P NMR, following a method reported by Wang et al. (2014), with minor modifications. About 0.2 g of lipid extracted from WPPC was dissolved in 12 mL of chloroform/methanol (2:1 v/v) and then washed with 3 mL of K-EDTA (0.1 M, pH 7.0). The denser chloroform phase, which contains the lipid, was collected and the residual water was removed by using 0.5 g of anhydrous sodium sulfate. Then, the solution was filtered through a 0.45 µm polytetrafluoroethylene filter disk and dried using a rotary evaporator. About 200 mg of the dried lipids were dissolved in 1 mL of chloroform-*d* and 1 mL of methanol with the addition of 100 to 150 mg of triphenyl phosphate (TPP) as an internal standard. Cs-EDTA (1 mL, 0.2 M, pH 8.5) was added to the sample solution, and the mixture was centrifuged at 1,800 X g for 2 min after 10 sec of vigorous mixing. The denser chloroform-*d* phase was then collected into NMR tubes and subjected to ^{31}P NMR analysis. The NMR spectra were obtained with a Bruker Avance III NMR Spectrometer (Billerica, MA and Karlsruhe, Germany) using a Bruker narrow bore 14.1 tesla superconducting magnet operating at 162 MHz. Samples were analyzed with an inverse gated decoupling pulse

sequence. The NMR spectroscopic scan conditions were as follows: probe temperature of 30ºC; pulse width of 22 μs; sweep width of 9718 Hz; acquisition time of 1.2 sec; relaxation delay of 10 sec; and a total of 384 scans. The chemical shifts were recorded relative to TPP (δ −17.8). The relative composition percentage was expressed in molar percentage relative to the sum of all PL classes.

Statistical Analysis

All temperature and alcohol concentration treatments were randomized in the order of extraction, with two replicates for each treatment. Data collected were analyzed by using a Statistical Analysis System (SAS 9.1) (SAS institute, Cary, NC), and a Tukey test was used to determine significant differences at P = 0.05. The relative average deviation from the mean for all treatments was calculated and presented as the error bar on the charts.

Results and Discussions

WPPC Composition

The composition of the initial liquid WPPC used for the extraction is shown in Table 1. It contained 20.2% total solids, with a total lipid content of 5.5% (27.0% on a dry basis), and a PL content of 1.6% (29.1% on a fat basis). The protein content of WPPC is approximately 65% on a dry basis, which equates to 13.1% as-is, with the remainder of the product being ash. Since WPPC contains such a high amount of protein, the denaturation of the protein is vital for the success of the PL extraction. The effective precipitation of the protein and dissolution of PLs during the extraction are necessary.

The major PL classes in the initial liquid WPPC (Table 1), characterized by ^{31}P NMR, were phosphatidylethanolamine (PE; 47.8% of the total PLs) and phosphatidylcholine (PC; 31.0% of the total PLs). Lower amounts of PS, SM, and phosphatidylinositol (PI) were

measured at 8.2, 7.9, and 5.1% of the total PLs in WPPC, respectively. Similar results were found by other researchers for WPPC and whey based powders (Levin et al., 2016; Boyd, et al. 1999), with PE and PC being the most abundant PL classes. Since WPPC has a relatively low content of SM and PS, it is important for the extraction process to have a high affinity for these PL classes, so the final product can have the highest possible concentration of PL in the final product, as well as enriched SM and PS.

Effect of Extraction Temperature and Ethanol Concentration on Lipid Recovery Total Lipid Recovery

The total lipid recovery was calculated by adding all 5 sequential extractions together to obtain a complete recovery for each treatment (Figure 2). The yield of each extraction was calculated based on the total lipid present quantified by the Folch extraction. The Folch extraction yielded 5.5% total lipid, which equates to 5.5 g of lipid for each 100 g of WPPC injected into the extraction system. This extraction resulted in a complete lipid recovery and a 100% yield was based on recovering 5.5 g of lipid from the initial WPPC.

The effect of extraction temperature on total lipid recovery was examined and results are shown in Figure 2. Temperature was a significant factor in the recovery of total lipid ($P < 0.05$). Total lipid recovery increased as the extraction temperature increased. Increasing the temperature from 60 to 70°C and 60 to 80°C significantly increased the total lipid recovery ($P < 0.05$). However, increasing the temperature from 70 to 80°C did not significantly affect total lipid recovery ($P > 0.05$), as shown a plateau or even a slight decrease in total lipid recovery when the 70 and 80% ethanol concentrations were compared. This is due to temperatures above 70°C not being as effective at further increasing the degree of protein denaturation. The denaturation temperature of milk whey proteins is near 70°C (Walstra et al., 2006). Overall, it

was determined that the optimum temperature for protein denaturation is 70°C for WPPC and total lipid recovery values were 40.7, 56.3, and 93.8% at 70, 80, and 90% ethanol concentrations.

The effect of ethanol concentration on total lipid recovery was also examined. There was a significant increase in total lipid recovery as the ethanol concentration increased ($P < 0.05$; Figure 2). It was observed that 90% ethanol concentration resulted in the highest total lipid recovery for all the temperature treatments and a total lipid recovery of 101.7% was achieved at 80°C. The higher total lipid recovery with increasing ethanol concentrations is probably due to a higher degree of protein denaturation along with increased lipid solubilization in higher concentration of ethanol. Another factor that may have contributed to the near complete extraction with 90% ethanol is that for the first extraction, different quantity of 100% ethanol had to be used to achieve the concentration desired. Therefore, the solvent and WPPC ratios were 1.9, 3.2, and 7.2 for the 70, 80 and 90% ethanol treatment, respectively. The higher amount of ethanol use at 90% treatment may have led to a better separation of lipid from protein in the denatured WPPC protein.

Total PL Recovery

The total PL recovery was calculated by adding all 5 sequential extractions of PLs to obtain a complete recovery for each treatment (Figure 3). The yield of each extraction was calculated based on the total PL content quantified by the Folch extraction. The Folch extraction yielded 1.6% total PL, which equates to 1.6 g of PL for each 100 g of WPPC (29.1% PL of total lipid fraction) injected into the extractor.

The PL recovery displayed a trend similar to that of total lipid, where higher total PL recoveries were obtained at higher ethanol concentrations and temperatures (Figure 3). The temperature of the extraction had a significant effect on the total PL recovery ($P < 0.05$).

Increasing the temperature from 60 to 70°C led to a significant increase in total PL recovery for all three ethanol concentrations ($P < 0.05$), while increasing the temperature from 70 to 80°C led to an unchanged (70 and 90% ethanol) or a decrease in total PL recovery (80% ethanol). The reduction may be due to the high degree of protein denaturation and the entrapment of PLs in the protein matrix. Because of the higher amount of solvent used for the 90% ethanol treatment, this reduction was not as obvious. These results were similar to the recoveries of total lipid. Overall, the 70°C treatment resulted in the highest PL recovery, and the values are 58.1, 75.4, and 100.8% at 70, 80, and 90% ethanol concentration, respectively.

The concentration of ethanol was a significant factor for total PL recovery ($P < 0.05$). The total PL recovery increased as the ethanol concentration increased. Similar effect of ethanol concentration on PL recovery was also reported by Wang et al. (2017). Overall, for the most complete recovery of PLs, the optimal ethanol concentration for total PL recovery was determined to be 90% ethanol and at 70ºC if not considering the PL content in the final lipid extract.

Effect of Ethanol Concentration and Temperature on the Efficiency of Lipid Recovery Total Lipid Recovery Profile

A total of 5 sequential extractions were completed to simulate industrial multiple stage extraction and determine the "speed" of lipid depletion from the WPPC. The amount of total lipid recovered by each extraction was quantified to determine the extraction efficiency or speed. Figures 4a-4c indicate that the first extraction always resulted in significantly more total lipid recovery than the other extractions ($P < 0.05$). The majority of the lipids were recovered with the first two extractions and there was no significant increase in the amount of total lipid recovered in extractions numbers 3, 4, and 5 ($P > 0.05$). Figures 4a-4c also show that 90% ethanol resulted

in higher total lipid recovery efficiency than 70 and 80% ethanol, while the 80°C temperature treatment resulted in the highest total lipid recovery with 73.2% of the total lipid being recovered during the first extraction. It can also be observed that for all the temperature treatments, less total lipid was recovered during the first extraction as the ethanol concentration and temperature decreased. It was expected that the first extraction would result in the most total lipid recovery since the majority of the protein denaturation and lipid solubilization occurs during this extraction. Figure 4a shows that the 70% ethanol at 60°C treatment resulted in the lowest total lipid recovery, 5.4%, for the first extraction, and the total lipid recovery was significantly increased when the temperature increased above 60°C. This is most likely due to poor protein denaturation at 60°C, while increasing the temperature significantly improved protein denaturation and lipid solubility. Overall, statistical analysis showed that both temperature and ethanol concentration were significant factors for the total lipid extraction efficiency or speed of the process ($P < 0.05$).

Total PL Recovery Profile and PL Content in the Product

The efficiency of the PL recovery is shown in Figure 5a-c and Table 2. A trend similar to that of total lipid extraction was observed. However, as the ethanol concentration increased from 70 to 90%, the content of PL in each of the lipid fraction decreased in general (Table 2), although total PL recovery increased. This indicates that lower ethanol concentrations have a stronger affinity for PLs, while higher ethanol concentration extracted more total lipid. The treatment that resulted in the highest PL content in the lipid fraction for all 5 extractions was the 70% ethanol at 70°C treatment (36.8-65.6% PL). The optimum temperature for PL recovery from WPPC was 70°C and it was also observed that 60°C had a slight advantage for PL content in general than

that at 80°C. This is because the higher temperatures resulted in a significantly higher neutral lipid recovery, which relatively lowered the proportion of PL in the final lipid fraction.

The first extraction always resulted in significantly more PL recovery than the other extractions ($P < 0.05$). The majority of the PLs were recovered with the first two extractions and there was no significant increase in the amount of PL recovery in extractions numbers 3, 4, and 5 ($P > 0.05$). All 5 extractions were completed to maximize overall recovery and to determine if a complete PL recovery could be obtained. The highest PL recovery in the first extractions was seen during the 90% ethanol at 80°C treatment. In this treatment 84.2% of the total PL was recovered, but the PL content in this lipid fraction was only 33.9%, which is similar to the WPPC's PL content of 29.1%. The PL content of the four remaining extractions were all less than 16.3%. Even though the 5 extractions in this treatment resulted in a total PL recovery of 100.8%, higher amount of neutral lipids was also recovered, leading to an overall lipid fraction with a lower PL content. Similar recoveries were observed with the 90% ethanol at 70°C treatment.

The highest PL content lipid fraction was obtained during the 70% ethanol at 70°C treatment. The 5th extraction of this treatment resulted in a lipid fraction with a PL content of 65.6% but, only 2.6% of total PLs was recovered during this extraction. The PL content in the extracted lipid fraction increased relatively as less total lipid was recovered. Ethanol at this temperature and concentration also seemed to have a higher affinity for PL during the later stages of these extractions. For the combined 5-fraction product, the 70% and 70°C conditions also gave a lipid fraction that contains 45.8% PLs, and it represented 40.7 and 58.1% of total lipid and PLs in the original WPPC as discussed previously.

Overall, the extraction method is capable of producing a high PL content lipid fraction, but total PL recovery also needs to be considered to make this a feasible method for industrial application. The optimal treatment should give a high PL recovery while also producing a high PL content lipid fraction. From an industry standpoint, the number of extractions could potentially be reduced to as few as one or two extractions due to these extractions being able to recover majority of lipid. If one extraction is utilized, the 90% ethanol at 70°C treatment will likely lead to optimal efficiency; in the present study it resulted in a high total lipid recovery (62.1%) and PL content of 39.8% (Table 2). However, if higher PL content lipid fraction is needed, ethanol concentration of 70% and 70 °C should be selected, as such conditions produced a lipid fraction with the highest average PL content (Table 2). There is a delicate balance among all of these parameters. These results should provide an insightful direction for further industrial scale optimization trials.

Effect of Temperature and Ethanol Concentration on PL Class Composition of the Products

The PL class composition of each extracted lipid was determined to evaluate the effect of temperature and ethanol concentration (Table 2). SM and PS are the PL classes of specific interest due to their health benefits, and the enrichment of these PLs during the extraction process is desired. Table 2 shows that the 70% ethanol at 60°C treatment resulted in a lipid fraction with the highest SM content (17.5-23.8%). This is a significantly higher SM enrichment when compared to the 70 and 80°C treatments ($P < 0.05$). When the temperature was 60°C, a more aqueous alcohol solvent system had a higher affinity towards SM compared to other classes of PL. Compared to the starting SM content in WPPC, which is 7.9% of PL, an increase in SM content of about 3 times was achieved with the 70% ethanol at 60°C extraction. However, the total lipid and PL yield (13.6 and 15.7%) and the PL content (33.7%) are all very low.

Table 2 also shows that SM percentage decreased when ethanol concentration was increased from 70 to 80% and 80 to 90%. Although not as significant as temperature, ethanol concentration played an important role. With a lower ethanol concentration, the solvent system had a higher polarity, which favors the extraction of SM, since this PL class is the most polar class found in WPPC (Christie and Han, 2010). Lower ethanol concentrations and extraction temperatures tended to produce lipid fractions with higher SM content.

Regarding PS, it was observed (Table 2) that only the extraction number had a significant impact on the enrichment of PS ($P < 0.05$). There was a slight increase in PS concentration as the extraction temperature increased, but it was not statistically significant. According to Table 2, higher PS enrichment can be achieved during the later extractions. This is due to other PL classes being extracted more efficiently during the first extractions, leading to less competition for PS during the later extractions. For all ethanol concentrations and extraction temperature, PS concentration tended to increase with the extraction number. The highest overall PS recovery was achieved by using the 70% ethanol at 80°C treatment, and PS content of the resulted lipid fraction was 1.3 times higher than the starting WPPC. PS did not become as concentrated as SM during extraction, which indicates that ethanol had a higher affinity toward SM than PS. Since PS was extracted more efficiently during the later extractions, this extraction process may not be feasible to target this PL class from WPPC.

Other PL classes such as PC, PI and PE all responded to ethanol concentration, extraction temperature, and extraction numbers differently. Lower ethanol concentration and temperature tend to result in a higher PC enrichment. Higher ethanol concentration led to higher enrichment of PI. Higher temperature promoted PE recovery, but ethanol concentration seemed to have little influence.

Overall, recovery from multiple stage extraction with 90% ethanol and at 70 or 80°C will give near complete extraction of total lipid and PLs, but the product will not have PL enrichment. Such total lipid maybe fractionated further to separate the two lipid classes. If PL content is a primary concern, the 70% ethanol at 70°C will give a product with 45.8% PLs, but with 40.7 and 58.1% total and PL recovery from the WPPC, and this incomplete lipid recovery may cause a concern.

Conclusions

The modified STEP method was shown to be effective and efficient in producing dairy PL concentrate. Higher ethanol concentration and extraction temperature led to a higher total lipid recovery, while lower ethanol concentration solvent had a greater affinity for PLs. Different classes of PL responded to ethanol concentration and extraction temperature differently. The optimum processing condition is dependent on the desired PL content and recovery yield. The highest total lipid recovery can be achieved with 90% ethanol at 70 and 80°C, and lipid fraction with highest PL content can be obtained using 70% ethanol at 70°C. The highly nutritionally valuable PL classes, SM, was favored during the 70% ethanol at 60 °C while PS enrichment required removal of other lipids first. Overall, a dairy PL concentrate comparable to the commercial lecithin product can be produced with extraction method demonstrated in this study.

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Figures and Tables

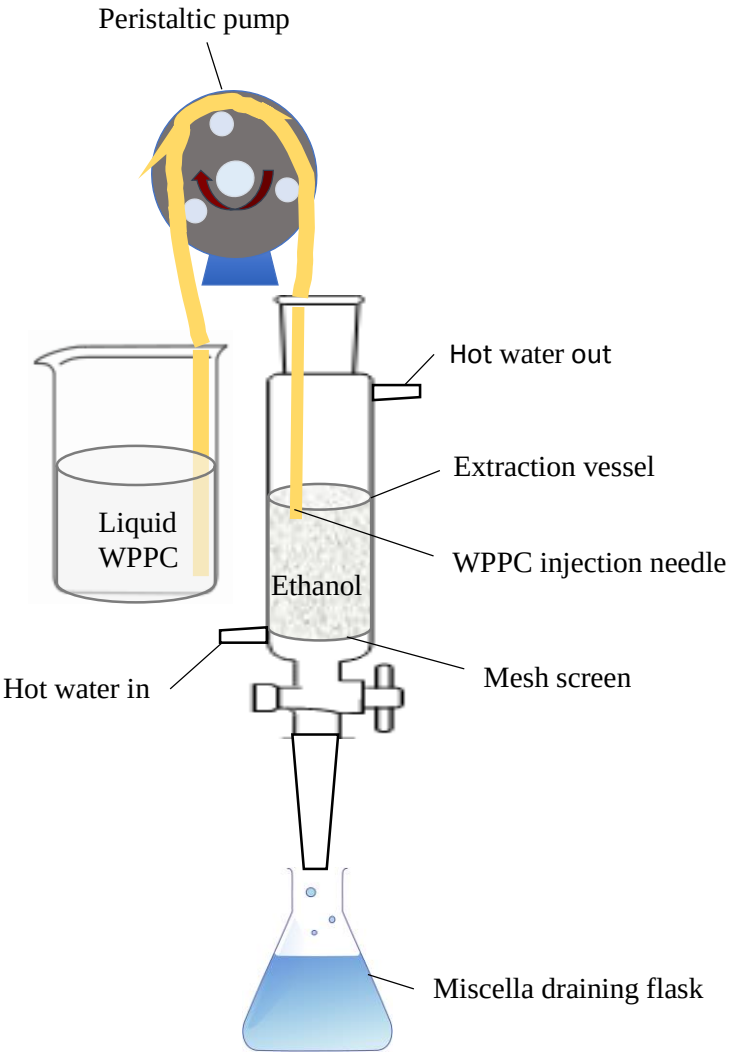
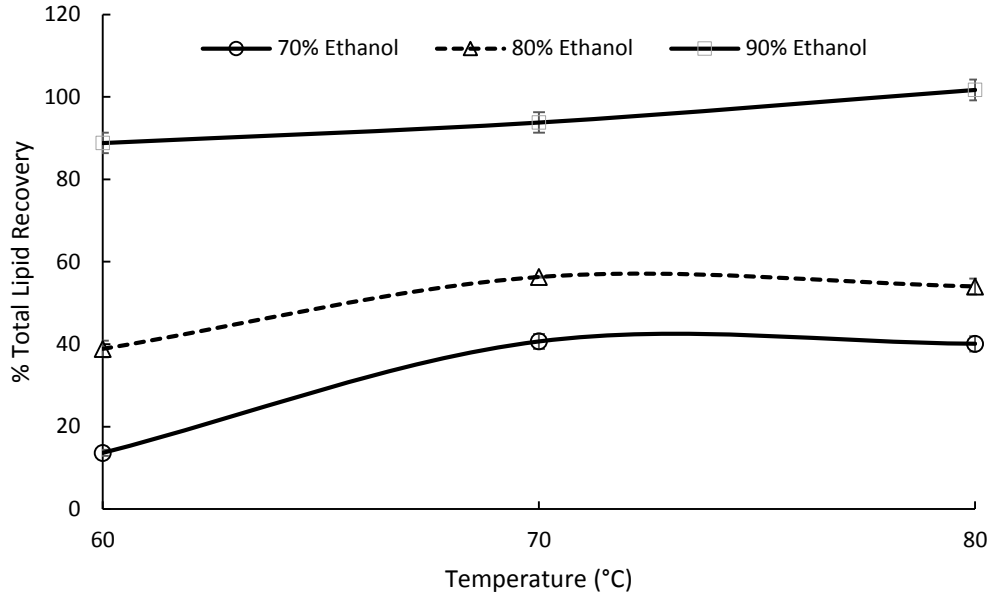
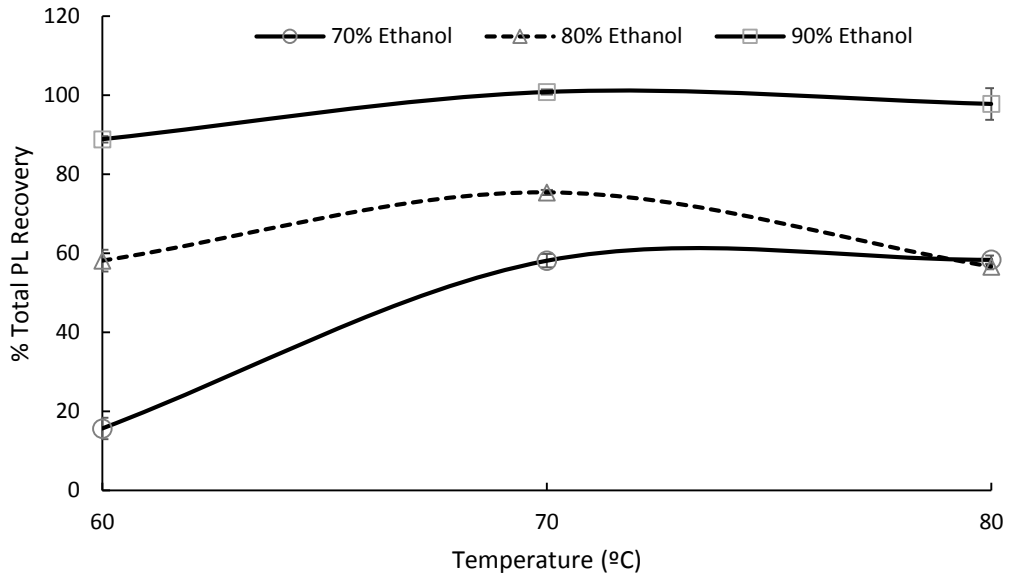


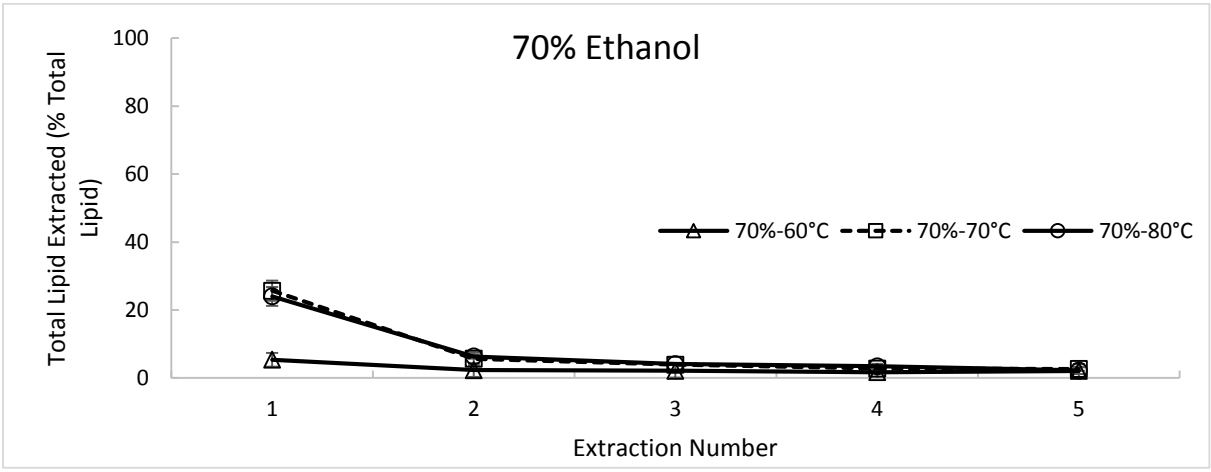
Figure 1. Solvent extraction system used for extraction of phospholipids from WPPC.

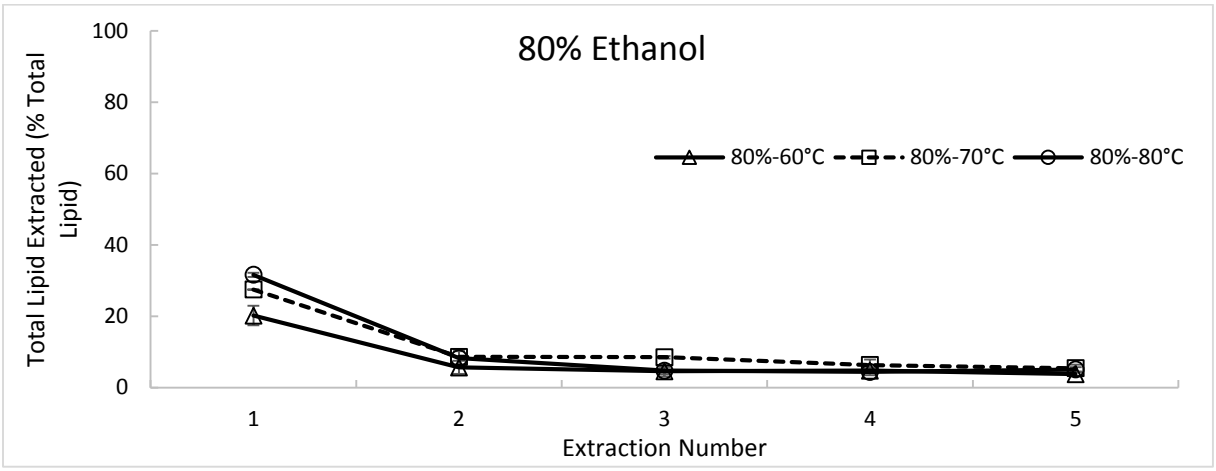


Figures 2. The effect of extraction temperature and ethanol concentration on total lipid recovery from WPPC.



Figures 3. The effect of extraction temperature and ethanol concentration on total PL recovery from WPPC.





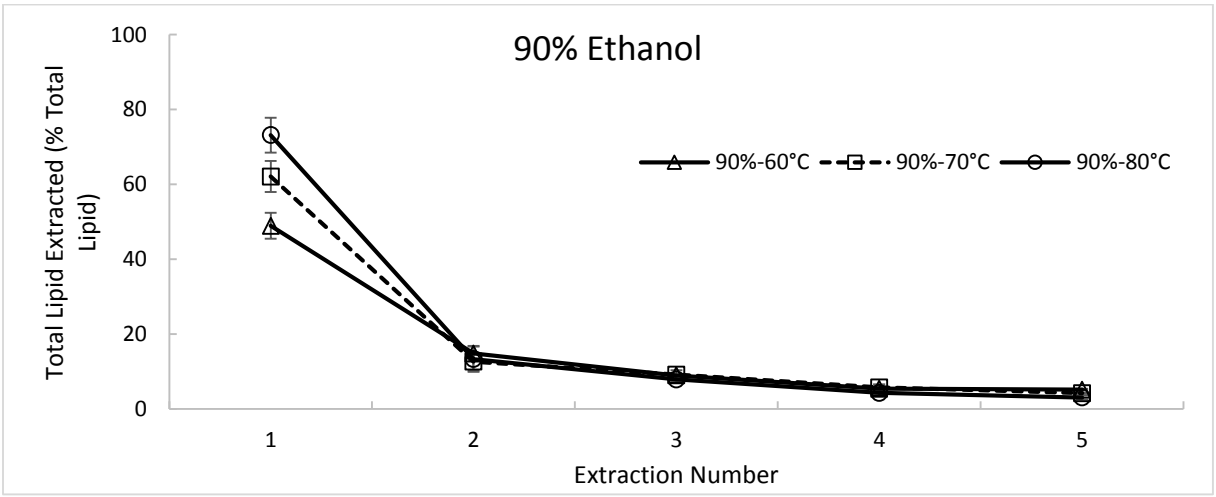


Figure 4. Effect of extraction number, temperature, and ethanol concentration on total lipid recovery.

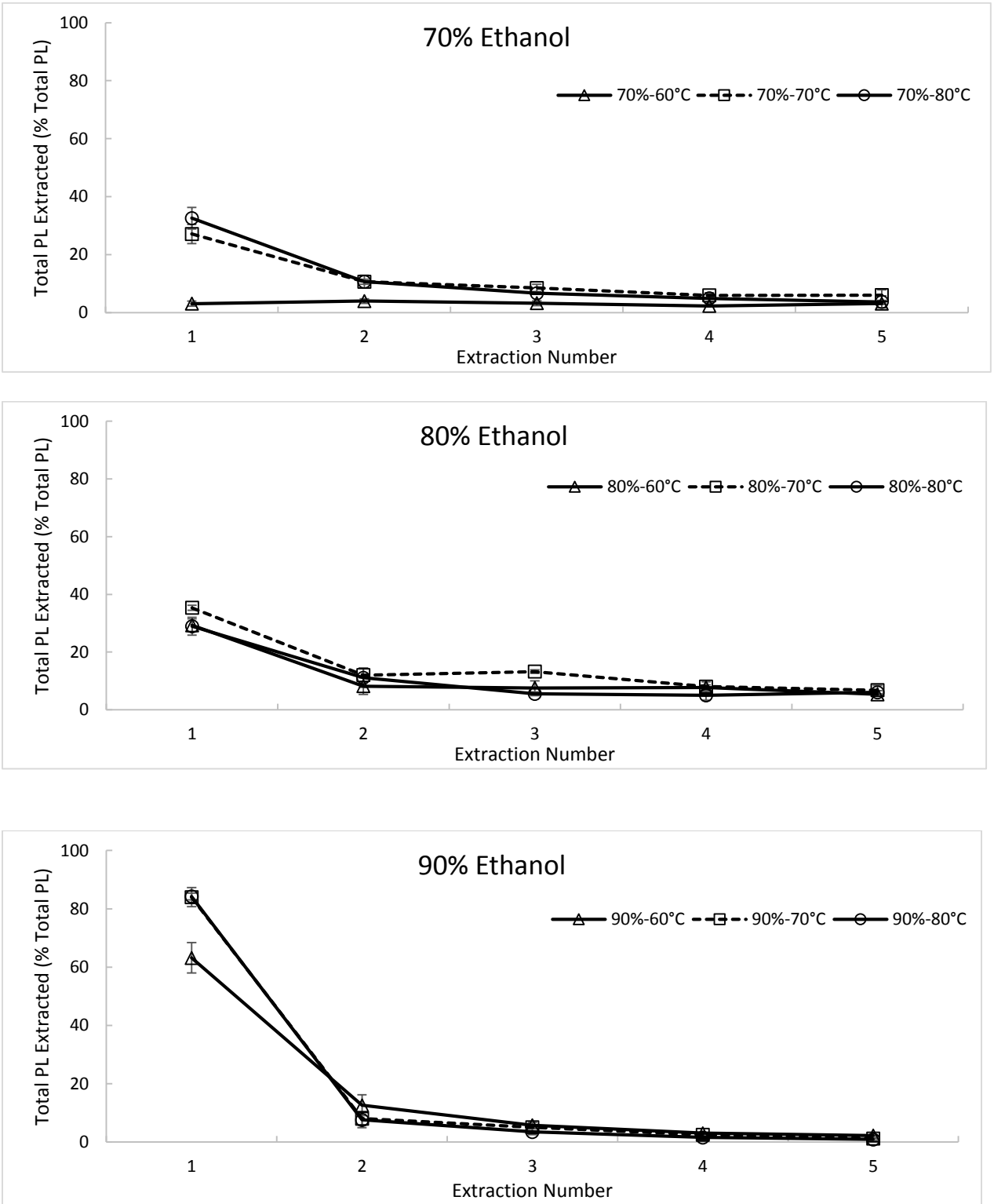


Figure 5. Effect of extraction number, temperature, and ethanol concentration on total PL recovery (relative to total PLs recovered).

Table 1. The PL class distribution of the liquid whey protein phospholipid concentrate compared to other studies on whey protein concentrate powder and milk fat.

	SM	PE	PS	PI	PC	PL (% Total Fat)
WPPC	7.9 ± 0.1	47.8 ± 1.4	8.2 ± 0.1	5.1 ± 0.2	31.0 ± 0.7	29.1 ± 0.7
Whey Powder*	3.9	36.0	8.9 (PS+PI)		51.2	31.4
Milk#	21.3	32.6	5.3	7.6	33.2	0.5-1.0

* Results from Boyd et al. (1999); PS and PI results were quantified together
# Results from Fong et al. (2007) on milk fat

Table 2. Total phospholipid content of each and combined extraction and phospholipid class composition.

Extraction #	Treatment	Relative Composition of PLs (% Total PL)					Total PL (% total Lipid)
		SM	PE	PS	PI	PC	
1	70%-60°C	21.0	34.1	3.5	5.2	36.2	16.7
2	70%-60°C	18.9	33.8	5.9	5.6	35.8	50.2
3	70%-60°C	21.8	30.5	6.0	6.9	34.8	43.9
4	70%-60°C	23.8	29.0	5.7	7.3	34.2	39.3
5	70%-60°C	22.2	29.6	7.6	7.4	33.2	44.0
	Combined	21.3	32.2	5.2	6.1	35.2	33.7
1	70%-70°C	11.5	38.7	4.8	8.7	36.3	36.8
2	70%-70°C	8.3	43.5	8.7	8.0	31.5	57.6
3	70%-70°C	9.4	43.1	8.6	9.1	29.8	63.5
4	70%-70°C	8.6	41.5	12.2	8.9	28.8	61.6
5	70%-70°C	9.5	42.3	11.8	9.0	27.4	65.6
	Combined	10.5	40.2	6.7	8.7	33.9	45.8
1	70%-80°C	8.3	42.6	8.1	6.9	34.1	39.8
2	70%-80°C	8.2	45.3	9.5	7.4	29.6	49.5
3	70%-80°C	9.0	44.2	11.0	7.9	27.9	47.8
4	70%-80°C	11.4	41.9	12.7	8.0	26.0	40.4
5	70%-80°C	9.9	45.8	12.3	7.4	24.6	45.4
	Combined	8.7	43.3	9.2	7.2	31.5	42.5
1	80%-60°C	13.0	37.9	4.5	6.4	38.2	42.9
2	80%-60°C	15.0	38.0	5.7	6.6	34.7	42.1
3	80%-60°C	11.6	40.8	7.8	7.7	32.1	48.7
4	80%-60°C	15.9	38.4	8.3	8.1	29.3	48.3
5	80%-60°C	12.4	41.3	10.4	8.5	27.4	42.5
	Combined	13.4	38.6	6.1	7.0	34.9	44.1

Table 2. (continued)

1	80%-70°C	9.4	43.8	3.5	6.8	36.5	37.7
2	80%-70°C	8.4	46.1	6.6	6.7	32.2	41.4
3	80%-70°C	12.7	43.3	5.9	6.9	31.2	45.7
4	80%-70°C	10.1	45.4	7.1	6.4	31	37.8
5	80%-70°C	15.5	44	12.2	9.5	18.8	36.3
	Combined	10.4	44.3	5.6	7	32.7	39.3
1	80%-80°C	7	43.6	7.5	6.2	35.7	26.9
2	80%-80°C	12.2	42	8.8	7.3	29.7	39
3	80%-80°C	9.8	43.1	10.9	8.4	27.8	35
4	80%-80°C	9.9	42.1	12.6	9	26.4	32.5
5	80%-80°C	10.5	41.9	13.9	9.2	24.5	35.2
	Combined	8.6	43	9	7.1	32.3	30.7
1	90%-60°C	14.8	36.3	7.7	7.1	34.1	37.9
2	90%-60°C	12	45.7	6.9	6.7	28.7	24.5
3	90%-60°C	16.5	40.4	9	8.7	25.4	18.9
4	90%-60°C	15.7	38.9	13.6	11.4	20.4	16.5
5	90%-60°C	11.6	42.4	16.2	14.5	15.3	12.7
	Combined	14.3	39	8.6	7.9	30.1	30.5
1	90%-70°C	9.6	43.8	7.4	6.9	32.3	39.8
2	90%-70°C	14	43.5	7.9	7.3	27.3	18.4
3	90%-70°C	15	39	12.8	10.6	22.6	16.2
4	90%-70°C	13.7	34.2	21.4	15.2	15.5	12.7
5	90%-70°C	16.2	45.1	0	21.3	17.4	8.7
	Combined	11.3	42.8	8.5	8.5	29	31.6
1	90%-80°C	7.7	46.1	6.6	7.3	32.3	33.9
2	90%-80°C	14.7	41.9	9.1	8.7	25.6	16.3
3	90%-80°C	14.1	36.7	13.9	13.2	22.1	12.7
4	90%-80°C	17.1	43.6	0	19.8	19.5	10.1
5	90%-80°C	20.6	39.7	0	12.3	27.4	8.3
	Combined	9.9	44.5	7	8.6	29.9	28.2
	WPPC Folch Extraction	7.9	47.8	8.2	5.1	31	29.1

*The relative average deviation from the mean for all values ranged from 0.0-6.9.

CHAPTER 3: THE USE OF SURFACTANT TO DISRUPT CREAM EMULSION AND INCREASE PHOSPHOLIPID CONCENTRATION IN BUTTERMILK

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Interpretive Summary

The phospholipids (PLs) content in cow's milk is very low (0.5 to 1% of the total lipids), but buttermilk (BM), a by-product of butter production, has a much higher PLs content (13-30% of total lipids). It is a feasible starting material for extracting dairy PLs. With the destabilization of the oil-in-water emulsion of cream, the movement of PLs from the interface to the aqueous BM can occur with the use of food-grade surfactants. The effect of hydrophilic-lipophilic balance (HLB) and concentration of surfactants (Tween 80 and Span 80 mixtures), churning temperature, and cream handling conditions on the PLs distribution in BM was investigated. Using a surfactant mixture with HLB value of 12.9 and at concentration of 750 ppm, a BM fraction with increased PLs concentration can be produced and potentially utilized for further PLs extraction.

Abstract

Buttermilk (BM) is a by-product of butter production from cream that contains a relatively high level of phospholipids (PLs). Success in further increasing the concentration of PLs of BM can make it a good feedstock for producing an economical dairy PLs product. The effect of various hydrophilic-lipophilic balance (HLB) and concentration of surfactants, churning temperature, and cream handling conditions on the PLs content in BM was evaluated, and the optimal conditions for shifting the PLs from butter to BM during cream churning were identified. A mixture of Tween 80 and Span 80 with HLB value of 12.9 was the most successful in disrupting the oil in water cream emulsion. The surfactant concentration of 750 ppm resulted in a BM with the highest PLs content (36.7%), although it was not statistically significantly higher than the control BM (30.4%). Churning at 10°C resulted in a BM with higher PLs content compared to churning at 35°C and 60°C. At pH of 4.6 and 5.1, cream emulsions were significantly destabilized, and significantly larger particle sizes (24.8 and 26.4 μm compared to an average particle size of 4 μm in native cream) were observed. Overall, the use of a combination of surfactants with HLB of 12.9 at 750 ppm, and with churning temperature of 10°C improved PLs content in BM, but opportunities exist to further explore a more effective means of changing PLs distribution.

Key Words: Surfactants, dairy, particle size, emulsion disruption, polar lipids

Introduction

Phospholipids (PLs) only account for 0.5-1.0% of the total lipid in raw milk (Bourlieu et al., 2018; Conway et al., 2014). Thus, raw milk is not ideal for producing a dairy lipid fraction with a high concentration of PLs, as it would take 2,500 to 3,300 kg of raw milk to produce one kg of pure PLs. Selecting a better starting material for the production of a dairy PLs concentrate is important if dairy is to compete with plant-based PLs products.

Cream is an emulsion with approximately 40% fat, which is encompassed by the milk fat globular membrane (MFGM). In recent years, the MFGM has attracted significant interest due to its potential health benefits and functional properties (Contarini and Povolo, 2013). Because of its cellular membrane origin, MFGM is the richest source of PLs, glycolipids, gangliosides, and glycoprotein in milk (Jimenez-Flores et al., 2008) and the approximate ratio of proteins, polar lipids, and carbohydrates within the membrane is 4:3:1 (Spence et al., 2009). Using cream as the feedstock for producing high purity PLs could be an option, however, it would still be uneconomical due to the high value of cream.

Buttermilk (BM) is produced when cream is churned into butter; BM has a much higher concentration of PLs (13-30% of total lipids) than raw milk and cream (Bourlieu et al., 2018). Therefore, BM is a potential starting material for producing PLs concentrate. However, a significant amount of dairy PLs remain in the butter fraction because only 40% of the total PLs flow to the aqueous BM (Rombaut et al., 2006). Shifting the distribution of PLs to the BM fraction during the churning of cream can make BM an even better feedstock for producing a PLs concentrate. A potential method to shift PLs distribution from cream to BM is by using food grade surfactants such as Tween 80 and Span 80 that may disrupt the MFGM. Surfactants that can lower the interfacial tension between two insoluble phases may displace the MFGM's

components and send more PLs to the aqueous BM. A similar concept was used in the recovery of corn oil from thick stillage and it was shown to be effective in destabilizing emulsions to increase the recovery of corn oil (Fang et al., 2015).

In this study, food grade surfactants with various hydrophilic-lipophilic balance (HLB) values and concentrations were used to disrupt the MFGM. The effect of churning temperature, pH adjustment, tempering, additional shear, churning speed, and without pasteurization were examined in an attempt to improve the distribution of PLs to BM. We hypothesized that food grade surfactants with certain HLB can disrupt the MFGM and move a higher fraction of PLs to the aqueous BM phase. We also hypothesized that modifying cream handling conditions would result in the production of butter oil and a BM fraction containing all the PLs from the original cream. With the resulting aqueous BM phase containing a higher concentration of PLs, the production of a PLs concentrate from cream can be more economical.

Materials and Methods

Unstabilized heavy whipping cream (Anderson Erickson Dairy; Des Moines, IA) was purchased from a local super market (Walmart in Ames, IA). Reagent grade solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Surfactant Preparation

Surfactants with different HLB values were obtained by mixing Span 80 and Tween 80 in different ratios. Span 80 is a lipophilic surfactant with HLB value of 4.3, while Tween 80 is a hydrophilic surfactant with HLB value of 15.0. The following weight ratios of Span 80 to Tween 80 were utilized to obtain the HLB values that were tested in this study: 1:0, 4:1, 2:1, 1:1, 1:2,

1:4, and 0:1 (w/w; Fang et al., 2015). The corresponding HLB values of the mixtures are 4.3, 6.4, 7.8, 9.7, 11.3, 12.9, and 15.0. The mixtures were vortexed prior to use to ensure a homogeneous surfactant mixture.

Sample Handling and Polar Lipid Quantification by ^{31}P NMR

To determine the optimum HLB value for cream emulsion disruption, surfactants with HLB values of 4.3, 6.4, 7.8, 9.7, 11.3, 12.9, and 15.0 were added to the cream at a fixed concentration of 1,000 ppm (w/w). After the optimum HLB value was determined, the effect of surfactant concentration (250, 750, 1,000, and 3,000 ppm) was investigated under three different churning temperatures of 10, 35, and 60°C. The optimum HLB value, concentration, and churning temperature were then used for studying the effect of pH adjustment, tempering, additional shear, modified churning speed, and pasteurization of cream on PLs distribution.

Figure 1 shows the flow chart of how the cream was handled prior to churning according to industrial practices. To mimic the steps, a vat pasteurization step was completed in a ThermoScientific hot water bath shaker (model #3582) at 67°C, under 200 rpm for 45 min, and the pasteurized cream was allowed to cool overnight under refrigeration (2-4°C). After cooling, the cream was churned at 10°C using a Kitchen Aid Classic 5 Speed Standard Mixer (Model # K45SSOB) on speed number six (135 rpm) until butter was produced (in approximately 7 minutes). A 5-quart wire whip attachment was used to agitate the cream for the churning process. After butter and BM were produced, the butter was worked with a metal spatula to remove BM that was trapped within the butter matrix. The BM was then drained from the mixing bowl and centrifuged at 3,500 rpm for 10 minutes at 51°C in a 50 mL centrifuge tube. After centrifugation, the BM was allowed to cool overnight. A fat pad formed on top of the BM after cooling and the defatted BM was then collected by draining the BM from the bottom of the centrifuge tubes.

This centrifuge step was done to remove excess or escaped neutral lipid during draining and produce an industrial type of BM with a fat content on a dry basis ranging from 5-10%. The lipid in the defatted BM was then extracted using the Folch extraction procedure (Rodriguez-Alcala and Fontecha, 2010; Folch et al., 1957) for PLs quantification using ^{31}P NMR following a method reported by Wang et al. (2014) and Price et al. (2018). Triphenyl phosphate (TPP) was used as the internal standard for quantification.

The effect of churning temperature was evaluated by churning the cream at 35°C and 60°C. The production procedure of this BM varied slightly from the 10°C churning. Prior to churning, the cream was heated in a hot water bath (ThermoScientific model #3582) to the designated churning temperature and then agitated in the Kitchen Aid at 135 rpm for 7 min prior to centrifugation (3,500 rpm for 10 min). After centrifugation the cream was decanted to separate the aqueous BM from the upper high-fat cream layer. The lower BM layer was collected. The remaining lipid in BM was extracted and prepared for ^{31}P NMR quantification of PLs as described previously. Centrifugation was used instead of the normal churning because the melting point range of milk fat is 32-36°C (Kaylegian and Lindsay, 1995) and the fat was liquid at these elevated temperatures.

Other treatments were also evaluated to potentially aid in the disruption of the MFGM. These additional treatments included no pasteurization of cream, additional sheer, cream tempering, pH adjustment, and churning speed. The additional shear was performed using a Bamix Handmixer (Type M 133; Switzerland) for 3 and 6 min at 10,000 rpm after the pasteurization step. Tempering was applied after the pasteurization step, for which the cream was heated at 50°C and 65°C for 1 hour in an oven. To determine the effect of pH, the pH of the cream was adjusted to 4.6, 5.1, and 8.3 prior to pasteurization and surfactant addition, using 2 M

sulfuric acid or 2 M NaOH (native cream pH was 6.7). The churning speed was also varied by adjusting the speed level of the Kichen Aid Mixer. The lowest speed of 60 rpm (setting 2) and highest speed of 280 rpm (setting 10) were used. The resulting BM from those treatments was collected as described above. All these BMs were analyzed for total lipid, PLs content, and PLs class composition.

Particle Size Distribution of the Oil Droplet

To evaluate the effect of surfactant on emulsion characteristics, the particle size of the O/W emulsion was determined using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The cream emulsion was analyzed after being stored at refrigeration temperatures (2-4°C) for 24 hours to prevent the emulsion destabilization. The sample was dispersed in water and a laser obscuration between 10 and 20% was used. The particle size distribution, surface weighted mean (D [3,2]) and the volume weighted mean (D [4,3]).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

During the low pH treatment, a pellet was formed in the BM samples after centrifugation. The protein composition of this pellet was assessed by SDS-PAGE using reducing conditions as described by Primacella et al. (2018). Individual proteins were identified using a pre-stained SDS-PAGE MW broad range standard (Bio-Rad laboratories, Hercules, CA, USA) and the protein bands were identified according to a report of Mather (2000).

Statistical Analysis

All the treatments were randomized with three replicates. The data were analyzed using Statistical Analysis System (SAS 9.1) (SAS institute, Cary, NC), and a Tukey test was used to

determine significant differences among means at P = 0.05. The standard deviation from the mean was calculated for all treatments.

Results and Discussions

Determination of Optimum HLB of Surfactant to Disrupt MFGM

The effect of surfactant with various HLB values on the cream emulsion disruption and BM PLs content after churning at 10˚C was examined and the results are summarized in Table 1 and Table 2. It was observed that the control (no surfactant addition) and HLB value of 12.9 resulted in significantly larger particle size than the other treatments ($P < 0.05$; Table 1), indicating that HLB value of 12.9 was relatively more effective in disrupting the MFGM than the other surfactant treatments. The larger particle size is an indication of a less stable emulsion, as the size of the droplets in an emulsion have a strong impact on stability (McClements, 2007). The particle size distribution had a strong linear regression ($R^2 = 0.783$) with the BM PLs content (Figure 2); as the particle size (D [4, 3] data) of the cream increased, the PLs content in the resulting BM also increased.

The surfactant combination with HLB value of 12.9 interacted more with the continuous aqueous phase along with the polar interface of the MFGM. Cream is an oil in water (O/W) emulsion and consists of a complex dispersion of fat globules (diameter ranging from 0.1 µm to 15 µm, with an average of 4 µm) and casein micelles (diameter ranging 60-120 nm) in an aqueous serum containing whey proteins, soluble caseins, lactose, and salts (Dickinson, 2001; Walstra, 1995; Huppertz et al., 2017). This type of emulsion made it feasible for the more hydrophilic surfactant with a HLB value of 12.9, to interact with the MFGM, while displacing more PLs to the aqueous BM. This complies with Bancroft's rule, which states that the phase in which the emulsifier is most soluble will form the continuous phase of an emulsion

(Ruckenstein, 1996). Others also reported that non-ionic water-soluble surfactants are generally more effective at displacing milk proteins from O/W interfaces than oil-soluble surfactants (Dickinson, 2001).

One interesting observation occurred with the most hydrophilic surfactant combination, having an HLB value of 15.0 (100% Tween 80). This HLB treatment yielded a significant decrease in BM PLs content and significantly smaller particle size compared to the 12.9 HLB value ($P<0.05$). This indicates that the more hydrophobic surfactant, Span 80, played a role in helping displace the MFGM components along with Tween 80. Span 80 was reported to displace the protein β-lactoglobulin from the interface because of its low molecular weight and high surface activity, which can reduce the interfacial tension to a lower value than proteins can (Zhang and Wang, 2016; Wilde et al., 2004; van Aken, 2003). Our results show that the mixture of Span 80 and Tween 80 (1:4 w/w) with HLB of 12.9 enhanced the displacement of MFGM PLs in the cream system. Since the HLB value of 12.9 resulted in a BM lipid fraction with significantly higher PLs content (25.1%) than all the other surfactant treatments, it was determined to be the optimum for further experiments. However, the PLs content of the BM obtained with this HLB value was slightly lower than the control (29.2%), and the concentration of the surfactant could be another important factor to consider. Different surfactant concentrations were then investigated, and their effect on PLs content in BM using the surfactant with HLB of 12.9 is discussed in a later section.

The five main PLs classes found in BM are sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). The PLs class composition of the resulting BM lipid fractions is summarized in Table 2. There were no significant differences in the five main PLs classes found in BM under different

surfactant HLB values (P>0.05), indicating that these surfactant combinations did not have any preference for displacing specific PLs classes found in the MFGM.

Optimum Surfactant Concentration and the Effect of Churning Temperature on PLs Distribution

The effect of surfactant concentration (at HLB of 12.9) as well as the churning temperature were investigated to determine if a BM lipid fraction with a PLs content higher than the control could be obtained. The particle size distribution resulting from the different concentrations of surfactants can be seen in Table 3. The particle size of the cream was determined prior to churning, so the churning temperature treatment was not a factor in these results. The concentration of the surfactants did not have a significant effect on the particle size of the cream (P >0.05). There was also no strong correlation between the particle size and PLs content of the resulting BM (R = 0.326). It is possible that the modified churning temperatures and additional cream handling treatments eliminated the relationship between particle size and BM PLs content.

Regarding the PLs content of the BM, the churning temperature and surfactant concentration were both significant factors (P <0.05) and these results can be seen in Table 4. The churning temperature of 10°C yielded a significantly higher PLs content in BM (14.3-36.7%) when compared to 35°C (12.2-20.1%) and 60°C (14.5-23.7%) (P <0.05). The 35°C treatment resulted in the BM lipid fraction with the lowest PLs content, while 60°C only led to a slightly higher content compared to 35°C, indicating that elevated churning temperature is not desirable. At such elevated temperatures, PLs may have been more fully dissolved in the oil phase, or they were strongly interacting with proteins, which did not allow flow to the aqueous BM fraction. The 10°C churning resulted in a phase inversion (O/W to W/O) as expected, while

churning temperatures of 35°C and 60°C did not, as the melting point of milk fat is about 32 to 36°C (Walstra et al., 2006). The phase inversion of cream emulsion plays a major role in the release of MFGM proteins and PLs into the BM. Such phase inversion decreases the surface area of the MFGM and allows membrane material to flow to the aqueous BM (Walstra et al., 2006). The absence of this phenomenon during the 35°C and 60°C churning temperature also potentially led to the lower BM PLs content.

Regarding the effect of surfactant concentration, the lower concentrations were more effective for moving the PLs to the BM. Under all the churning temperatures, the surfactant concentration of 750 ppm resulted in BM lipid fraction with significantly higher PLs content when compared to 1,000 and 3,000 ppm ($P < 0.05$). Although the 750 ppm treatment was not statistically significantly better than the 250 ppm and control ($P > 0.05$), it resulted in a BM lipid fraction with the highest PLs content (36.7%) when churned at 10°C. Surfactants are able to reduce the interfacial tension to values lower than the ones proteins can achieve, and relatively low concentrations of surfactant can affect the surface tension of protein solutions (Wilde et al., 2004; Dickinson et al., 1990; Kragel et al., 1995). Wilde (2004) demonstrated that at lower concentrations, the surfactant could adsorb alongside the protein and weaken the interfacial layer. The MFGM consists principally of a single inner layer of polar lipids, a dense protein coat, and an outer bilayer of polar lipids with embedded specific proteins (Vanderghem et al., 2010). More than 130 specific proteins have now been identified within the MFGM (Affolter et al., 2010; Conway et al., 2014). This large degree of diversity helps to stabilize the MFGM, while the use of surfactants led to some destabilization and displacement of MFGM components from the membrane. A diagram of this potential mechanism of action of the surfactant is shown in Figure 3. The diversity of the proteins in MFGM may have limited the effectiveness of the

surfactant for displacing PLs. Also, with high concentration of surfactants, it may have helped disperse more PLs on interfaces of oil droplets or other dispersed protein particles, such as casein. Since the oil droplet size did not change dramatically, it is more likely that PLs bound tightly with other cream components at the high surfactant concentration, resulting in a reduction of PLs level in the BM.

The PLs class distribution of the BM lipid fraction is shown in Table 4. The churning temperature and surfactant concentration had no significant effect ($P > 0.05$). SM was recovered at a significantly higher level during the 10°C churning when compared to 35°C and 60°C ($P < 0.05$). Similar results were reported by Price et al. (2018), when SM was recovered more efficiently during a lower temperature ethanol extraction. Similar trend was observed with PS, where the lower churning temperatures resulted in higher PS recovery. The 10°C and 35°C treatments resulted in significantly more PS recovery when compared to the 60°C treatment ($P < 0.05$). SM and PS are of a particular interest because of the health benefits associated with them and their higher content in dairy compared to other lecithin sources such as soybean and egg yolk (Burling & Graverholt, 2008; Contarini & Povolo, 2013). For PE, the higher churning temperatures of 35°C and 60°C resulted in significantly higher recovery than the 10°C treatment ($P < 0.05$). There was no significant difference in the relative content of PI and PC among all treatments ($P > 0.05$).

Optimization of Treatments at Fixed Surfactant Concentration (750ppm) and HLB value (12.9)

Because the BM's PLs content was not significantly higher than the control under the optimal HLB value and surfactant concentration, modified cream handling conditions were evaluated. The effect of additional shear, cream tempering, pH adjustment, churning speed, and

the effect of no pasteurization were evaluated. The PLs class composition and content in the BM obtained under the various cream handling conditions are summarized in Table 5.

The treatments that resulted in the BM lipid fraction with significantly higher PLs content were the surfactant control, and surfactant with pH of 5.1 when compared to the other treatments ($P < 0.05$). The PLs content in these BM lipid fractions were 36.7 and 35.0%, respectively. The particle size of the samples treated with these conditions can be seen in Table 6, and it shows that pH adjustment of 4.6 and 5.1 (with and without surfactant) resulted in significantly larger particle sizes ($P < 0.05$). This indicates that pH adjustment alone resulted in more coalescence of the fat globules and MFGM disruption. Visually, these treatments led to a thicker cream layer and a pellet was formed after the BM was centrifuged. In line with these findings, the isoelectric point of many MFGM proteins is between 4.8 and 5.3, and that for casein and whey proteins is 4.9 to 5.5 and 4.2 to 5.5, respectively (Fong et al., 2007). The pH of 4.6 and 5.1 led to a neutral charge of the proteins, which subsequently led to precipitation of many milk proteins in the pellet. This was an interesting fraction with slimy and stringy texture.

The protein composition in the pellet found at lowered pH conditions was determined using SDS-PAGE, and the result is shown in Figure 4. The pellet contained mostly caseins and whey proteins. Visually, a higher amount of whey protein was precipitated during the pH 4.6 treatment, while the pH 5.1 pellet contained greater amounts of casein. Faint MFGM protein bands can also be observed in both of the pH conditions, indicating that these treatments impacted the stability of the MFGM. The MFGM proteins represent only 1 to 2% of the total protein found in milk (Riccio, 2004), so only faint MFGM bands were observed.

The surfactant with HLB value of 12.9 at 750 ppm and pH of 5.1 led to a significant increase in the BM PLs content (35.0%; $P<0.05$), which could be related to the larger particle

size found with surfactant addition. An investigation to determine if the elevated churning temperature of 60°C would result in an increase in PLs content of the pH 5.1 treatment (with surfactant) was also done since a large particle size was observed. The resulting BM had a PLs content of 21.4%, which is significantly lower than the 10°C treatment ($P<0.05$), indicating that even with an unstable emulsion, increasing churning temperature was not effective. This could suggest that higher temperature facilitated the solubilization of more PLs in the neutral lipid fraction, or caused stronger associations of PLs with proteins.

Also, a harsher shear treatment may be required to mimic the industrial scale processes used for butter oil production. Therefore, other processing treatments including additional shear, cream tempering, alkaline pH, churning speed, and no pasteurization were examined. They all resulted in PLs contents that were significantly lower than that of the control (no surfactant; $P<0.05$). However, the 50°C tempering and churning speed of 280 rpm resulted in PLs content of 30.5, and 25.6% respectively, which was similar to the control (28.5%). Tempering of the cream was done to potentially denature the MFGM proteins to produce a viscoelastic film of protein so that PLs could be displaced or released. The denaturation temperature of milk whey proteins is near 70°C (Walstra et al., 2006). However, tempering at 65°C led to a BM lipid with a PLs content significantly lower than the 50°C treatment ($P<0.05$). This is probably due to more interaction of PLs with proteins or neutral lipids at 65˚C. Several studies have demonstrated the interaction of specific PLs with milk protein (Barratt & Rayner, 1972; Patrick et al., 1972; Korver & Meder, 1974; McCrae, 1999) and PLs may exert an effect on heat stability by targeting the membrane proteins through either displacing the protein or strongly interacting with the protein at the lipid surface (McCrae, 1999). The 50°C tempering treatment was likely not severe enough for these interactions to occur, thus resulting in PLs content in BM similar to the control.

The effect of no pasteurization (with surfactant mixture and with or without pH adjustment on PLs distribution) was also investigated. It was found that no pasteurization led to a significant decrease in PLs content in BM ($P < 0.05$). The pasteurization step (Figure 1) includes agitation at 200 rpm, which was not applied when the pasteurization step was skipped. The surfactant may require time to interact with the MFGM to be effective at displacing PLs. Skipping the pasteurization step did not allow enough time for these interactions to occur, which likely led to a suppression of PLs to flow to BM.

To determine if the phase inversion time played a role in the release of PLs, the effect of churning speed on PLs distribution to BM was investigated. The 60 rpm churning speed led to a decrease in BM PLs content while the 280 rpm speed resulted in a BM lipid fraction similar to the control. The slower phase inversion at lower churning speed apparently resulted in less MFGM components distributing to the BM fraction.

No significant difference in particles size was observed when different processing treatments were used (Table 6; $P > 0.05$), except for the additional shear. This treatment resulted in significantly smaller particle size when compared to the other treatments ($P < 0.05$). The additional shear acted as a homogenization step, which is known to reduce the size of fat globules and prevent fat separation during storage (McCrae, 1999). The additional shear did not result in additional displacement of PLs, with a PLs content of 19.0 and 18.1% for the 3 and 6 min shear intervals. The PLs content in these BM samples was significantly lower than the control ($P<0.05$; Table 5). The additional shear resulted in an increase in membrane surface area, which potentially led to more PLs interaction with fat globule, thus reducing their distribution to BM. The class composition of PLs from these additional processing treatments is shown in Table 5. There was no significant difference in SM and PI content among all the treatments ($P>0.05$).

PS and PC content were observed to be significantly higher when low pH and additional shear were applied ($P<0.05$). However, one exception occurred when pH of 4.6 was used, and it resulted in the lowest PS content. All these PLs class composition data are interrelated or relative, so caution should be given when evaluating such relative composition data.

Conclusions

The use of food grade surfactants was shown to be effective in improving the PLs distribution to BM during cream churning and the optimum HLB value was determined to be 12.9 (1 part Span 80: 4 part Tween 80). Surfactant concentration and churning temperature significantly impacted the PLs content of the resulting BM. The surfactant concentration of 750 ppm led to the highest PLs content BM when combined with the churning temperature of 10°C. The combination of 750 ppm and 12.9 HLB surfactant at pH of 5.1 resulted in the weakest cream emulsion but did not lead to increased PLs content in BM compared to control. Although the process did not provide a dramatic enrichment of PLs as initially hypothesized, this work is a proof of concept, and the experiments may provide insights for future work with practical or theoretical importance.

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Figures and Tables

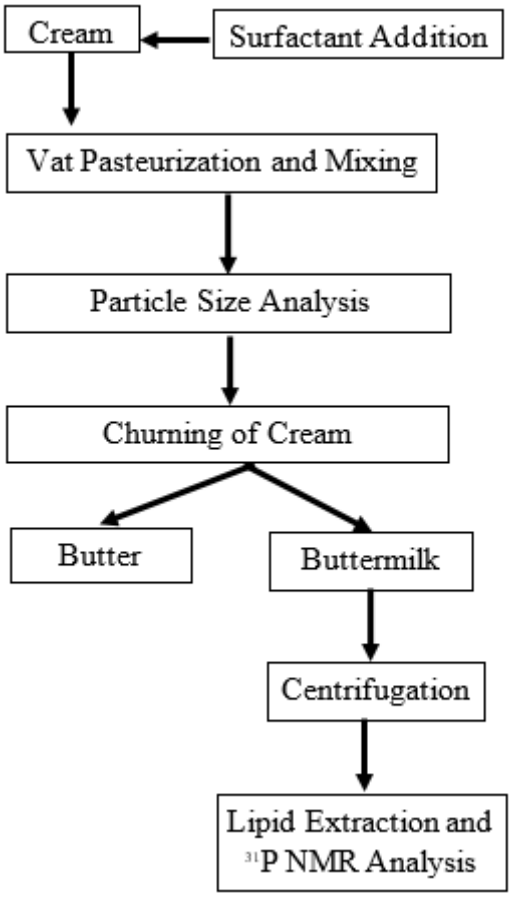

Figure 1. Processing flow of cream and BM handling.

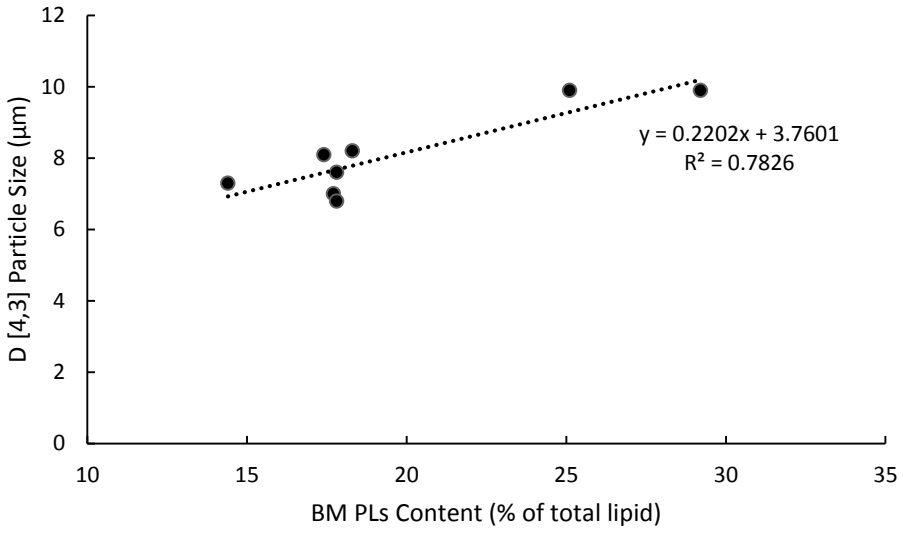

Figure 2. Regression of the particle size (D [4, 3]) as a function of the BM PLs content of the HLB surfactant results.

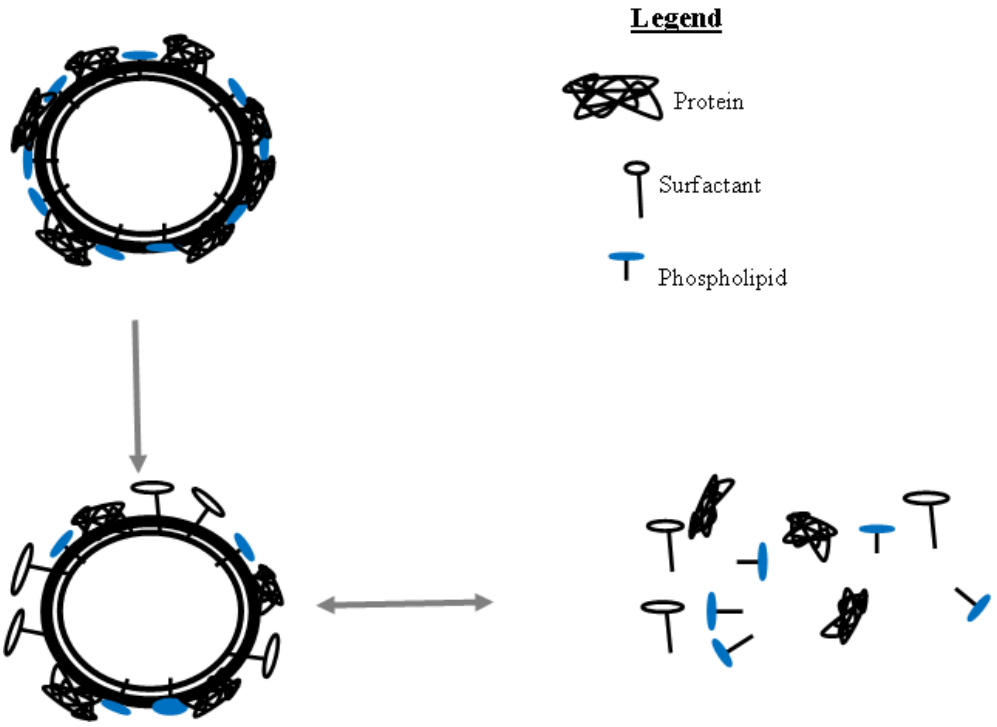

Figure 3. The potential mechanism for surfactant interaction with the MFGM within cream.

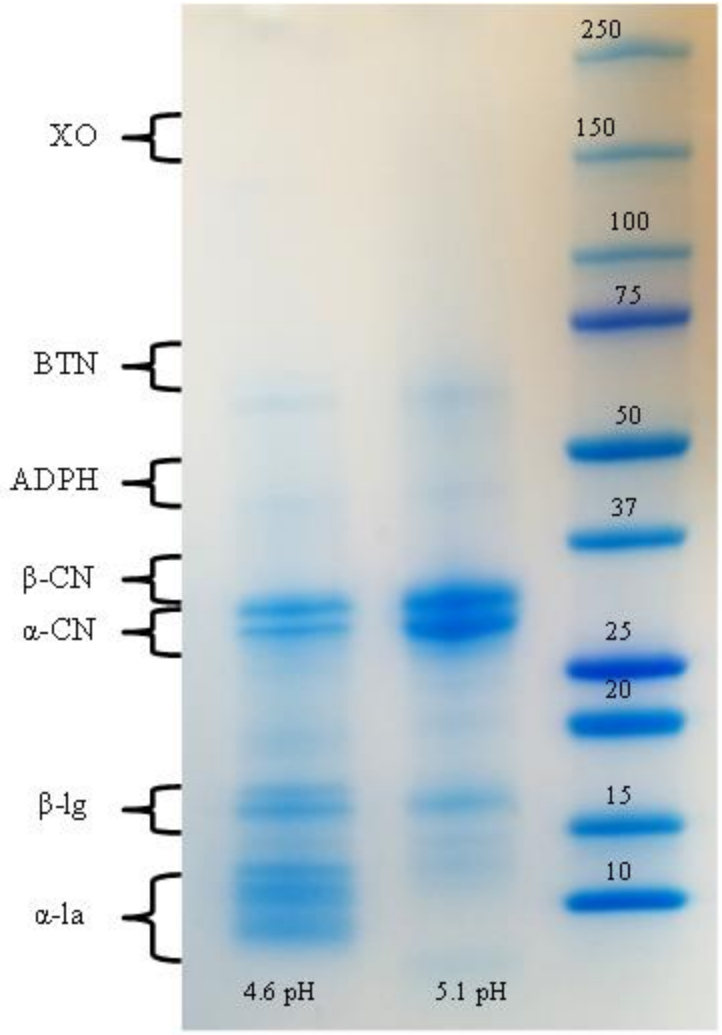

Figure 4. SDS PAGE gel on the BM pellet recovered from the 4.6 and 5.1 pH optimization treatments with 750 ppm 12.9 HLB value surfactant. Protein Bands: β-lactoglobulin (β-lg), α-lactalbumin (α-la), β-casein (β-CN), α-casein (α-CN), xanthine oxidas (XO), butyrophilin (BTN), and adipophilin (ADPH).

Table 1. Particle size analysis to determine the optimum HLB of surfactant used at 1,000 ppm concentration for MFGM disruption

	HLB Value							
Particle Size (μm)	Control	4.3	6.4	7.8	9.7	11.3	12.9	15.0
d (0.1)	2.1 a	1.6 b	1.8 b	1.6 b	2.0 a	1.8 b	1.7 b	2.0 b
d (0.2)	2.7 a	2.4 a	2.5 a	2.4 a	2.7 a	2.5 a	2.4 a	2.6 a
d (0.5)	5.0 a	4.5 a	4.6 a	4.5 a	5.1 a	4.7 a	4.8 a	4.4 a
d (0.8)	12.9 a	9.7 b	10.0 b	9.2 b	11.5 a	11.1 ab	12.9 a	8.4 b
d (0.9)	25.1 a	16.2 bc	16.6 bc	14.4 c	18.1 bc	18.9 bc	22.7 ab	12.9 c
D [3, 2] - Surface weighted mean	4.2 a	2.5 c	2.9 c	2.5 c	4.1 ab	3.0 bc	3.8 ab	3.0 bc
D [4, 3] - Volume weighted mean	9.9 a	7.3 b	7.6 b	7.0 b	8.1 b	8.2 b	9.9 a	6.8 b

* d (0.1-0.9) represents the percentile distribution; Ex: d (0.1) represents 10% of the particle size is smaller than size reported.
- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

Table 2. BM PLs content and composition as affected by HLB value of the surfactant at 1,000 ppm concentration

		PLs Classes (% of total PL)				
HLB Value	PLs (% Total Lipid)	SM	PE	PS	PI	PC
Control	29.2 a	28.7 a	25.2 a	6.7 a	8.7 a	26.6 a
4.3	14.4 c	29.1 a	24.9 a	6.2 a	8.9 a	30.9 a
6.4	17.8 bc	31.3 a	26.6 a	4.4 a	8.4 a	29.3 a
7.8	17.7 bc	33.9 a	26.6 a	2.6 a	8.6 a	28.5 a
9.7	17.4 c	25.3 a	31.2 a	5.9 a	8.8 a	28.9 a
11.3	18.3 bc	29.3 a	27.9 a	4.2 a	8.6 a	29.9 a
12.9	25.1 ab	30.6 a	28.9 a	3.9 a	8.5 a	28.2 a
15.0	17.8 bc	26.4 a	30.7 a	5.1 a	8.8 a	29.1 a

- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

Table 3. Particle size distribution of cream under various surfactant concentration at 12.9 HLB

Particle Size (μm)	Surfactant Concentration (ppm) Control	250	750	1,000	3,000
d (0.1)	1.2 ab	1.6 a	1.8 a	1.2 ab	0.9 b
d (0.2)	2.3 a	2.5 a	2.5 a	2.4 a	2.2 a
d (0.5)	4.3 a	4.7 a	4.6 a	4.5 a	4.2 a
d (0.8)	9.4 a	12.0 a	11.1 a	11.7 a	8.3 a
d (0.9)	16.0 b	20.1 a	19.7 a	20.5 a	12.3 b
D [3, 2] - Surface weighted mean	2.3 a	3.3 a	3.6 a	2.6 a	2.2 a
D [4, 3] - Volume weighted mean	7.2 a	8.4 a	8.3 a	8.3 a	5.9 a

* Storage at 2°C prior to particle size analysis

- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

Table 4. BM PLs content and composition as affected by surfactant of 12.9 HLB at various concentration and at different churning temperature

Treatment		PLs (% Total Lipid)	PLs Classes (% of Total PLs) SM	PE	PS	PI	PC
Control		30.4 ab	34.1 a	18.4 a	9.8 a	8.5 a	29.1 a
250		27.4 abc	33.1 a	19.2 a	9.8 a	8.5 a	29.4 a
750	10°C	36.7 a	34.4 a	18.6 a	9.5 a	8.6 a	28.9 a
1000		22.7 bcde	32.6 a	22.9 a	6.2 a	9.1 a	29.2 a
3000		14.3 de	32.8 a	18.2 a	10.6 a	8.9 a	29.5 a
Control		25.0 abcd	30.0 a	22.4 a	9.5 a	8.0 a	30.1 a
250		20.1 bcde	32.3 a	22.8 a	7.3 a	8.2 a	29.4 a
750	35°C	16.9 cde	32.8 a	22.6 a	7.2 a	8.3 a	29.1 a
1000		17.9 cde	31.1 a	19.9 a	10.3 a	8.7 a	30.0 a
3000		12.2 e	31.1 a	20.0 a	10.3 a	8.4 a	30.2 a

Table 4. (continued)

Control		26.5 abc	31.8 a	23.8 a	6.0 a	8.4 a	30.0 a
250		23.7 bcd	31.5 a	23.8 a	6.0 a	8.7 a	30.0 a
750	60°C	22.2 bcde	30.8 a	24.9 a	6.0 a	8.4 a	29.9 a
1000		17.9 cde	30.6 a	27.2 a	5.2 a	8.4 a	28.6 a
3000		14.5 de	30.5 a	24.3 a	6.1 a	8.6 a	30.5 a

- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

Table 5. BM PLs content and composition as affected by various processing conditions with the use of 750 ppm of 12.9 HLB surfactant.

Treatments	PLs (% of Total Lipid)	PLs Classes (% of Total PLs)				
		SM	PE	PS	PI	PC
Control	36.7 a	34.4 a	18.6 f	9.5 abc	8.6 a	28.9 ab
Control no Surfactant	28.5 bcde	34.3 a	25.5 abcde	6.3 bcdefg	7.7 a	26.2 ab
Control no Pasteurization	24.0 defg	31.8 a	22.9 def	5.9 cdefgh	10.5 a	28.9 ab
pH 5.1	35.0 a	29.8 a	24 cdef	8.1 abcde	7.2 a	30.9 a
pH 5.1 no Surfactant	25.8 cdef	33.4 a	18.9 f	10.3 a	7.1 a	30.3 a
pH 5.1 60°C	21.4 fg	33.3 a	28.7 abcd	1.8 ij	6.5 a	29.7 ab
pH 5.1 60°C no Surfactant	22.2 efg	33.3 a	18.7 f	9.9 a	7.6 a	30.5 a
pH 4.6	32.2 abc	35.2 a	23.2 def	3.6 fghij	7.2 a	30.8 a
pH 4.6 No Pasteurization	21.8 efg	35.7 a	24.3 cdef	4.4 efghij	6.4 a	29.2 ab
pH 8.3	24.3 defg	29.1 a	31.6 a	5.0 defghi	10.9 a	23.4 b
Temper 50°C	30.5 abcd	32.4 a	30.0 abc	2.2 hij	9.4 a	26.0 ab
Temper 65°C	17.7 g	32.0 a	24.8 bcdef	5.4 defghi	9.0 a	28.8 ab
Shear 3 min	19.0 fg	33.0 a	23.5 def	7.4 abcdef	8.7 a	27.4 ab
Shear 6 min	18.1 g	32.9 a	19.7 ef	8.4 abcd	8.7 a	30.3 a
60-rpm Churn	22.6 efg	31.2 a	30.9 ab	3.0 ghij	7.6 a	27.3 ab
280-rpm Churn	25.6 cdef	33.5 a	29.1 abcd	1.0 j	9.6 a	26.8 ab

*****Control indicates surfactant concentration of 750 ppm at a HLB value of 12.9

- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

Table 6. Particle size distribution as affected by various processing treatments.

Particle Size (μm)	Temper 50°C	Temper 65°C	Shear 3 min	Shear 6 min	pH 8.3	pH 4.6	pH 5.1	pH 5.1 Control*	Churn Speed	Control*
d (0.1)	2.1 a	2.1 a	1.6 b	1.4 c	2.0 a	2.4 a	2.5 a	2.1 a	2.0 a	2.1 a
d (0.2)	2.6 cd	2.8 c	2.6 cd	2.9 bc	2.5 d	4.1 a	4.5 a	3.4 b	2.6 cd	2.6 cd
d (0.5)	4.5 b	5.1 b	5.1 b	5.8 b	4.2 b	12.3 a	13.8 a	9.6 ab	4.6 b	4.5 b
d (0.8)	9.5 b	12.9 b	8.7 b	9.8 b	7.5 b	25.5 ab	37.0 a	22.2 ab	9.1 b	9.1 b
d (0.9)	17.6 b	23.4 b	11.1 b	12.4 b	10.7 b	49.7 a	56.5 a	35.0 ab	14.4 b	15.7 b
D [3, 2] - Surface weighted mean	4.0 b	4.3 ab	2.1 d	2.0 d	3.7 bc	4.5 a	4.6 a	3.4 cd	3.8 cd	3.9 cd
D [4, 3] - Volume weighted mean	8.2 bc	10.0 bc	6.2 c	6.5 bc	6.3 c	24.8 a	26.4 a	16.4 ab	7.0 bc	7.9 bc

*No surfactant addition

- a, b, c unshared letters beside means (within a column) indicate significant differences were found ($p < 0.05$).

CHAPTER 4: APPLICATION OF ZINC AND CALCIUM ACETATE TO PRECIPITATE MILK FAT GLOBULE MEMBRANE COMPONENTS FROM A DAIRY BY-PRODUCT

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Interpretive Summary

The milk fat globule membrane (MFGM) has been of a particular interest because of its potential health benefits and functional properties (or milk fat globule membrane (MFGM) components have been of a particular interest because of their potential health benefits and functional properties). The use of salts to precipitate MFGM components is shown to be a successful method to isolate MFGM proteins and phospholipids (PLs) from whey by-products. The application of this technology to the beta stream, which is a by-product of anhydrous milk fat production and is similar to buttermilk, has great potential to obtain MFGM proteins and PLs. With an additional ethanol extraction step on the precipitate, a valuable dairy lipid with high PLs content can be produced from this low-value processing by-product.

Abstract

There has been a great interest in developing isolated dairy lipid fractions that are rich in phospholipids (PLs) due to their health benefits and functional properties. Dairy by-products that contain elevated content of PLs and milk fat globule membrane (MFGM) proteins can be an excellent source for these isolates. Beta stream (a by-product from anhydrous milk fat production) is an excellent candidate since it contains a higher concentration of PLs than many other dairy by-products. In this study, we investigated an economically feasible processing method to obtain these valuable components from the beta stream. The use of zinc acetate and calcium acetate, along with mild heat treatment and pH adjustment, was effective in precipitating PLs and proteins into a pellet fraction. With an additional extraction from the pellet using ethanol (90% at 70˚C), a PLs-enriched lipid fraction was obtained. The effective precipitation conditions were zinc acetate of 25 mM concentration at pH of greater than 6.5 at 30°C, and calcium acetate of greater than 75 mM concentration at pH of greater than 6.5 at 60°C. With the ethanol extraction, PLs recovery of 97.7±1.7% from the zinc acetate precipitate and 94.9±3.7% from calcium acetate precipitate were achieved.

Key words: dairy lipid, ^{31}P NMR, sphingomyelin, phospholipids, ethanol extraction.

Introduction

The milk fat globule membrane (MFGM) has attracted significant interest because of its potential health benefits and functional properties (or milk fat globule membrane (MFGM) components have been of a particular interest because of their potential health benefits and functional properties). This membrane encompasses the triacylglycerol fat globule and is the richest source of phospholipids (PLs), glycolipids, gangliosides, and glycoproteins in milk (Jimenez-Flores & Brisson, 2008). Although dairy PLs represent only 0.5-1.0% of total milk lipids (Fong et al., 2007), they are of a particular interest because of their higher content of sphingomyelin (SM, 4.1-29.2% of total PLs) and phosphatidylserine (PS, 2.0-16.1% of total PLs) compared to other PLs sources such as soybean and egg yolk (Burling & Graverholt, 2008; Contarini & Povolo, 2013). Sphingomyelin (SM) is reported to play important roles in cell regulation and is referred to as a tumor suppressor (Contarini & Povolo, 2013; Dewettinck et al., 2008; Rombaut et al., 2006). Cognitive performance improvement, which is of a particular significance to Alzheimer's disease treatment, has been attributed to phosphatidylserine (PS; Burling & Graverholt, 2008; Contarini & Povolo, 2013; Dewettinck et al., 2008; Pepeu et al., 1996; Rombaut & Dewettinck, 2006).

The use of salts, such as calcium chloride and zinc acetate, to precipitate MFGM components has been reported for dairy whey by-products (Damodaran, 2010; Rombaut & Dewettinck, 2007). These salts can precipitate MFGM components such as PLs and proteins prior to ultra-filtration (UF) to increase processing efficiency and prevent fouling of membranes during whey protein concentrate (WPC) production (Rombaut & Dewettinck, 2007). If the MFGM components are not removed from whey, the resulting WPC can develop a stale or oxidized off-flavor and brown discoloration during storage due to a series of complex and inter-

related chemical reactions that include lipid oxidation and Maillard browning (Morr & Ha, 1991). These off-flavors can limit the use of WPC in the food industry (Carunchia Whetstine et al., 2005) and reduce the functionality of WPC by leading to high turbidity, impaired foaming, and reduced emulsification properties (Damodaran, 2005). These drawbacks have led to research focusing on the removal of MFGM components from whey. The application of salts to dairy by-products other than whey has yet to be investigated.

The beta stream is a by-product anhydrous milk fat (AMF) production that has a composition similar to buttermilk and butter serum. The starting material for the beta stream is cream, which is further processed into AMF. There are differences in the composition of the beta stream compared to whey by-products, in that the beta stream contains a higher protein content and different protein profile. Casein is present in the beta stream but not in whey by-products, as it is removed during the cheese making process. There is also less lactose in the beta stream (43.6%). Working with the higher amount of protein present in the beta stream will provide insight into how well the beta stream proteins and lipid fractions are precipitated during the salt precipitation step of the extraction process.

The application of calcium chloride (Rombaut and Dewettinck, 2007) and zinc acetate (Damodoran, 2010) have shown a great potential to remove dairy PLs and MFGM components from whey by-products. Both salts were used in combination with temperature and pH adjustments to precipitate the MFGM components present in whey by-products. However, an efficient and economical lipid extraction for the precipitated pellet has yet to be developed to obtain PLs. Rombaut and Dewettinck (2007) attempted to use a water wash to remove lactose and other water-soluble components from the pellet but PLs losses ranging from 16.6 to 49.7% were observed. A novel approach to extract the PLs in the pellet is to use ethanol for solubilizing

the lipid and to precipitate the protein (Price et al., 2018). With such an ethanol extraction of the precipitate, it is important to understand the apparent solubility of each salt in ethanol. Calcium chloride has high ethanol solubility, while acetate salts have much lower ethanol solubility (Sheftel, 2000). Considering these factors, an investigation into the use of zinc acetate and calcium acetate on the beta stream was performed to determine how efficiently the total lipid, total PLs, and protein are precipitated, and then the effectiveness of PLs extraction with ethanol.

The specific aim of this investigation was to determine the practical and effective salt concentration and pH for the precipitation of both PLs and proteins. Each individual PLs class was quantified to evaluate the effect of these treatments on their recovery. The apparent solubility of each salt under various ethanol concentrations and temperatures was also determined to understand what ethanol extraction parameters are best suited to limit the amount of salt being solubilized during the extraction. Then the application of ethanol to remove the total lipid and PLs from the precipitate was evaluated.

Materials and Methods

The beta stream powder used in this study was provided by Valley Queen Cheese (Milbank, SD). The extracted lipid was quantified for PLs using ^{31}P NMR (Wang et al., 2014; Price et al., 2018). Reagent-grade solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Sample preparation

The beta stream powder was dispersed in deionized water (5% w/w) and allowed to hydrate for 4 hr under constant agitation (450 rpm). The pH of the dispersion was 4.7 and 2 M sodium hydroxide solution was used to adjust the pH to a specific value (pH monitored using Thermo Scientific Orion Star A111 pH meter). After hydration, powdered zinc acetate or

calcium acetate were added at the designed molar concentration (0, 25, 50, 75, 100, 150, and 200 mM). An exception was made for studying the effect of pH on the efficiency of lipid recovery, where the pH was adjusted after zinc acetate or calcium acetate addition since these salts can lower the pH of the beta stream dispersion (Figure 1). The samples were then placed in a hot water bath (ThermoScientific model 3582) at 30 or 60°C for 30 min. After this heat treatment, the samples were centrifuged (IEC Centra CL-2) at 1800×g for 5 min. The samples were then allowed to cool overnight in the refrigerator (2-4°C), and the supernatant was then decanted and the lower pellet was collected for total lipid extraction and PLs quantification. The transmittance of the supernatant was measured at 500 nm using a Beckman Coulter DU 720 spectrophotometer (Brea, CA) as an indicator of effective MFGM precipitation.

Analytical methods

The moisture and ash contents were determined by drying at 95°C for 24 h in a drying oven, and incineration in a muffle furnace (Barnstead Thermolyne 48000 Furnace Model #F48015) at 600°C for 24 h. The Dumas (LECO CHN-2000; St. Joseph, MI) method was used to quantify protein content in the initial beta stream powder and in the precipitated pellet. The protein concentration in the supernatant was assayed using the Bradford method (Bradford, 1976). Bovine serum albumin was used to establish a standard curve and the protein concentrations were reported as mg/mL. Protein distribution (initial beta stream, zinc acetate and calcium acetate precipitated pellets, and supernatant) were assessed qualitatively using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Primacella et al. (2018). Individual proteins were identified using a SDS-PAGE molecular weight broad range standard (Bio-Rad laboratories, Hercules, CA) and the protein bands were identified according to the profile reported by Mather (2000). The MFGM-salt

complex would not dissolve in water, so an ethylenediaminetetraacetic acid (EDTA) solution of 0.2% was used to release the MFGM into the dispersion prior to SDS-PAGE. Total lipid extraction using the Folch method (Rodriguez-Alcala & Fontecha, 2010; Folch and Stanley, 1957) was performed to quantify the lipid content in the beta stream and the precipitates. The PLs content and the class composition of the lipid extracts were determined by using ^{31}P NMR, following a method reported by Price et al. (2018) and Wang et al. (2014).

Zinc acetate and calcium acetate apparent solubility in ethanol

The apparent solubility of each salt was determined by adding 10 mL of ethanol (70, 80, and 90% v/v) to 5 g of zinc acetate or calcium acetate. The sample vials were then capped and placed into a Thermo Scientific MaxQ 4450 heated shaker at 30, 50, and 70°C under constant agitation (200 rpm) for 24 hr. The solubilized salt in ethanol were then decanted and the ethanol was removed to determine how much salt was solubilized in the ethanol. The results were reported as g of salt dissolved in 100 g of ethanol under the various ethanol concentrations and temperatures investigated.

Ethanol extraction of lipids from the pellets

Once the pellet was recovered, the lipid was extracted using ethanol, according to Price et al. (2018). The optimum conditions for total lipid and PLs recovery using a one-step extraction were reported as 90% ethanol at 70°C, and these conditions were used to extract the lipid from the beta stream pellet. The pellet (25 g) was placed in 206 mL of neat ethanol for zinc acetate and 198 mL of 100% ethanol for calcium acetate. The difference in total solids percentage between the zinc acetate and calcium acetate pellets led to slightly different volumes of ethanol required to achieve a final ethanol concentration of 90%. After the pellet was in contact with the ethanol, incubation was allowed for 6 min before the ethanol with solubilized lipid was filtered

to remove the residual pellet fraction. The ethanol was then rotary evaporated (Buchi Rotavapor R-205) to collect the lipid fraction. The residual pellet fraction was also recovered and saved for further analysis.

Statistical analysis

All the treatments were randomized, with three replicates. The data were analyzed using Statistical Analysis System (SAS 9.1) (SAS institute, Cary, NC) software, and the difference in treatment means and significance were evaluated at P = 0.05 among all the treatments analyzed. The standard deviation from the mean was calculated for all treatments and presented as the error bar in the figures. Tukey test was used to analyze statistical differences among means.

Results and Discussions

The beta stream powder contained 18.1% total lipid, of which 35.0% was PLs (6.3% on product basis). There was 29.1% protein, 6.1% ash, 43.6% lactose, and 3.1% moisture. The major PLs classes found in the beta stream were phosphatidylcholine (PC; 34.2% of total PLs), phosphatidylethanolamine (PE; 28.0%), and SM (27.0%). The minor PLs classes were PS (2.1%) and phosphatidylinositol (PI; 8.7%). The PLs composition of the beta stream was similar to that of native milk fat (Contarini and Povolo, 2013).

The effect of calcium acetate and zinc acetate concentration on the precipitation of total lipid, PLs, and protein

The total lipid recovery, PLs recovery, PLs content, and PLs class composition for the zinc acetate and calcium acetate precipitated samples are shown in Table 1. The concentration and type of salt were both significant factors for total lipid and PLs recoveries ($P<0.05$). All the concentration treatments resulted in significantly more total lipid and PLs recoveries when compared to the control treatments ($P<0.05$). There were no significant differences among the

total lipid recoveries for the zinc acetate concentration treatments (P>0.05), which resulted in total lipid recovery ranging from 94.5 to 99.9% (25-200 mM). The treatment of calcium acetate (25-100 mM) at 30°C resulted in significantly less total lipid and PLs recovery compared to the zinc acetate treatment, with total lipid recovery ranging from 41.6 to 61.8%, and PLs recoveries ranging from 36.5 to 65.8%. The concentration of 150 to 200 mM resulted in significantly higher total lipid and PLs recovery than the lower salt concentration. At 60°C, the 75-200 mM concentration range of calcium acetate led to similar total and PLs recoveries compared to zinc acetate (88.4 - 97.9% total and 84.9 - 92.9% PLs recoveries). The 30°C calcium acetate treatment resulted in significantly lower total and PLs recoveries compared to zinc acetate and 60°C calcium acetate overall treatments means (P<0.05).

There was no significant difference in the PLs content in the precipitated total lipid fraction among any of the zinc acetate and calcium acetate treatments (P>0.05). All the treatments resulted in a significantly higher PLs content (24.5 to 34.4%) than the control treatments (8.4 to 12.3%). The PLs class compositions for all three types of treatment were similar, with a few significant variations in the content of SM, PS, and PE. The 30°C calcium acetate treatment as a whole resulted in significantly more SM recovery and less PS recovery than the 60°C calcium acetate and zinc acetate treatments. The 60°C calcium acetate treatment resulted in seemingly higher PS recovery when compared to zinc acetate. Zinc acetate led to a slightly higher PE recovery than both of the calcium acetate treatments. SM and PS are the PLs classes of specific interest because of their health benefits, and enrichment of these PLs during extraction is desired. Therefore, the 30°C calcium acetate treatment would be desired for good SM recovery, while the 60°C calcium acetate treatment would be desired for maximum PS recovery. The difference in recovery of different PLs classes may be attributed to the location of

these PLs within the MFGM, which may lead to a preference for complexing with calcium compared to zinc. SM, the most polar PLs class, is located on the outer bilayer of the MFGM, while PS, a more non-polar PLs class, is more closely associated with the inner membrane (Deeth, 1997; Dewettinck et al., 2008; Christie and Han, 2010). Zinc acetate may interact more with the integral hydrophobic proteins of the MFGM and have a preference to bind to the MFGM inner membrane, as more PE was recovered with zinc acetate. Calcium acetate may not have a preference to specific areas in the MFGM due to variations in PLs class recovery under the temperature treatments investigated.

The supernatant transmittance and protein concentration were analyzed to give an indication of how the salt concentration affects precipitation of protein, and the results are summarized in Figures 2 and 3. A higher transmittance indicates that more protein and/or lipoprotein were precipitated into the pellet. As shown in Figure 4, there was a strong correlation between the transmittance and protein concentration in the supernatant. As the transmittance increased there was less protein in the supernatant. The transmittance of all the supernatant from the zinc acetate treatments was significantly higher than in the calcium acetate treatments, while the protein concentration in the supernatant was also significantly lower ($P<0.05$). The zinc acetate treatment was more effective in removing the lipoproteins from the supernatant and precipitating them into the pellet compared to the calcium acetate treatments. When the temperature of the calcium acetate treatment was increased from 30 to 60°C, significantly higher transmittance and lower protein concentration in the supernatant were observed ($P<0.05$). Increasing the temperature resulted in a better binding affinity of calcium with protein and lipid, but not to the extent of zinc acetate. This indicates that zinc acetate had a stronger affinity towards protein, while calcium acetate had a lower affinity towards protein but stronger affinity

towards lipid. These results may be due to how zinc and calcium interact with the MFGM components. Zinc forms a tetracoordination complex with membrane phospholipids in a tetrahedral geometry, while calcium forms a hexacoordination complex in an octahedral geometry (Binder et al., 2001; D'Acapito et al., 2002; Damodaran, 2010). The differences in the geometry of the salt-MFGM complexes may lead to more opportunity for zinc to interact with the protein present in the beta stream.

Effect of pH on the efficiency of total lipid, PLs, and protein precipitation

An investigation to determine which pH is ideal for the effective and most practical zinc acetate treatment was performed using the salt concentration of 25 mM at 30°C. At this concentration, a pH range of 5.0 to 8.0 was investigated to determine if the pH of the beta stream had a significant effect on the recovery of total lipid, PLs, and protein. There were no significant differences in the total lipid and PLs recoveries among all the pH investigated for zinc acetate ($P>0.05$). The numerically highest lipid recovery was seen for the 8.0 pH treatment, with 98.1% total lipid and 98.2% total PLs recovery (Table 2). These results indicate that zinc acetate had no preference for neutral lipid or PLs under all pH conditions investigated. This can also be observed in the PLs content of the overall lipid fraction, which were similar to the initial beta stream (35.0% PL). The highest PLs content fraction was the pH 7.5 treatment, with a PLs content of 36.2%, but this was not statistically significant. Additionally, there was no significant difference in PLs classes composition under the various pH conditions ($P>0.05$).

The supernatant transmittance and protein concentration, as affected by pH, results can be seen in Figure 5. As the pH increased, there was less protein in the supernatant and a higher transmittance was observed. The pH of 6.5 and above yielded significantly higher transmittance than the lower pH treatments ($P<0.05$). The lower transmittance resulting at pH below 6.5

indicates that the proteins were not precipitated as effectively under the lower pH conditions. Similar results were observed by Damodaran (2010), who only investigated the pH range below 6.5, and concluded that charge variations of membrane proteins as a function of pH can contribute to the net charge of MFGM and its interaction with zinc. The isoelectric point of most milk proteins is about 4.6-5.2 (Fong et al., 2007) and at pH above this range (5.5 through 8.0) the charge on the proteins would be negative. The zinc ion has a positive charge, thus better precipitation of protein and PLs can be expected at pH above 5.2. The transmittance results observed in this study were higher than the results observed when the concentration of the salt was studied. During this study the salt was added prior to pH adjustment (versus salt addition after pH adjustment during the salt concentration study) so there was a longer interaction time between the salt and MFGM components. This longer interaction time may have led to a supernatant with increased transmittance and lower protein concentration.

For calcium acetate of 25 mM at 60°C, the pH significantly affected the total lipid and PLs recoveries (Table 2). As the pH of the beta stream increased, higher total lipid and PLs recoveries were observed, and the pH range of 6.5-8.0 resulted in significantly higher total lipid, PLs recovery, and PLs content in the lipid fraction ($P<0.05$). The PLs content of calcium acetate treatments were significantly lower compared to the zinc acetate treatment ($P<0.05$), indicating that calcium acetate did not bind PLs as effectively as zinc acetate, or binding was stronger to other non-PLs components. With calcium acetate, pH also had a significant effect on PLs class composition. It was observed that significantly more SM was recovered in the pH range of 5.0-6.0, and significantly more PC was recovered at the pH range of 5.0-6.0; significantly more PE was recovered at the pH range of 6.5-8.0.

The protein concentration and transmittance results for the calcium acetate treatment under various pH conditions can be seen in Figure 5. The transmittance of the calcium acetate supernatant was significantly higher in the pH range of 7.0-8.0 ($P<0.05$). This pH range also resulted in significantly lower protein concentration in the supernatant ($P<0.05$). The pH range of 5.0-6.0 resulted in a supernatant with significantly higher protein concentration ($P<0.05$). The protein charge under the various pH treatments had a profound effect on how efficiently the MFGM proteins were precipitated into the pellet.

The apparent solubility of zinc acetate and calcium acetate in ethanol for its use to extract lipids from the pellet

The apparent solubility of zinc acetate and calcium acetate under three different ethanol concentrations (70, 80, and 90% v/v) and three different temperatures (30, 50, and 70°C) were investigated to determine the potential quantity of each salt that can be solubilized during the ethanol lipid extractions. The results are shown in Table 3. It is apparent that the temperature and type of salt were significant factors for their solubility in ethanol ($P<0.05$). Zinc acetate is significantly more soluble in ethanol compared to calcium acetate ($P<0.05$). The temperature of 70°C solubilized significantly more salt when compared to 30 and 50°C ($P<0.05$), whereas the concentration of ethanol was not a significant factor. Since ethanol at higher concentration had a slightly lower salt solubility, an ethanol concentration of 90% was used for the ethanol extraction. Previous work with whey protein phospholipid concentrate showed that an ethanol concentration of 90% at 70°C was optimal for a one-step total lipid extraction (Price et al., 2018). Under such conditions, 0.5 g of calcium acetate and 22.7 g of zinc acetate can be solubilized in 100 g of ethanol.

The precipitation treatments used for the ethanol extraction from the beta stream pellets were 25 mM, 7.5 pH, and 30°C for zinc acetate and 100 mM, 7.5 pH, and 60°C for calcium acetate. The zinc acetate-precipitated pellet contained of 0.9% total lipid (10.5% dry-basis (DB)), 0.3% PLs (3.6% DB), 3.2% protein (37.2% DB), 2.6% lactose (31.4% DB), and 1.8% ash (20.9% DB), with a total solids content of 8.6%. The PLs content of the pellet was 36.2% of its total lipid, which was composed of 35.9% PC, 33.7% PE, 11.5% SM, 11.3% PI, and 7.6% PS. The calcium acetate-precipitated pellet contained 0.8% total lipid (6.6% DB), 0.3% PLs (2.3% DB), 4.6% protein (38.0% DB), 2.8% lactose (23.1% DB), and 3.9% ash (32.2% DB), with a total solids content of 12.1%. The PLs content of this pellet was 31.2% of its total lipid, which was composed of 38.3% PE, 34.2% PC, 10.0% SM, 9.7% PI, and 7.8% PS. The calcium acetate-precipitated pellet contained significantly higher ash and total solids content than the zinc acetate pellet ($P<0.05$). The usage of calcium acetate was 100 mM, which equates to 1.58% in the beta stream dispersion, while zinc acetates usage was 25 mM and 0.46% in molar and weight concentration. This means that calcium acetate usage was 3.43 times higher than zinc acetate (in weight concentration), which may have resulted in a higher concentration of ash in the calcium acetate pellet.

The weight distribution of the pellet and supernatant was calculated from the weight of supernatant divided by the initial mass of the beta stream. The higher total solids content seen in the calcium acetate treatment is due to the lower weight distribution to the pellet (23.8% of initial mass), which means there was a larger volume of supernatant (76.2%). The weight distribution for zinc acetate was 56.8% to the pellet and 43.2% to supernatant. The calcium acetate-precipitated pellet contained 12.1% total solids compared to 8.6% for zinc acetate. These results indicate that calcium acetate produced a denser pellet with significantly higher total solids

(P<0.05). These differences in the total solids content resulted in the zinc acetate-precipitated pellet requiring more ethanol for the ethanol extraction (8 mL more required for zinc acetate as stated in methods section). Also, the calcium acetate supernatant had a higher total solids (4.4%) content when compared to the supernatant produced by zinc acetate (3.0%), mainly due to higher salt concentration usage. This could also indicate that more proteins were dissolved in the calcium acetate supernatant, which was confirmed by the protein concentration results.

Ethanol extraction of lipids from the precipitated pellets

A total lipid and PLs recovery of 96.7% and 97.7% were achieved from the zinc acetate precipitated pellet, while 88.2% and 94.9 were achieved from the calcium acetate precipitated pellet (Table 4). There was no significant difference in the total lipid recovery, PLs recovery, and PLs class composition between the lipid fractions extracted from the zinc acetate- and calcium acetate-precipitated pellets (P>0.05). The PLs percentage relative to the total lipid fraction was 38.7% for calcium acetate and 34.5% for zinc acetate, which are similar to the initial 35.0% of the beta stream lipid fraction. The PLs content in the pellet was 31.2% for calcium acetate and 36.2% for zinc acetate. There was a significant enrichment in PLs content found in the ethanol extracted lipid from the calcium acetate lipid fraction compared to zinc acetate (P<0.05). The binding geometry and spacing of the complex between zinc and calcium with the MFGM components may have affected how efficiently the PLs were extracted. Zinc binds with MFGM components in a tetrahedral geometry, leading to the closest distance (2.04 Angstrom) between the zinc-MFGM complexes, which is smaller than the diameter of a water molecule (Damodaran, 2010). This short distance would not easily allow for the diffusion of water into the complex as well as ethanol. In contrast, the calcium-MFGM complex is about 3.43 Angstrom (Damodaran,

2010), which may have provided more opportunity for ethanol to penetrate and extract the PLs present in the calcium acetate pellet.

After the lipid was extracted from the pellets with ethanol, the ash content was quantified to determine the quantity of salt that was solubilized during the extraction. The lipid fraction from calcium acetate treatment had a significantly higher ash content (27.9%) than that from the zinc acetate treatment (18.5%). Under the extraction conditions (90% ethanol at 70°C), 206 mL of ethanol was used for zinc acetate pellet and 198 mL of ethanol for calcium acetate pellet. Such volume of ethanol could potentially solubilize 36.8 g of zinc acetate and 0.8 g of calcium acetate. The amount of ash in the zinc acetate-precipitated and calcium acetate-precipitated pellet was 1.8% and 3.9%, respectively, which equates to 0.05 g and 0.10g of ash in the 25g of pellet, respectively. These quantities of salt are well below the apparent solubility limit for zinc acetate and calcium acetate, meaning that all the salt present in the pellet was dissolved in ethanol. With this current extraction procedure, reducing the amount of salt being dissolved in ethanol will be difficult and additional processing to remove salt may be required.

Zinc acetate may have health concerns because greater than 225 mg zinc intake may suppress copper and iron absorption (Sheftel, 2000), but our newly identified calcium salt is safe and may provide additional benefit.

Protein distribution under optimal precipitation conditions

The protein distribution of the beta stream, supernatant, and pellet were investigated using SDS-PAGE (Figure 6). The major protein class in the initial beta stream was casein (28-35 kD), whey (<25 kD) and a small quantity of MFGM proteins (>50 kD; Mathers, 2000). The MFGM proteins represent only 1-2% of the total protein found in milk (Riccio, 2004), and the beta stream seems to have a similar protein profile to native milk. The zinc acetate supernatant

contained only whey proteins, which indicates that the casein and MFGM proteins were effectively precipitated into the pellet. The calcium acetate supernatant displayed all protein classes, which confirmed our results that calcium acetate had a lower binding affinity toward dairy proteins. The protein composition in the pellet was similar for zinc acetate and calcium acetate, with casein and whey being the predominant proteins. The concentration of MFGM proteins in these protein fractions were very low compared to casein and whey proteins as shown on the SDS-PAGE gel. Nonetheless, two membrane proteins were visible in the samples (Figure 6).

Conclusions

The use of zinc acetate and calcium acetate was effective for precipitating the total lipid, PLs, and some proteins found in the beta stream. The lowest salt concentration treatment for zinc acetate was 25 mM, at pH above 6.5 at 30°C; for calcium acetate, the effective conditions were salt concentration of 75-200 mM, pH above 6.5 at 60°C. For better recovery of SM, the 30°C calcium acetate treatment would be desirable, while the 60°C calcium acetate treatment would be desirable for PS recovery. The aqueous ethanol extraction was an effective way to remove the lipid from the precipitated pellet, and PLs recovery of as high as 98% can be achieved. Although high ash content was observed in the ethanol extracted lipid fraction, a simple further processing step can remove the salt. This work demonstrates that calcium acetate precipitation has a great potential for producing valuable lipid fractions from the beta stream or other similar dairy processing by-products.

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Figures and Tables

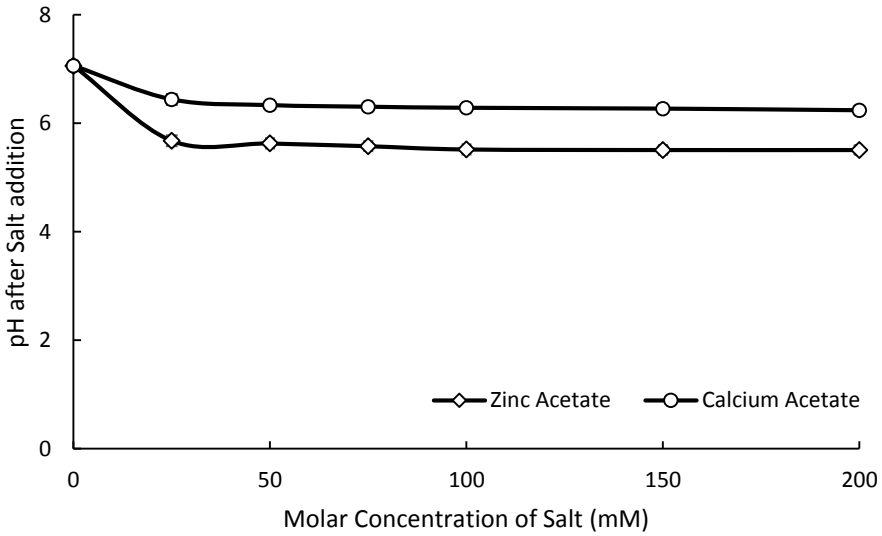


Figure 1. The pH of the beta stream (5% dispersion) as affected by salt concentration prior to heat treatment.

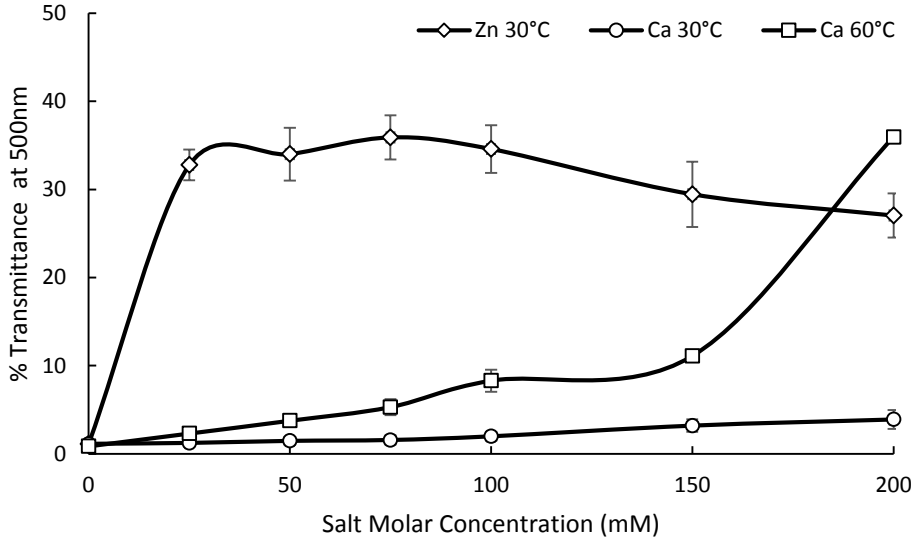


Figure 2. The effect of salt concentration and temperature on transmittance of the supernatant of the 5% beta stream dispersion.

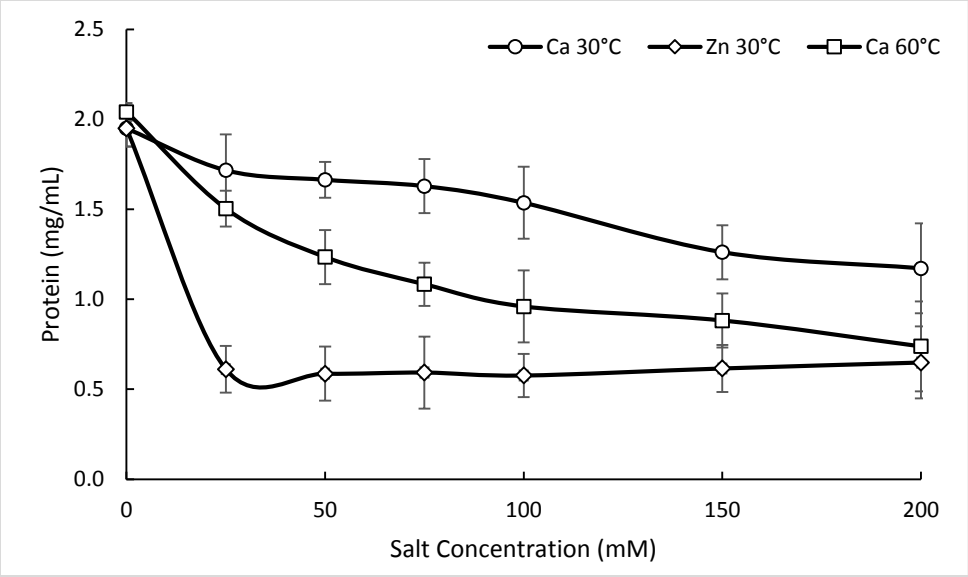


Figure 3. The effect of salt concentration and temperature treatment on the supernatant protein concentration (assayed with Bradford method) of the 5% beta stream dispersion.

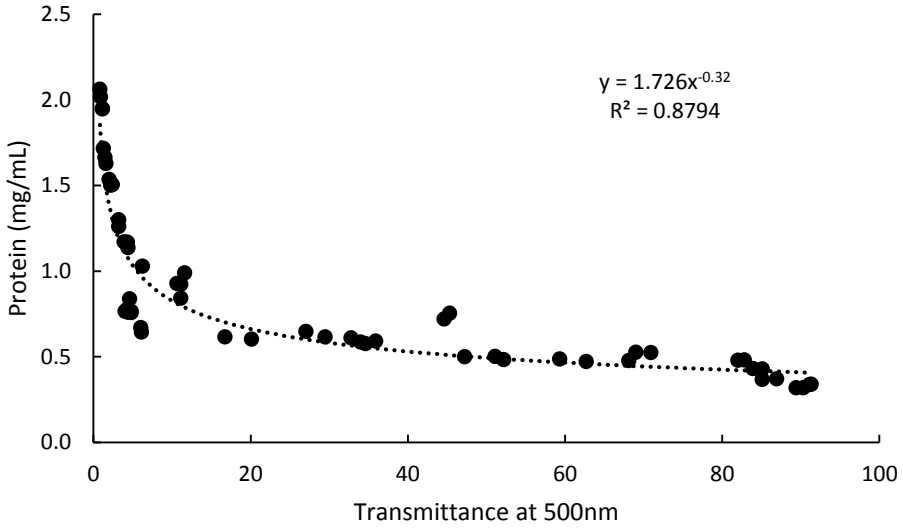
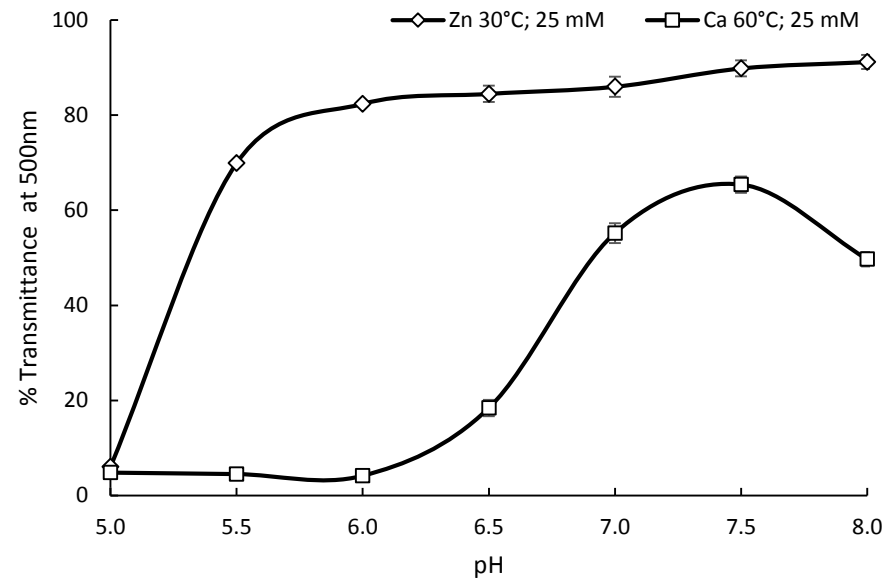
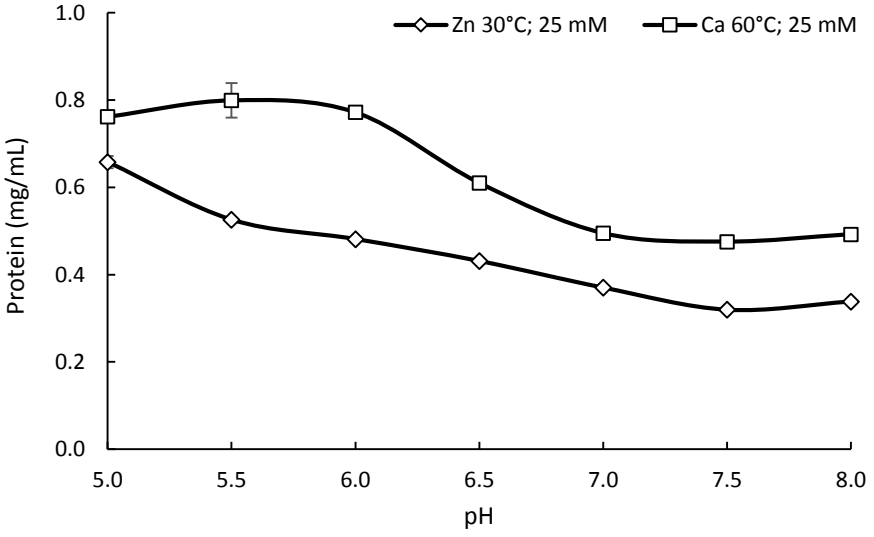


Figure 4. Correlation (Power-Law) between the protein concentration and transmittance of the supernatant from all treatments examined in this study.



a)



b)

Figure 5. The transmittance (a) and protein concentration (b) of the beta stream supernatant treated with zinc acetate at 25 mM and 30°C and calcium acetate at 25 mM and 60°C under various pH conditions.

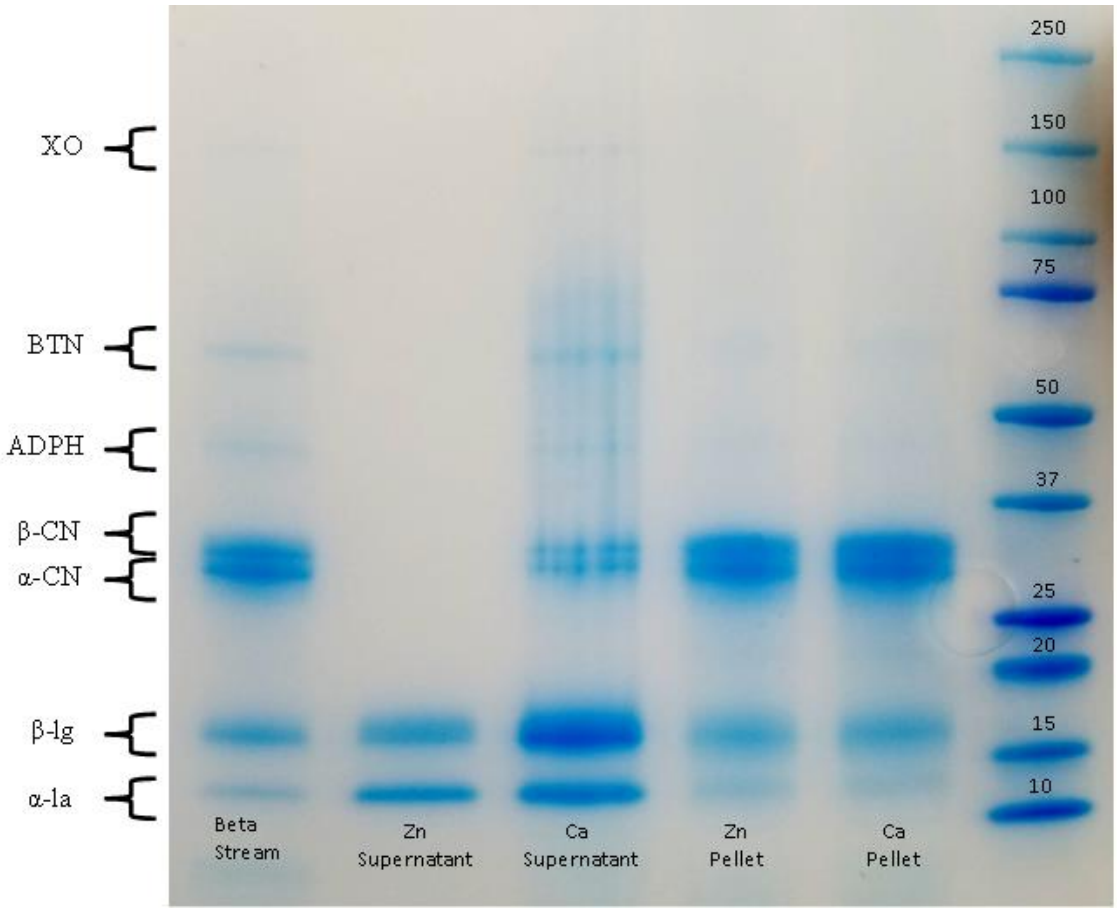


Figure 6. SDS-PAGE of the initial beta stream and supernatant and pellet samples from zinc acetate and calcium acetate treatments.

Table 1. Total lipid recovery, PLs recovery, and PLs class composition for zinc acetate at 30°C and calcium acetate at 30 and 60°C treatments under various salt concentrations.

Treatment	Total Lipid Recovery (%)	PL Recovery (%)	SM	PE	PS	PI	PC	PL % of Total Lipid
Control; 30°C	12.9 ef	10.0 gh	32.2 a	24.3 c	2.4 cd	6.9 a	34.3 a	12.3 bc
Zn 25mM; 30°C	96.2 a	97.4 a	11.2 de	36.8 ab	8.6 bcde	9.8 a	33.5 b	31.3 a
Zn 50mM; 30°C	94.5 a	97.3 a	11.7 cde	37.2 ab	8.0 bcde	9.4 a	33.7 b	31.9 a
Zn 75mM; 30°C	97.3 a	97.5 a	10.8 e	40.5 a	7.1 bcdef	9.0 a	32.6 b	31.6 a
Zn 100mM; 30°C	99.0 a	96.7 a	10.7 e	40.3 ab	7.5 bcdeg	8.7 a	32.8 b	30.6 a
Zn 150mM; 30°C	98.9 a	93.8 ab	9.9 cde	41.9 a	1.5 bcdef	10.3 a	36.4 ab	34.4 a
Zn 200mM; 30°C	96.1 a	91.2 abc	10.6 e	40.7 a	5.1 bcdef	9.3 a	34.4 b	29.8 a
Ca 25mM; 30°C	41.6 de	36.5 fg	27.4 abc	29.2 abc	0.8 f	10.3 a	32.3 b	24.5 abc
Ca 50mM; 30°C	51.4 cd	48.6 ef	27.7 ab	27.2 bc	0.6 f	10.7 a	33.4 b	27.0 ab
Ca 75mM; 30°C	51.6 cd	56.6 def	27.8 ab	28.1 abc	1.1 def	10.3 a	32.7 b	30.4 a
Ca 100mM; 30°C	61.8 bcd	65.8 cde	27.0 abcd	28.6 abc	1.8 cdef	10.0 a	32.6 b	31.1 a
Ca 150mM; 30°C	73.5 ab	76.7 abcd	20.2 abcde	31.3 abc	6.9 bcdef	10.0 a	31.6 b	33.3 a
Ca 200mM; 30°C	78.6 ab	80.3 abc	21.4 abcde	32.2 abc	3.3 bcdef	10.3 a	32.8 b	32.3 a
Control; 60°C	10.7 f	3.6 h	35.0 a	3.9 d	18.4 a	0.0 b	42.7 a	8.4 c
Ca 25mM; 60°C	71.5 abcd	66.2 bcd	11.6 bcde	33.4 abc	7.6 bcdef	11.1 a	36.2 b	30.3 a
Ca 50mM; 60°C	82.3 ab	78.8 abcd	9.3 de	35.9 ab	8.2 bcdef	11.0 a	35.6 b	31.9 a
Ca 75mM; 60°C	88.4 ab	84.9 abc	10.1 bcde	36.9 ab	8.4 bcdef	10.7 a	34.0 b	32.3 a
Ca 100mM; 60°C	90.9 ab	86.7 abc	9.9 cde	39.1 ab	9.0 bcde	9.7 a	32.3 b	31.3 a
Ca 150mM; 60°C	91.2 ab	89.9 abc	11.2 bcde	36.1 ab	9.1 bcde	10.0 a	33.7 b	33.2 a
Ca 200mM; 60°C	97.9 a	92.9 abc	11.2 bcde	37.5 ab	9.9 ab	9.6 a	31.9 b	32.1 a

Means followed by different lowercase letters within the same column are significantly different at P=0.05.

Table 2. Total lipid recovery, PLs recovery, and PLs composition from the 25 mM zinc acetate at 30°C and 25mM calcium acetate at 60°C treatments under various pH.

Treatment	Total Lipid Recovery (%)	PL Recovery (%)	SM	PE	PS	PI	PC	PL % of Total Lipid
Zn-5.0	89.3 abcd	86.9 abc	11.7 abcd	32.6 a	7.5 a	11.6 ab	36.6 ab	34.9 a
Zn-5.5	92.7 abc	87.9 abc	10.6 bcd	34.2 a	7.5 a	12.0 a	35.7 ab	33.9 ab
Zn-6.0	93.9 abc	90.9 ab	12.6 abcd	34.6 a	8.1 a	10.4 ab	34.3 b	34.6 a
Zn-6.5	94.7 ab	93.1 a	12.3 abcd	34.7 a	6.8 a	10.6 ab	35.6 ab	35.2 a
Zn-7.0	96.6 ab	95.3 a	11.9 abcd	33.8 a	7.5 a	10.9 ab	35.9 ab	34.9 a
Zn-7.5	96.7 ab	96.9 a	11.5 bcd	33.7 a	7.6 a	11.3 ab	35.9 ab	36.2 a
Zn-8.0	98.1 a	98.2 a	10.5 bcd	34.0 a	7.0 a	11.1 ab	37.4 ab	34.6 a
Ca-5.0	53.1 e	27.0 e	16.0 ab	24.1 b	8.2 a	11.2 ab	40.5 a	19.6 de
Ca-5.5	50.3 e	24.4 e	17.0 a	24.5 b	6.5 a	10.7 ab	41.4 a	19.0 e
Ca-6.0	54.6 e	32.8 e	17.1 a	24.0 b	7.1 a	10.9 ab	40.8 a	22.4 d
Ca-6.5	80.0 d	63.9 d	12.0 abcd	34.0 a	7.4 a	10.5 ab	36.1 ab	28.5 c
Ca-7.0	81.9 d	75.3 bcd	14.5 abc	33.4 a	6.5 a	9.5 b	36.0 ab	31.2 bc
Ca-7.5	83.9 cd	73.6 cd	10.1 cd	36.3 a	6.7 a	9.7 b	37.2 ab	31.1 bc
Ca-8.0	84.3 bcd	68.8 d	7.9 d	35.5 a	7.0 a	10.9 ab	38.7 ab	28.4 c

Means followed by different lowercase letters within the same column are significantly different at P=0.05.

Table 3. The apparent solubility (g salt/100 g of ethanol) of zinc acetate and calcium acetate under various ethanol concentrations (70, 80, and 90%) and temperatures (30, 50, and 70°C).

	Ethanol Concentration (%)		
Salt	70	80	90
Calcium Acetate 30°C	3.1 c	1.2 c	0.2 c
Zinc Acetate 30°C	3.2 c	2.2 c	1.6 bc
Calcium Acetate 50°C	3.2 c	1.6 c	0.4 c
Zinc Acetate 50°C	6.9 b	6.2 b	3.4 b
Calcium Acetate 70°C	3.3 c	1.8 c	0.5 c
Zinc Acetate 70°C	23.6 a	23.4 a	22.7 a

Means followed by different lowercase letters within the same column are significantly different at P=0.05.

Table 4. Total lipid and PLs yield and class distribution from ethanol extraction (90% ethanol at 70°C).

Salt	Total Lipid Recovery (%)	PLs Recovery (%)	% of Total PL					PLs % of Total Lipid
			SM	PE	PS	PI	PC	
Zinc Acetate	96.7	97.7	11.8	36.2	2.6	11.4	37.9	34.5
Calcium Acetate	88.2	94.9	11.7	40.5	4.0	8.0	35.8	38.7

There is no significant difference between the two treatments for all parameters.

CHAPTER 5: FRACTIONATION OF PHOSPHOLIPIDS AND BRANCH CHAIN FATTY ACIDS FROM DAIRY TOTAL LIPIDS USING SOLVENTS

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Interpretive Summary

The extraction of phospholipids (PLs) from dairy products has been of particular interest for the utilization of by-products and the production of a dairy lecithin. Branch chain fatty acids (BCFAs) and PLs are unique lipids found in dairy products, which are highly desired due to their health benefits. With the use of solvent fractionation, a dairy lecithin with elevated concentrations of PLs is achievable. In this study, a readily scalable method to produce a dairy lecithin from a low-value source (beta stream) is described.

Abstract

Solvent fractionation has commonly been used in the dairy industry to fractionate milk fat but has not been used to concentrate the phospholipids (PLs). Dairy PLs are of particular interest due to their health benefits and functional properties. Research has been conducted to increase the concentration of PLs in dairy products but limited concentration has been achieved using current methods. The beta stream is a by-product of anhydrous milk fat production that is currently being discarded. By applying solvent fractionation to the total lipid fraction of the beta stream, the production of a dairy lecithin can be achieved. A fixed lipid solvent ratio (1:10 g/v) was utilized along with various fractionation temperatures (-20, 2, 15, 23, 40, and 60°C) and solvent polarities (0.8-6.3 polarity index). These combinations led to a dairy lecithin product with 70.3% PLs being produced with the use of acetone. Acetone effectively precipitated PLs, while the more polar solvent ethanol was able to solubilize them. Branch chain fatty acids (BCFAs) are another unique dairy lipid fraction that has health benefits. BCFA distribution was also explored with solvent fractionation. However, no significant improvement in concentration of BCFAs was achieved because of overlapping melting points of the fatty acids with different chain lengths and degree of saturation.

Key Words: beta stream, lecithin, sphingomyelin, phosphatidylserine, branch chain fatty acids

Introduction

Phospholipids (PLs), which are generally referred to as lecithin in the food industry, are a unique class of polar lipids. Because PLs are amphiphilic molecules, they have excellent emulsification properties (Contarini & Povolo, 2013; Donato et al., 2011), and are important functional ingredients for the food, pharmaceutical, and cosmetic industries. There has been considerable interest in dairy PLs because of their nutritional value, in particular because of their high concentrations of sphingomyelin (SM) and phosphatidylserine (PS), which are deficient in commercial lecithin sourced from soybean and egg yolk (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Nejrup et al., 2017). Dairy PLs represent only 0.5-1.0% of total lipid found in milk (Fong et al., 2007), which make extraction from raw milk uneconomical. Thus low-value dairy by-products that contain elevated concentrations of PLs have been utilized for PLs concentration. One low-value by-product that has not been explored extensively is the beta stream, a by-product of anhydrous milk fat, which has a similar composition to buttermilk and butter serum. This product is currently being discarded, but it has great potential to be utilized due to its high PLs content.

Two methods have been developed (Price et al., 2018; Price et al., under review for publication) to produce an isolated lipid fraction with the use of aqueous ethanol. However, ethanol extracts both neutral lipid and PLs, and the content of PLs in the extracted fraction was similar to the starting material (25-35% PL). There has yet to be an industrial process feasible of producing an isolated dairy lipid fraction with a PLs content comparable to commercial lecithins (>50% PL). Milk fat is a mixture of triacylglycerols (TAGs) with fatty acids of varying molecular weight and degree of unsaturation. Thus, this lipid fraction exhibits a broad, variable melting range with varying physical properties (Boudreau and Arul, 1992; Mulder and Walstra, 1984).

Milk fat fractionation methods have capitalized on this variability to produce distinct categories of fat with a broad range of melting points. Solvent fractionation is a potential method to either precipitate or solubilize PLs since the selection of solvent can have a significant effect on the crystallization behavior of milk fat, largely resulting from the lower viscosity of the milk fat-solvent solution (Kaylegian and Lindsay, 1995). Due to differences in polarity and melting point between PLs and neutral lipids (NLs), solvent fractionation may be used as a feasible method to separate these two lipid classes.

Another unique type of lipid found in dairy lipid that can potentially be recovered during solvent fractionation is branch chain fatty acids (BCFAs), and they are desired because of their nutritional benefits such as anticancer activity (Yang et al., 2000; Wongtangtintharn et al., 2004). These fatty acids are derived in the dairy cow's rumen and have low melting points relative to a linear chain fatty acid with equivalent carbon number (Enser, 1984). These properties make it possible to recover BCFAs along with the concentration of PLs. Although solvents have been widely used to separate different triacylglycerol (TAG) classes based on melting point, they have yet to be utilized for PLs and BCFA concentration. SM is a highly sought dairy PLs class because of its unique composition of highly saturated long chain fatty acids (C22-24) while being the most polar dairy PLs class (Dewettinck et al 2008; Christie and Han, 2010). These unique properties may make it difficult to extract SM using solvent fractionation and identifying specific solvents suitable for SM extraction is essential for the success of an industrial methodology.

The aim of this study was to develop an economical and industrially scalable solvent fractionation technique that can be utilized to produce dairy lecithin and a product with elevated BCFA concentration. In this work solvents such as ethanol, acetone, and hexane:isopropanol (H:IP) were tested to fractionate the lipid fraction extracted from the beta stream because these

solvent systems have a wide range of polarity that will interact differently with PLs. The fractionation temperature is important for these solvent systems due to the potential for different TAG classes being recovered. Acetone has been widely used in the soybean industry to precipitate PLs and it has also been used in dairy research to precipitate PLs, specifically SM (Puente et al., 1992). The use of acetone at low temperatures was expected to crystallize the higher melting point TAGs while precipitating PLs and solubilize the other NLs. A mixture of hexane and isopropanol was expected to solubilize PLs along with BCFAs and TAGs with low melting point. Due to the different solvent property, we hypothesized that the more aqueous solvent systems would provide a solvent that solubilizes PLs while precipitating the NLs fraction at lower temperatures. The differences in PLs and NLs solubility within these various solvent systems were expected to provide an effective means to separate these lipid classes. The objective of this work was to identify a solvent system to produce a dairy lipid fraction that is comparable to a commercial lecithin with a PLs content greater than 50%, along with elevated BCFA.

Materials and Methods

The beta stream used in this study was provided by Valley Queen Cheese (Milbank, SD). The extracted lipid was quantified for PLs using ^{31}P NMR (Wang et al., 2014; Price et al., 2018). Reagent-grade solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Lipid fractionation

The total lipid was extracted from the beta stream using the Folch method (2:1 v/v chloroform-methanol; Rodriguez-Alcala & Fontecha, 2010; Folch and Stanley, 1957). The pure beta stream lipid was then added to a fixed volume of solvent (1 part lipid: 10 parts solvent, w/v). The solvents were ethanol, acetone, and H:IP. Two concentrations of ethanol (70 and 90% v/v

with deionized water) and H:IP at 1:4, 2:3, 3:2, and 4:1 v/v were investigated. After the solvent was added to the lipid fraction, the samples were heated to 60°C under 150 rpm agitation for 1 h in an incubator (Thermo Scientific MaxQ 4450). This ensured that all lipid was dissolved in solvent. The milkfat solution was then allowed to crystallize under -20, 2, 15, 23, 40, and 60°C conditions for 12 h. After crystallization the samples were centrifuged (IEC Centra CL-2) at 1800×g for 2.5 min to create a hard pellet of the precipitated lipid. The lipid in the supernatant was then decanted from the solid fat into a separate vial. The supernatant and precipitate were saved for assays.

Sequential extractions were performed after the initial extraction in an attempt to increase the concentration of PLs and BCFAs. These were specifically performed under the optimum fractionation conditions (acetone 23°C precipitate and 95% ethanol/23°C supernatant). The resulting supernatant and precipitate underwent another fractionation with the desired solvent, at a 1:10 w/v ratio. After the fractionation steps, the solvent was removed from each fraction with nitrogen. Then the samples were further dried using a vacuum oven at 50°C for 3 h. After the solvent was removed from each lipid fraction, the weights of the supernatant and precipitate fraction were recorded to determine the mass distribution. The lipid fractions were then prepared for further PLs characterization.

PLs quantification by ^{31}P NMR

The lipid from the resulting precipitate and supernatant were analyzed to determine the PLs content and class composition. This was completed using ^{31}P NMR, following a method reported by Wang et al. (2014) and Price et al. (2018) using triphenyl phosphate (TPP) as the internal standard.

Thin layer chromatography for lipid class separation

The individual PLs classes and neutral lipids (NLs) fraction were separated using thin layer chromatography (TLC; Christie, 1993). Preparative silica TLC plates (Analtech Uniplate; Newark, DE) were first activated at 120°C overnight to remove any residual moisture. Approximately 70 mg lipid in solvent was then streaked onto each plate. The polar lipids were separated using a solvent consisting of chloroform:methanol:denionized water (65:25:4 v/v/v) as described by Spence et al. (2009). After the polar lipid classes had been separated, they were visualized using 2', 7'-dichlorofluorescein spray (0.01% in ethanol). Each individual PLs class along with the NLs fraction were removed from the TLC plate and prepared for direct transesterification to fatty acid methyl esters (FAME). FAME was made using acid catalysis; 5 mL of 3% sulfuric acid in methanol (v/v) was added to each lipid fraction in a glass vial. The vial was then capped and wrapped in Teflon tape and placed in an oven at 80°C for 8 h. The reaction was stopped using 2 mL of deionized water and the FAME was extracted with 1 mL of hexane. The samples were vortex mixed and then centrifuged at 3,500 rpm for 5 min. The top hexane layer was collected and subjected to gas chromatography (GC) for determination of the fatty acid composition.

GC analysis to determine BCFA concentration and fatty acid profiles

FAME samples were analyzed by using an Agilent 7890A GC with an SPB-5 capillary column, which was 30 m length x 0.25 mm diameter with a 0.25 μm film thickness (Supelco; Bellefonte, PA). The injector and flame ionized detector were at 250 °C and 300 °C, respectively. The oven temperature program was initially 160 °C for one min, increased by 20 °C/min to 200 °C and held for one min, then increased by 10 °C/min to 240 °C and held for one min, and lastly increased by 4 °C/min to a final temperature of 300 °C and held for two min. Chromatographic peaks were identified by comparing the retention time with FAME standard mixture (17A; Nu-

Chek-Prep, Elysian, MN). For BCFA identification, several BCFA standards including *iso*-15:0, *anteiso*-15:0, *iso*-17:0 and *anteiso*-17:0 (Larodan, Solna, Sweden) were used.

Statistical analysis

All the treatments were randomized, with three replicates. Data were analyzed using a Statistical Analysis System (SAS 9.1) (SAS institute, Cary, NC), and the difference in treatment means and significance were evaluated at P = 0.05.

Results and Discussions

Beta stream PLs and BCFAs composition

The initial beta stream lipid fraction contained 35.2% PLs, which was composed of phosphatidylethanolamine (PE; 35.3%), phosphatidylcholine (PC; 30.9%), SM (19.0%), phosphatidylinositol (PI; 8.0%), and PS (6.8%). The BCFAs concentration in the initial beta stream was 1.0% of the total lipid fraction, which was composed of 13.1% *iso* 15:0, 28.9% *anteiso* 15:0, 24.0% *iso* 17:0, and 34.0% *anteiso* 17:0.

Fractionation of neutral and polar lipids

The fat distribution, PLs distribution, PLs content, and class composition for each solvent used for fractionation are shown in Table 1. It can be seen that the type of solvent and fractionation temperature were significant factors for fat (used as the term for total lipids) and PLs distribution ($p<0.05$).

Acetone: Acetone yielded significantly more PLs being recovered in the precipitated lipid fraction and the highest PLs content when compared to the other solvent treatments. The PLs content observed in the acetone treatment ranged from 57.6% to 70.3% and fractionation temperature was found to be a significant factor for the fat distribution and PLs content. The 23°C treatment resulted in a significantly higher PLs content in the precipitate compared to the 2°C and

15°C treatments. The majority of PLs found in the initial beta stream were distributed to the precipitate resulting in 98.0% to 98.7% recovery, while only 53.7% to 66.3% of the total fat was distributed to the precipitate. Lower temperature precipitated more NLs because of the saturated nature of the dairy fat, and reduced the PLs content in the precipitate. This differentiation of NLs to the supernatant and distribution of PLs to the precipitate led to the significant increase in PLs content. The fractionation temperature did not affect any of the PLs classes.

Ethanol: Unlike acetone, which effectively precipitated the PLs, ethanol was able to solubilize PLs. The 70% and 95% ethanol treatments effectively solubilized PLs into the supernatant and resulted in a supernatant lipid fraction with the higher PLs content compared to the precipitates from the treatment. The 70% ethanol supernatant fraction had the highest PLs content, ranging from 65.8% to 76.7%, while the 95% ethanol resulted in a PLs content of 38.5% to 59.9%. Although ethanol solubilized PLs into the supernatant, both ethanol treatments resulted in significantly more fat and PLs distribution to the precipitate in most cases. The 70% ethanol treatment only yielded a supernatant fat distribution ranging from 4.6% to 13.0%, while the 95% ethanol treatments resulted in significantly more PLs distribution to the supernatant. Due to the limited solubility of PLs in ethanol, the 70% treatment is not feasible. Similar results were observed where more aqueous ethanol solutions had a strong affinity towards PLs but had limited ability to solubilize high quantity of fat (Price et al., 2018).

The fractionation temperature within the ethanol treatment was a significant factor for PLs distribution, SM, and PE recovery. As the temperature of the fractionation increased, more PLs were distributed to the supernatant. The -20°C treatment resulted in significantly lower PLs distribution to the supernatant and the 95% ethanol treatment yielded significantly higher PLs distribution compared to the 70% treatment. Significantly more SM and PS were recovered in the

70% ethanol supernatant compared to all the solvents analyzed, however, it should be noted that these values are relative percentages. The fractionation temperature of -20°C resulted in higher SM recovery than all other temperature treatments analyzed for ethanol. Meanwhile, less PE was found in the -20°C fractionation temperature and 95% ethanol recovered PE at a higher rate. Ethanol effectively solubilized PLs into the supernatant and also recovered the highly-sought PLs classes.

H:IP: The polarity index of H:IP of different combinations ranged from 3.1 to 0.8, with the ratio of 1:4 resulting in the highest polarity, and the ratio of 4:1 the lowest. The increase in polarity limited the solubility of the lipid fraction. The polarity of the solvent was a significant factor for fat distribution during the fractionation ($p<0.05$). Overall, the H:IP treatments resulted in significantly less preferential PLs distribution to the precipitate or supernatant compared to the ethanol and acetone treatments. When higher concentration of hexane was used, higher fat distribution was seen in the supernatant due to the better fat solubilization. Hexane was able to solubilize both the NLs and PLs with no differentiation between the two fractions. The PLs concentration in final products was not significantly affected by the polarity of the H:IP solvent ($p>0.05$). Therefore, this solvent cannot be used for fractionation since it does not have a preference between neutral and polar lipids.

Fractionation of BCFAs

In regard to the total BCFAs content, there was difference between the precipitate and supernatant fractions as shown in Table 2. Based on the differences in melting point between BCFAs and major saturated fatty acids (e.g. C16:0, C18:0) found in dairy lipids (as shown in Table 3), the BCFAs containing TAGs was recovered effectively, as expected. It was also observed that more BCFAs were recovered from the supernatant of the acetone treatments,

indicating that the BCFAs are more esterified more into the NLs molecules than in the PLs. However, during these fractionations there was not a dramatic increase in the total BCFAs content when compared to the initial beta stream content.

There are some general trends of relative content of BCFAs in the various lipid fractions under different solvent and temperature treatments, and the percentage distribution is more related to their melting temperature. With lower melting points, higher relative content of *iso* 15:0 and *anteiso* 15:0 was observed in the supernatant, while higher *iso* 17:0 and *anteiso* 17:0 content was found in the precipitate due to their relatively higher melting points compared to *iso* 15:0 and *anteiso* 15:0. Under 95% ethanol fractionation, relative content of *anteiso* 15:0 was found to be linearly correlated with the fractionation temperature ($p<0.05$), in which decreased temperature led to increased *anteiso* 15:0 content. Similar trend was shown in C18:1 content under 95% ethanol fractionation: C18:1 content is linearly correlated with temperature ($p<0.05$), and decreased temperature results in higher C18:1 content. Moreover, under 95% ethanol fractionation, a positive linear correlation was observed between C18:1 and *anteiso* 15:0 ($p<0.05$).

BCFAs and other fatty acids are esterified randomly in the TAGs structure and a clear separation and enrichment of BCFAs has proved to be very difficult. The best treatments for PLs concentration which were 23°C acetone and 95% ethanol were selected for subsequent fractionations to examine if further concentration of the BCFAs is possible.

Fatty acid profile of fractionated products under various solvents and temperature

The fatty acid profile of fractionated products from different solvents and temperatures is shown in Supplementary Table 1. The type of solvents and fractionation temperatures were found to significantly affect the fatty acid profile of the fractionated lipids ($p<0.05$). With decrease in fractionation temperatures, ethanol yielded a significant increase in the percentage of saturated

fatty acids (C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C23:0, C24:0) in the precipitate ($p<0.05$). While higher concentration of unsaturated fatty acid (C18:1) was observed in the supernatant due to the lower melting point of C18:1 (Table 3). Fractionation at -20°C using 95% ethanol resulted in the highest C18:1 content (45.8%) in the supernatant compared to all the other solvent and temperature combinations. This corresponds to the high PLs content (59.9%) (Table 1) in which PC, with a high concentration of C18:1 (as discussed later), was found to be one of the dominant PLs (47.5%) in the supernatant from the treatment of 95% ethanol at -20 °C.

As discussed in the early section, unlike ethanol which solubilizes PLs, acetone is able to partition NLs to the supernatant while distribute PLs to the precipitate. This differentiation capability of acetone resulted in significantly higher concentration of C18:1, C20:0, C22:1, C23:0 and C24:0 in the precipitate ($p<0.05$) corresponding to the high PLs content (57.6% - 70.3%) in acetone precipitate. SM likely contributed to the higher content of C20:0, C22:1, C23:0 and C24:0 in the precipitate as it contains significantly higher amount of these fatty acids compared to the other PLs classes and NLs (Table 8). Moreover, increasing fractionation temperature significantly increased the concentration of C18:1, C20:0, C22:1, C23:0 and C24:0 coinciding with the significant increase in PLs content in the precipitate at higher temperatures ($p<0.05$) (Table 1). At 23°C, the highest PLs content (70.3%) was observed along with the highest concentration of C18:1 (39.4%), C20:0 (0.4%), C22:1 (1.9%), C23:0 (2.1%) and C24:0 (1.5%) under the three temperatures applied.

For H:IP fractionation, similar to ethanol, significantly ($p<0.05$) higher content of saturated fatty acids (C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C23:0, C24:0) was observed in the precipitates while significantly ($p<0.05$) higher amount of unsaturated fatty acid (C18:1) was found in the supernatant. Polarity did not significantly affect the concentration of C18:1, C22:1,

C23:0 and C24:0 in the supernatant. Overall, polarity of the solvent seems to have minimal effects on the fatty acid profile of H:IP fractionated products. This agrees with the results shown in Table 1 which demonstrates that H:IP solvent did not have a preference to NLs or PLs, thus led to minimal effects on the fatty acid profile of the fractionated products as well.

Sequential fractionation at different temperatures

The 95% ethanol and acetone treatments at 23°C were selected for the sequential fractionations to concentrate BCFAs, as they are most promising in PLs concentration. In addition, the 95% ethanol treatment was also investigated in an attempt to further increase the PLs concentration. The additional subsequent fractionation results for BCFAs can be seen in Figure 1, 2 and Table 4, while the PLs content and class composition of the ethanol treatment can be seen in Table 5. The additional fractionation did not further concentrate the BCFAs. These results indicate that concentration of BCFAs within the TAG structure of dairy fat may not be feasible. A BCFA may constitute only one of the three fatty acids on the glycerol backbone and the other two fatty acids could have a drastic effect on the overall melting points of the TAG, resulting in less difference between the non-BCFA- and BCFA-containing TAGs. The theoretical overlap of these BCFAs, with a wide variety of fatty acids found in dairy products, had a strong effect on where the BCFAs went during the fractionation. Thus, it may not be feasible to concentrate fatty acids when part of the TAG structure.

The non-branch fatty acid profile of sequential fractionated products is shown in Supplementary Table 2. The additional fractionation treatments were found to significantly affect the fatty acid profile of the lipid fractions ($p<0.05$). Content of C18:1 was significantly increased in the supernatant-supernatant (S-S) of the 95% ethanol treatment. Highest C18:1 (44.6%) content was observed in S-S at -20°C due to the low melting point of C18:1. Moreover, higher amount of

PC was recovered in S-S after sequential 95% ethanol treatment as shown in Table 5. This could also contribute to the higher C18:1 content in S-S as C18:1 is one of the dominant fatty acids in PC. Significant increase in C18:1 content was also observed in the supernatant-supernatant (S-S) of the acetone treatment.

During the sequential fractionations using 95% ethanol, no practically significant increase was found in the PLs content of the lipid fractions compared to the initial fractionation. However, the PLs class composition was significantly affected by the additional fractionation and content of SM was significantly increased in the precipitate-supernatant (P-S). This PLs fraction contained 56.2% to 57.8% SM, which represents a 2.7 to 2.8 times increase in concentration of SM compared to the initial precipitated fraction. However, this fraction only contained 2.9% to 3.7% of the initial lipid and 2.8% to 3.4% of initial PLs, which would be a major limiting factor in an industrial application because of the low recovery. Since SM is a highly-sought PLs class, this information may be used to develop plans to specifically target SM recovery for future studies. PS and PI were recovered at significantly higher percentage in the precipitate-precipitate (P-P) fractionation with a 1.3 to 1.5 times increase in content. Under these conditions 52.8% to 53.6% of the initial lipid and 36.9% to 37.5% of the PLs remained in this fractionation. PC was recovered at a significantly higher percentage in the supernatant-supernatant (S-S) treatment with a 1.2 to 1.6 times increase in concentration. This fraction contained 8.1% to 26.5% the initial lipid and 13.5% to 40.0% of PLs. The higher lipid distribution may make these conditions more feasible for practical applications.

Fatty acid profile of PLs products

The fatty acid profile of the initial beta stream, PLs product from acetone precipitation at 23°C, and PLs product from ethanol extraction at 23°C is shown in Table 6. The original lipid,

individual PLs classes and the NLs fraction were analyzed to determine their fatty acid composition. This analysis was conducted to determine what fractionation condition could lead to a PLs product with more saturated fatty acids, because the fatty acid composition profile could affect the oxidative stability of the final products and be used to predict oxidative stability of PLs products. Studies have shown that fatty acid composition is the dominant factor affecting lipid oxidative stability of oils and the reaction rate of linoleic acid was 20 to 40 times faster than oleic acid (Wang and Wang, 2008; McClements et al., 2000; Decker et al., 2005; Osborn and Akoh, 2003).

In general, PLs are more unsaturated than NLs, as seen in the PLs bands and the NLs band. SM contained the highest amount of long chain fatty acids, with a significantly higher content of C20:0, 22:0, 22:1, 23:0, and 24:0 compared to all the other PLs and NLs fractions. The high content of long chain fatty acids found in the SM lipid portion is similar to what other researchers have reported (Rombaut and Dewettinck, 2006; Smith & Lowry, 1962). The combination of long chain and a lower content of unsaturated fatty acids should make SM a stable PLs class under oxidative stress conditions. Similar to SM, PE contained significantly lower content of unsaturated fatty acids and significantly higher content of C18:0. The high degree of saturation in the PE fatty acid profile should make it a stable PLs class. PS and PI contained a significantly higher content of C18:1 and 18:0 compared to the other lipid fractions. PC also contained a high content of C18:1, which was significantly lower than PS and PI but higher than the other lipid fractions.

There is another interesting observation, i.e., the presence of the C23:0 fatty acid. This is an unusual odd and long chain fatty acid, which was found primarily in the PLs, not in the NL fraction from the TLC separated lipid bands. It is notable that this molecule is highly concentrated in SM (18-22%) and PE (up to 12.6%) fraction. Others also reported the C23:0's presence in the

MFGM (Le et al., 2015). Lopez et al. (2008) has reported about 1.5% C23:0 in MFGM of cream. Park et al. (2014), Schwendel et al. (2015), Frelich et al. (2009), and Kim et al. (2016) also reported C23:0 in milk fat samples.

The acetone precipitated PLs product was less saturated than the 95% ethanol extracted PLs. This may be due to the difference in PLs content of the two products. It is also interesting that the BCFAs were primarily found in the NL fraction. PC is the only polar lipid that contained BCFAs, particularly the 17:0. The NL in the acetone precipitated PLs is also more saturated than the NL from the ethanol extracted PLs.

Overall, acetone precipitation produced PLs product with higher purity. Even though the PLs product was less saturated than the unfractionated fat, the main unsaturated fatty acid was oleic acid, and it is known to be very stable compare to other polyunsaturated fatty acids.

Conclusions

The use of solvents was shown to be an effective way to concentrate PLs from the beta stream lipid fraction. The solvent type and fractionation temperature were significant factors for PLs concentration. The use of acetone resulted in a precipitated lipid fraction with a high PLs content (70.3%), while 95% ethanol was able to solubilize PLs into the supernatant. Ethanol had a high affinity for SM, and a PLs fraction with elevated SM content was obtained (56.2-57.8%) in the sequential fractionations. BCFAs were not effectively concentrated with the use of solvent fractionation, likely because of their position within the TAGs structure.

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Figures and Tables

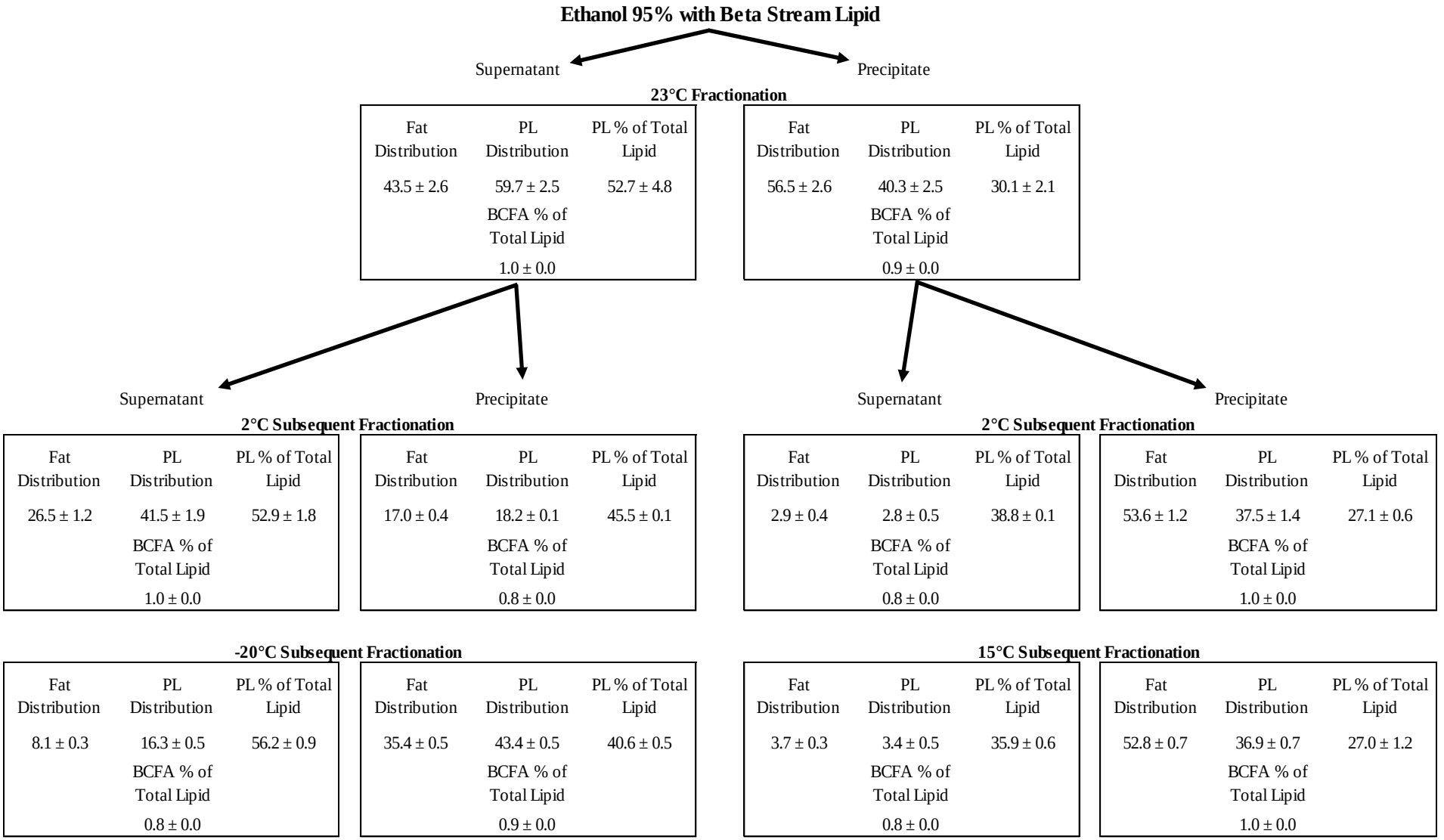

Figure 1. Phospholipids (PLs) and branch chain fatty acid (BCFA) fractionation by 95% ethanol sequential extractions.

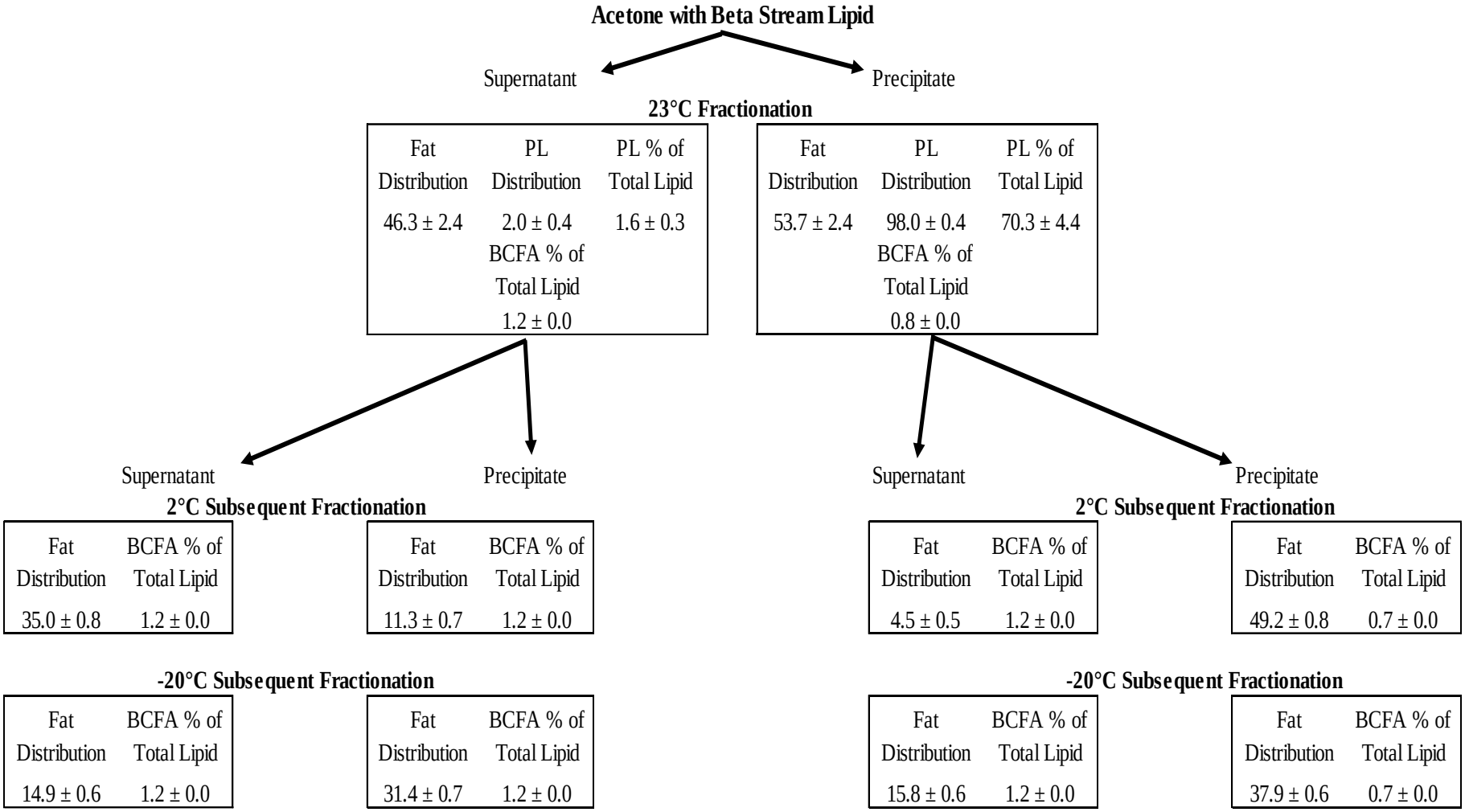

Figure 2. Phospholipids (PL) and branch chain fatty acid (BCFA) fractionation by acetone sequential extractions.

Table 1. The distribution of lipids using ethanol, acetone, and hexane:isopropanol under various fractionation temperatures and solvents.

Treatment	Fractionation Temp (°C)	Fat Distribution (%)	PLs Distribution (%)	% of Total PLs					PLs % of Total Lipid
				SM	PE	PS	PI	PC	
Original Beta Stream				19.0^{ab}	35.3^{ab}	6.8^{a}	8.0^{a}	30.9^{ab}	35.2^{bc}
70% Ethanol Precipitate	2	95.4^{a}	91.5^{a}	18.8^{ab}	40.4^{ab}	4.3^{a}	6.1^{a}	30.4^{a}	34.7^{bc}
70% Ethanol Supernatant		4.6^{c}	8.5^{c}	39.7^{a}	11.0^{bc}	5.8^{a}	5.1^{a}	38.4^{a}	65.8^{ab}
70% Ethanol Precipitate	23	93.7^{a}	88.9^{a}	15.1^{ab}	43.9^{a}	3.8^{a}	6.4^{a}	30.8^{a}	32.0^{bc}
70% Ethanol Supernatant		6.3^{c}	11.1^{c}	24.2^{ab}	22.8^{abc}	5.8^{a}	7.6^{a}	39.6^{a}	71.7^{a}
70% Ethanol Precipitate	40	90.5^{a}	78.3^{ab}	19.6^{ab}	39.6^{ab}	4.7^{a}	6.4^{a}	29.7^{a}	30.1^{c}
70% Ethanol Supernatant		9.5^{c}	21.7^{bc}	25.2^{ab}	25.2^{abc}	5.5^{a}	7.0^{a}	37.1^{a}	76.7^{a}
70% Ethanol Precipitate	60	87.0^{ab}	73.7^{ab}	16.1^{ab}	43.3^{a}	4.4^{a}	6.5^{a}	29.6^{a}	28.9^{c}
70% Ethanol Supernatant		13.0^{bc}	26.3^{bc}	21.7^{ab}	19.4^{abc}	3.4^{a}	5.8^{a}	49.7^{a}	74.7^{a}
Original Beta Stream				19.0^{ab}	35.3^{abc}	6.8^{a}	8.0^{a}	30.9^{a}	35.2^{bc}
95% Ethanol Precipitate	-20	95.3^{a}	90.5^{a}	13.8^{ab}	43.5^{a}	5.4^{a}	5.0^{a}	32.2^{a}	35.0^{bc}
95% Ethanol Supernatant		4.7^{c}	9.5^{c}	33.0^{a}	12.3^{c}	3.8^{a}	3.4^{a}	47.5^{a}	59.9^{ab}
95% Ethanol Precipitate	2	78.0^{ab}	66.6^{abc}	13.6^{ab}	44.9^{a}	5.5^{a}	10.2^{a}	25.8^{a}	33.6^{bc}
95% Ethanol Supernatant		22.0^{bc}	33.4^{bc}	9.9^{b}	40.1^{ab}	2.3^{a}	2.3^{a}	45.3^{a}	56.3^{ab}
95% Ethanol Precipitate	23	56.5^{abc}	40.3^{bc}	20.6^{ab}	33.2^{abc}	9.1^{a}	9.8^{a}	27.3^{a}	30.1^{c}
95% Ethanol Supernatant		43.5^{abc}	59.7^{ab}	12.7^{ab}	44.3^{a}	1.9^{a}	4.2^{a}	37.0^{a}	52.7^{ab}
95% Ethanol Precipitate	40	58.7^{abc}	55.6^{abc}	23.5^{ab}	29.5^{abc}	11.0^{a}	11.1^{a}	24.9^{a}	35.4^{bc}
95% Ethanol Supernatant		41.3^{abc}	44.4^{abc}	10.7^{ab}	43.9^{a}	1.3^{a}	3.4^{a}	40.7^{a}	38.5^{bc}
Original Beta Stream				19.0^{a}	35.3^{a}	6.8^{a}	8.0^{a}	30.9^{b}	35.2^{b}
Acetone Precipitate	2	66.3^{a}	98.7^{a}	13.4^{a}	43.1^{a}	4.2^{a}	7.9^{a}	31.4^{b}	57.6^{b}
Acetone Supernatant		33.7^{e}	1.3^{b}	21.2^{a}	5.2^{b}	3.9^{b}	8.1^{a}	61.6^{a}	1.5^{c}

Table 1. (continued)

Acetone Precipitate	15	57.3^{b}	98.3^{a}	11.0^{a}	45.0^{a}	5.1^{a}	7.9^{a}	31.0^{b}	63.7^{ab}
Acetone Supernatant		42.7^{d}	1.7^{b}	18.3^{a}	4.3^{b}	2.6^{ab}	6.3^{a}	68.5^{a}	1.5^{c}
Acetone Precipitate	23	53.7^{bc}	98.0^{a}	12.4^{a}	44.1^{a}	4.8^{a}	8.0^{a}	30.7^{b}	70.3^{a}
Acetone Supernatant		46.3^{cd}	2.0^{b}	19.3^{a}	3.2^{b}	0.4^{b}	1.7^{a}	75.4^{a}	1.6^{c}
Original Beta Stream				19.0^{b}	35.3^{ab}	6.8^{a}	8.0^{a}	30.9^{ab}	35.2^{a}
H: IP Precipitate (1:4 v/v)	-20	90.6^{a}	93.7^{a}	16.1^{b}	41.9^{ab}	3.5^{ab}	8.2^{a}	30.3^{ab}	38.1^{a}
H: IP Supernatant (1:4 v/v)		9.4^{g}	6.3^{c}	43.1^{a}	2.0^{b}	2.2^{b}	1.6^{b}	51.0^{a}	25.6^{a}
H: IP Precipitate (2:3 v/v)	-20	70.6^{b}	75.4^{ab}	15.1^{b}	43.7^{ab}	3.9^{ab}	8.6^{a}	28.8^{b}	38.9^{a}
H: IP Supernatant (2:3 v/v)		29.4^{f}	24.6^{bc}	10.9^{b}	38.5^{ab}	3.0^{ab}	5.3^{ab}	42.3^{ab}	30.3^{a}
H: IP Precipitate (3:2 v/v)	-20	60.8^{bc}	53.1^{bc}	13.6^{b}	44.7^{a}	4.6^{ab}	7.5^{a}	29.7^{b}	33.5^{a}
H: IP Supernatant (3:2 v/v)		39.2 ^{ef}	46.9 ^{bc}	16.5 b	32.4 ^{ab}	7.8 ^{a}	8.2 ^{a}	35.0 ^{ab}	43.9 ^{a}
H: IP Precipitate (4:1 v/v)	-20	46.8 ^{de}	47.5 ^{bc}	16.5 b	42.6 ^{ab}	3.9 ^{ab}	6.9 ^{a}	30.0 ^{ab}	37.7 ^{a}
H: IP Supernatant (4:1 v/v)		53.2 ^{cd}	52.5 ^{bc}	12.6 b	37.4 ^{ab}	8.0 ^{a}	8.3 ^{a}	33.6 ^{ab}	36.7 ^{a}

*Same lowercase letter indicates no significant difference among the samples under same solvent system including the original beta stream.

Table 2. Branch chain fatty acids (BCFAs) % relative to total BCFAs and total BCFA% under various fractionation temperatures and solvents.

Treatment	Fractionation Temp (°C)	*iso* 15:0 %	*anteiso* 15:0 %	*iso* 17:0 %	*anteiso* 17:0 %	Total BCFA%
Original Beta stream		13.1^{ab}	28.9^{a}	24.0^{ab}	34.0^{a}	1.0^{a}
70% Ethanol Precipitate	2	9.6^{c}	31.8^{a}	24.6^{a}	33.9^{a}	1.0^{ab}
70% Ethanol Supernatant		14.5^{a}	34.0^{a}	19.5^{c}	32.0^{a}	0.7^{c}
70% Ethanol Precipitate	23	9.1^{c}	33.5^{a}	24.1^{ab}	33.5^{a}	1.0^{ab}
70% Ethanol Supernatant		14.0^{a}	30.1^{a}	21.6^{bc}	34.3^{a}	0.6^{c}
70% Ethanol Precipitate	40	9.0^{c}	31.6^{a}	24.5^{a}	34.9^{a}	0.9^{ab}
70% Ethanol Supernatant		12.9^{b}	28.0^{a}	23.3^{ab}	35.7^{a}	0.8^{b}
Original Beta stream		13.1^{a}	28.9^{d}	24.0^{a}	34.0^{bc}	1.0^{a}
95% Ethanol Precipitate	-20	10.1^{c}	30.5^{c}	24.9^{a}	34.3^{ab}	1.0^{abc}
95% Ethanol Supernatant		11.6^{b}	37.8^{a}	18.8^{c}	31.7^{d}	0.8^{d}
95% Ethanol Precipitate	2	9.9^{c}	30.4^{c}	25.1^{a}	34.6^{a}	0.9^{c}
95% Ethanol Supernatant		11.2^{b}	34.6^{b}	20.5^{b}	33.7^{c}	1.0^{ab}
95% Ethanol Precipitate	23	10.1^{c}	31.0^{c}	24.2^{a}	34.6^{a}	0.9^{bc}
95% Ethanol Supernatant		10.3^{c}	31.1^{c}	24.4^{a}	34.2^{abc}	1.0^{ab}
Original Beta stream		13.1^{a}.	28.9^{c}	24.0^{b}	34.0^{c}	1.0^{b}
Acetone Precipitate	2	11.2^{bc}	25.8^{d}	26.8^{a}	36.2^{b}	0.9^{c}
Acetone Supernatant		13.0^{a}	34.7^{a}	21.9^{c}	30.3^{e}	1.2^{a}
Acetone Precipitate	15	10.7^{c}	24.4^{d}	27.6^{a}	37.3^{a}	0.8^{d}
Acetone Supernatant		13.0^{a}	34.1^{ab}	22.3^{c}	30.7^{de}	1.2^{a}
Acetone Precipitate	23	11.6^{b}	24.7^{d}	26.4^{a}	37.3^{a}	0.8^{e}
Acetone Supernatant		13.6^{a}	32.9^{b}	22.6^{bc}	30.9^{d}	1.2^{a}
Original Beta stream		13.1^{a}	28.9^{cd}	24.0^{b}	34.0^{b}	1.0^{c}
H:IP Precipitate (2:3 v/v)	-20	11.2^{c}	26.7^{d}	27.1^{a}	35.1^{a}	0.9^{d}
H:IP Supernatant (2:3 v/v)		12.5^{ab}	35.7^{b}	20.0^{c}	31.8^{c}	1.1^{a}

Table 2. (continued)

H:IP Precipitate (3:2 v/v)	-20	11.2^{c}	27.5^{d}	26.6^{a}	34.7^{ab}	0.9^{d}
H:IP Supernatant (3:2 v/v)		12.0^{bc}	34.6^{b}	21.0^{c}	32.4^{c}	1.1^{b}
H:IP Precipitate (1:4 v/v)	-20	11.5^{c}	29.9^{c}	24.4^{b}	34.2^{b}	0.9^{d}
H:IP Supernatant (1:4 v/v)		12.8^{ab}	38.3^{a}	18.2^{d}	30.7^{d}	1.1^{b}

*Same lowercase letter indicates no significant difference among the samples under same solvent system including the original beta stream.

Table 3. Melting point of the major fatty acids and BCFAs in milk fat.

	Melting point °C	Reference
C4:0	-7.9	(Budavari et al., 2001)
C6:0	-3.4	(Kirk-Othmer, 1993)
C8:0	16.3	(Lide, 2008)
C10:0	31.5	(Hawley & Lewis, 2001)
C12:0	43.8	(Lide, 2008)
C14:0	53.9	(Lide, 2008)
C16:0	62.5	(Lide, 2008)
C16:1	- 0.1	(Lide, 2008)
C18:0	69.3	(Lide, 2008)
C18:1	16.3	(Lide, 2008)
iso 15:0	51.0	(Knothe & Dunn, 2009)
anteiso 15:0	24.1	(Knothe & Dunn, 2009)
iso 17:0	60.4	(Gunstone et al., 2007)
anteiso 17:0	40.0	(Gunstone et al., 2007)

Table 4. BCFA content % relative to total BCFAs content and total BCFA% of lipid fractions from sequential extractions.

Sample ID	Fractionation Temp (°C)	*iso*15:0 %	*anteiso*15:0 %	*iso*17:0 %	*anteiso*17:0 %	Total BCFA%
95% Ethanol S-S	23-2	13.9^{a}	32.7^{cd}	20.8^{d}	32.6^{bcd}	1.0^{cd}
95% Ethanol S-S	23-(-20)	12.6^{a}	37.1^{b}	18.7^{e}	31.6^{def}	0.8^{ef}
95% Ethanol S-P	23-2	12.9^{a}	25.1^{gh}	28.2^{a}	33.7^{bcd}	0.8^{ef}
95% Ethanol S-P	23-(-20)	13.8^{a}	29.2^{ef}	23.9^{bc}	33.1^{bcd}	0.9^{d}
95% Ethanol P-S	23-2	13.0^{a}	34.0^{c}	23.5^{bc}	29.5^{gh}	0.8^{ef}
95% Ethanol P-S	23-15	12.5^{a}	30.6^{de}	23.1^{bc}	33.8^{bc}	0.8^{ef}
95% Ethanol P-P	23-2	13.0^{a}	27.1^{fg}	28.1^{a}	31.8^{cdef}	1.0^{c}
95% Ethanol P-P	23-15	13.5^{a}	27.0^{fg}	28.4^{a}	31.1^{efg}	1.0^{c}
Acetone S-S	23-2	14.2^{a}	33.3^{c}	23.3^{bc}	29.2^{gh}	1.2^{ab}
Acetone S-S	23-(-20)	13.9^{a}	40.5^{a}	17.1^{e}	28.4^{h}	1.2^{a}
Acetone S-P	23-2	14.0^{a}	25.0^{gh}	27.1^{a}	33.9^{bc}	1.2^{b}
Acetone S-P	23-(-20)	13.5^{a}	27.4^{fg}	26.5^{a}	32.6^{cde}	1.2^{b}
Acetone P-S	23-2	14.3^{a}	33.3^{c}	22.2^{cd}	30.1^{fgh}	1.2^{b}
Acetone P-S	23-15	13.7^{a}	30.6^{de}	24.5^{b}	31.2^{efg}	1.2^{b}
Acetone P-P	23-2	11.9^{a}	23.1^{hi}	27.2^{a}	37.8^{a}	0.7^{gh}
Acetone P-P	23-15	11.8^{a}	22.2^{i}	28.0^{a}	38.0^{a}	0.7^{h}

P-P: precipitate-precipitate
P-S: precipitate-supernatant
S-P: supernatant-precipitate
S-S: supernatant-supernatant
Same lowercase letter indicates no significant difference among the samples under same column.

Table 5. Class composition of PLs extracted by sequential fractionation from 95% ethanol at 23°C (see figure 1 flow chart for fractionation flow).

Sample ID	Fractionation Temp (°C)	SM	PE	PS	PI	PC	PLs % of Total Lipid
95% Ethanol S	23	12.7^{c}	44.3^{a}	1.9^{c}	4.2^{b}	37.0^{bc}	52.7^{ab}
95% Ethanol S-S	23-2	10.9^{c}	40.8^{a}	2.6^{c}	1.3^{c}	44.3^{b}	52.9^{ab}
95% Ethanol S-S	23-(-20)	31.9^{b}	6.9^{b}	2.0^{c}	1.1^{c}	58.1^{a}	56.2^{a}
95% Ethanol S-P	23-2	26.9^{ab}	35.2^{a}	4.6^{bc}	4.7^{b}	28.6^{d}	45.5^{bc}
95% Ethanol S-P	23-(-20)	15.4^{ab}	45.0^{a}	1.9^{c}	3.1^{bc}	34.6^{c}	40.6^{c}
95% Ethanol P	23	20.6^{ab}	33.2^{a}	9.1^{ab}	9.8^{ab}	27.3^{d}	30.1^{d}
95% Ethanol P-S	23-2	57.8^{a}	1.8^{b}	4.0^{bc}	1.9^{c}	34.4^{c}	38.8^{c}
95% Ethanol P-S	23-15	56.2^{a}	2.4^{b}	4.9^{bc}	1.8^{c}	34.7^{c}	35.9^{c}
95% Ethanol P-P	23-2	19.3^{ab}	33.2^{a}	13.7^{a}	14.2^{a}	19.5^{e}	27.1^{d}
95% Ethanol P-P	23-15	20.0^{ab}	34.8^{a}	9.7^{ab}	13.0^{a}	22.5^{e}	27.0^{d}

*S: supernatant; P: precipitate

Same lowercase letter indicates no significant difference within a column.

Table 6. Fatty acid profile the original lipid (OL), PLs class, and NLs fractions of the initial beta stream and PLs products from 23°C acetone precipitation and 23°C 95% ethanol extraction.

	Initial Beta Stream (35.2% PLs)							Acetone Precipitate 23°C (70.3% PLs)							95% Ethanol Supernatant 23°C (52.7% PLs)						
Fatty Acid	OL*	SM	PC	PS	PI	PE	NL	OL*	SM	PC	PS	PI	PE	NL	OL*	SM	PC	PS	PI	PE	NL
C10:0	3.6^{c}	-	-	-	-	7.8^{a}	5.2^{b}	0.9^{d}	-	-	-	-	-	3.1^{c}	2.4^{c}	-	-	-	-	-	5.6^{b}
C12:0	2.7^{bc}	-	-	-	-	4.0^{b}	5.8^{a}	1.3^{c}	-	0.5^{d}	-	-	2.3^{c}	4.2^{b}	2.9^{b}	0.3^{d}	0.6^{d}	-	-	-	6.0^{a}
C14:0	7.5b^{c}	2.2^{fg}	6.4^{bcd}	1.9_{fg}	1.3^{g}	11.6^{a}	14.1^{a}	5.9^{cde}	2.9^{fg}	6.5^{bcd}	1.7^{fg}	2.0^{fg}	7.8^{bc}	13.3^{a}	10.0^{ab}	2.7^{fg}	7.8^{bc}	2.6^{fg}	1.5^{fg}	5.6^{cde}	14.6^{a}
iso 15:0	0.1^{b}	-	-	-	-	-	0.1^{b}	0.1^{b}	-	-	-	-	-	0.3^{a}	0.1^{b}	-	-	-	-	-	-
*anteiso*15:0	0.3^{a}	-	-	-	-	-	0.3^{a}	0.2^{b}	-	-	-	-	-	-	0.3a	-	-	-	-	-	-
C16:0	27.3^{cd}	21.7^{de}	32.4^{bc}	20.0^{de}	18.6^{de}	44.1^{a}	39.7^{a}	25.1^{cd}	22.6^{de}	34.3^{ab}	17.2^{e}	18.2^{e}	33.2^{ab}	42.5^{a}	32.4^{bc}	25.2^{cd}	38.6^{ab}	23.8^{cd}	19.5^{de}	25.6^{cd}	39.4^{ab}
iso 17:0	0.2^{c}	-	-	-	-	-	0.2^{c}	0.2^{c}	-	0.6^{ab}	-	-	-	0.5^{ab}	0.2^{c}	-	0.7^{a}	-	-	-	0.5^{ab}
*anteiso*17:0	0.3^{a}	-	-	-	-	-	0.1^{c}	0.3^{a}	-	0.3^{a}	-	-	-	-	0.3^{a}	-	0.3^{a}	-	-	-	0.2^{b}
C18:1	34.4^{c}	6.1^{f}	40.5^{bc}	52.8^{a}	54.0^{a}	9.7^{f}	21.8^{d}	43.8^{ab}	11.0^{de}	37.3^{c}	45.1^{ab}	50.5^{a}	16.8^{de}	17.2^{de}	35.8^{c}	4.3^{f}	36.3^{c}	43.2^{ab}	52.4^{a}	2.1^{f}	22.4^{d}
C18:0	18.1^{cd}	12.4^{e}	19.9^{bc}	22.0^{bc}	20.9^{bc}	22.8^{bc}	12.5^{de}	15.4^{cd}	16.4^{cd}	18.3^{cd}	29.0^{a}	26.9^{ab}	27.2^{a}	17.6^{d}	10.8^{e}	12.7^{de}	14.4^{cd}	24.8^{ab}	22.8^{bc}	33.1^{a}	11.2^{e}
C20:0	0.5^{c}	1.5^{a}	0.2^{c}	-	-	-	0.1^{c}	0.4^{c}	1.3^{a}	0.3^{c}	0.7^{b}	-	-	0.2^{c}	0.2^{c}	1.4^{a}	-	-	-	-	0.1^{c}
C22:1	1.4^{ef}	18.3^{a}	0.3^{f}	1.8^{ef}	1.6^{ef}	-	-	2.1^{ef}	15.0^{a}	0.7^{ef}	2.6^{de}	0.8^{ef}	4.8^{c}	0.4^{f}	1.6^{ef}	17.8^{a}	0.4^{f}	3.1^{cd}	0.9^{ef}	13.1^{b}	-
C23:0	1.9^{e}	22.3^{a}	0.3^{e}	1.5^{cd}	2.6^{c}	-	-	2.4^{c}	18.1^{a}	0.7^{e}	2.3^{c}	0.9^{e}	4.7^{c}	0.4^{e}	1.8^{d}	21.1^{a}	0.5^{e}	2.5^{d}	2.1^{de}	12.6^{b}	-
C24:0	1.7^{e}	15.5^{a}	-	-	1.0^{e}	-	-	1.7^{e}	12.7^{b}	0.5^{e}	1.4^{e}	0.7^{e}	3.2^{d}	0.3^{e}	1.1^{e}	14.5^{a}	0.4^{e}	-	0.8^{e}	7.9^{c}	-
Total Saturated	63.9^{cd}	75.6^{b}	$59.2d^{e}$	45.4^{f}	44.4^{f}	90.3^{a}	78.1^{ab}	54.1^{f}	74.0^{bc}	62.0^{de}	52.3^{f}	48.7^{de}	78.4^{ab}	82.4^{ab}	62.7^{de}	77.9^{ab}	63.3^{de}	53.7^{ef}	46.7^{f}	84.8^{ab}	77.6^{ab}
Total Unsaturated	36.1^{cd}	24.4^{ef}	40.8^{bc}	54.6^{a}	55.6^{a}	9.7^{f}	21.9^{ef}	45.9^{bc}	26.0^{de}	38.0^{cd}	47.7^{bc}	51.3^{ab}	21.6^{ef}	17.6^{ef}	37.3^{cd}	22.1^{ef}	36.7^{cd}	46.3^{bc}	53.3^{a}	15.2^{ef}	22.4^{ef}

"-" as not detected; Same lowercase letter indicated no significant difference within a row.
*****Original lipid fraction that did not undergo TLC.

Supplementary Table 1. Fatty acid profile of lipids under various fractionation temperature and solvents

Sample ID	Fractionation Temp (°C)	C10:0	C12:0	C14:0	C16:0	C18:1	C18:0	C20:0	C22:1	C23:0	C24:0
Original Beta stream		2.1	2.0	8.5	29.2	33.1	12.4	0.2	1.0	1.1	0.8
70% Ethanol Precipitation	2	2.1^{a}	2.5^{a}	8.9^{a}	29.2^{a}	31.7^{a}	12.4^{a}	0.2^{a}	1.0^{a}	1.0^{a}	0.7^{a}
70% Ethanol Precipitation	23	2.1^{a}	2.5^{a}	8.9^{a}	29.0^{a}	31.9^{a}	12.3^{a}	0.2^{a}	1.0^{a}	1.1^{a}	0.7^{a}
70% Ethanol Precipitation	40	1.9^{a}	2.4^{a}	8.3^{a}	27.8^{a}	33.2^{a}	12.7^{a}	0.2^{a}	1.1^{a}	1.2^{a}	0.8^{a}
70% Ethanol Supernatant	2	1.0^{a}	1.6^{a}	6.3^{a}	21.2^{b}	38.3^{a}	7.3^{b}	0.1^{b}	0.8^{a}	0.8^{a}	0.5^{a}
70% Ethanol Supernatant	23	0.8^{a}	1.2^{a}	6.3^{a}	23.4^{ab}	35.5^{ab}	8.1^{b}	0.2^{a}	1.0^{a}	0.9^{a}	0.5^{a}
70% Ethanol Supernatant	40	1.5^{a}	2.0^{a}	7.9^{a}	27.7^{a}	33.0^{b}	10.3^{a}	0.2^{a}	1.1^{a}	1.0^{a}	0.5^{a}
95% Ethanol Precipitation	-20	2.1^{a}	2.5^{a}	8.9^{a}	29.1^{b}	31.5^{b}	12.4^{c}	0.2^{b}	1.0^{a}	1.1^{a}	0.8^{a}
95% Ethanol Precipitation	2	2.0^{b}	2.5^{a}	8.7^{b}	29.9^{a}	30.4^{c}	13.5^{b}	0.2^{b}	1.0^{b}	1.0^{b}	0.7^{b}
95% Ethanol Precipitation	23	2.0^{b}	2.6^{a}	8.8^{b}	28.5^{c}	31.9^{a}	13.8^{a}	0.3^{a}	0.7^{c}	0.7^{c}	0.5^{c}
95% Ethanol Supernatant	-20	2.2^{b}	2.4^{c}	6.3^{b}	18.9^{c}	45.8^{a}	5.5^{c}	0.1^{c}	0.5^{c}	0.6^{c}	0.4^{c}
95% Ethanol Supernatant	2	2.4^{a}	2.8^{a}	8.9^{a}	24.4^{b}	38.2^{b}	7.8^{b}	0.2^{b}	0.9^{b}	1.0^{b}	0.6^{b}
95% Ethanol Supernatant	23	2.2^{b}	2.6^{b}	9.0^{a}	29.2^{a}	32.2^{c}	9.7^{a}	0.2^{a}	1.4^{a}	1.5^{a}	1.0^{a}
Acetone Precipitation	2	1.3^{a}	1.8^{a}	7.1^{a}	26.8^{a}	34.1^{c}	13.9^{a}	0.3^{b}	1.4^{c}	1.5^{c}	1.1^{c}
Acetone Precipitation	15	0.9^{b}	1.3^{b}	5.8^{b}	24.2^{b}	37.6^{b}	14.1^{a}	0.4^{a}	1.8^{b}	1.9^{b}	1.3^{b}
Acetone Precipitation	23	0.8^{b}	1.2^{c}	5.3^{c}	22.6^{c}	39.4^{a}	13.8^{a}	0.4^{a}	1.9^{a}	2.1^{a}	1.5^{a}
Acetone Supernatant	2	3.4^{a}	4.0^{b}	12.1^{c}	32.6^{c}	26.9^{a}	9.4^{c}	0.1^{b}	-	-	-
Acetone Supernatant	15	3.4^{a}	4.0^{a}	12.4^{b}	34.0^{b}	24.8^{b}	9.9^{b}	0.2^{ab}	-	-	-
Acetone Supernatant	23	3.4^{a}	4.1^{a}	12.5^{a}	34.6^{a}	24.2^{c}	10.2^{a}	0.2^{a}	0.1	-	-
H:IP Precipitation (2:3 v/v)	-20	1.7^{c}	2.3^{b}	8.8^{b}	30.9^{a}	28.6^{b}	13.9^{a}	0.3^{a}	1.1^{b}	1.2^{b}	0.8^{b}
H:IP Precipitation (3:2 v/v)	-20	1.8^{b}	2.4^{a}	9.1^{a}	31.2^{a}	28.0^{b}	13.3^{b}	0.3^{b}	1.2^{a}	1.3^{a}	0.9^{a}
H:IP Precipitation (1:4 v/v)	-20	1.9^{a}	2.5^{a}	8.8^{b}	29.3^{b}	31.3^{a}	12.7^{c}	0.3^{b}	1.0^{c}	1.1^{b}	0.8^{c}
H:IP Supernatant (2:3 v/v)	-20	2.7^{b}	3.2^{b}	8.6^{a}	23.0^{ab}	38.9^{a}	8.2^{b}	0.2^{a}	0.6^{a}	0.7^{a}	0.5^{a}
H:IP Supernatant (3:2 v/v)	-20	2.4^{c}	2.8^{c}	8.4^{ab}	23.7^{a}	39.0^{a}	9.8^{a}	0.2^{a}	0.6^{a}	0.7^{a}	0.5^{a}
H:IP Supernatant (1:4 v/v)	-20	3.0^{a}	3.3^{a}	8.2^{b}	22.7^{b}	39.5^{a}	6.4^{c}	-	0.5^{a}	0.6^{a}	0.4^{a}

*Same lowercase letter indicates no significant difference among the samples fractionated under same solvent (of the same strength) system into the same fraction (precipitate or supernatant).

Supplementary Table 2. Fatty acid profile of lipid fractions from sequential extractions.

Sample ID	Fractionation Temp (°C)	C10:0	C12:0	C14:0	C16:0	C18:1	C18:0	C20:0	C22:1	C23:0	C24:0
95% Ethanol S	23	2.2^{b}	2.6^{b}	9.0^{a}	29.2^{c}	32.2^{c}	9.7^{c}	0.2^{ab}	1.4^{c}	1.5^{c}	1.0^{c}
95% Ethanol S-S	23-2	2.3^{a}	2.8^{a}	8.1^{b}	24.4^{d}	36.2^{b}	7.4^{d}	0.2^{b}	1.2^{d}	1.4^{d}	0.9^{d}
95% Ethanol S-S	23-(-20)	2.2^{b}	2.4^{c}	5.7^{c}	17.7^{e}	44.6^{a}	4.8^{e}	-	0.4^{e}	0.5^{e}	0.3^{e}
95% Ethanol S-P	23-2	1.8^{c}	2.0^{d}	7.9^{b}	32.8^{a}	22.8^{e}	11.6^{a}	0.3^{a}	2.2^{a}	2.7^{a}	1.9^{a}
95% Ethanol S-P	23-(-20)	2.1^{d}	2.6^{b}	9.2^{a}	30.4^{b}	28.1^{d}	10.4^{b}	0.3^{ab}	1.9^{b}	2.2^{b}	1.5^{b}
95% Ethanol P	23	2.0^{a}	2.6^{a}	8.8^{a}	28.5^{a}	31.9^{a}	13.8^{a}	0.3^{b}	0.7^{c}	0.7^{c}	0.5^{c}
95% Ethanol P-S	23-2	2.0^{a}	2.3^{ab}	6.9^{b}	22.2^{b}	29.7^{b}	8.0^{b}	0.2^{c}	1.0^{a}	1.2^{a}	0.9^{a}
95% Ethanol P-S	23-15	1.9^{a}	2.2^{b}	6.9^{b}	23.3^{b}	27.4^{c}	8.4^{b}	0.2^{c}	1.0^{a}	1.2^{a}	0.8^{a}
95% Ethanol P-P	23-2	2.0^{a}	2.5^{a}	8.5^{a}	27.4^{a}	31.2^{ab}	13.9^{a}	0.3^{a}	0.8^{b}	0.9^{b}	0.7^{b}
95% Ethanol P-P	23-15	2.0^{a}	2.5^{a}	8.6^{a}	27.6^{a}	31.5^{ab}	13.6^{a}	0.3^{ab}	0.9^{b}	0.9^{b}	0.7^{b}
Acetone S	23	3.4^{c}	4.1^{b}	12.5^{a}	34.6^{b}	24.2^{c}	10.2^{c}	0.2^{c}	0.1	-	-
Acetone S-S	23-2	3.5^{b}	4.0^{b}	12.3^{a}	33.0^{c}	26.8^{b}	9.2^{d}	0.1^{d}	-	-	-
Acetone S-S	23-(-20)	4.1^{a}	4.8^{a}	11.2^{c}	23.8^{d}	36.6^{a}	6.2^{e}	0.1^{d}	-	-	-
Acetone S-P	23-2	2.2^{e}	3.4^{c}	11.7^{b}	38.5^{a}	17.0^{e}	15.1^{a}	0.2^{a}	-	-	-
Acetone S-P	23-(-20)	2.6^{d}	3.3^{c}	11.9^{b}	38.3^{a}	19.8^{d}	13.1^{b}	0.2^{b}	-	-	-
Acetone P	23	0.8^{b}	1.2^{b}	5.3^{c}	22.6^{c}	39.4^{b}	13.8^{a}	0.4^{a}	1.9^{b}	2.1^{b}	1.5^{b}
Acetone P-S	23-2	3.1^{a}	3.8^{a}	11.1^{b}	33.1^{b}	28.1^{c}	10.2^{c}	-	-	-	-
Acetone P-S	23-15	3.1^{a}	3.8^{a}	11.4^{a}	35.4^{a}	24.9^{d}	11.4^{b}	-	0.3^{c}	0.3^{c}	0.3^{c}
Acetone P-P	23-2	0.5^{c}	0.9^{c}	4.3^{d}	21.1^{d}	39.7^{b}	13.9^{a}	0.4^{a}	2.3^{a}	2.6^{a}	1.8^{a}
Acetone P-P	23-15	0.5^{c}	0.7^{d}	4.0^{e}	20.3^{e}	41.3^{a}	13.9^{a}	-	2.4^{a}	2.7^{a}	1.9^{a}

*S: supernatant; P: precipitate

*Same lowercase letter indicates no significant difference among the samples from the same solvent and initial fraction.

CHAPTER 6: INVESTIGATION OF THE OXIDATIVE STABILITY OF WHEY PROTEIN PHOSPHOLIPID CONCENTRATE

A short communication submitted for publication in the *Journal of Dairy Science*

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Abstract

Dairy products contain phospholipids (PLs) which are of a particular interest in the food industry due to their health and functional properties. Products high in PLs have been reported to develop off flavors in applications and formulations. By investigating the oxidative stability of the lipids found in whey protein phospholipid concentrate (WPPC), it can be predicted how they will affect food quality. The lipids found in WPPC were analyzed for oxidative stability in the form of a powder and as an isolated lipid fraction. The addition of antioxidants to WPPC powder did not prevent secondary oxidation products from being formed. The isolated WPPC lipid fraction was compared to egg and soy gum lipid and the results indicate that it had a lower oxidative stability compared to the other PLs rich lipid fractions. Overall, the WPPC lipid fraction may have been oxidized prior to analysis and this may be a limitation in its utilization in food applications.

Key Words: phospholipids, dairy by-products, oxidation, dairy lipids

Introduction

Lipid oxidation and the generation of secondary oxidation products in foods systems are of a serious concern because of reduced shelf life and issues related to consumer health (Wang and Wang, 2008; Berliner et al., 2001). Extensive studies have been conducted on the stability of fats and oils as bulk and in emulsions. However, there has been limited research on the oxidative stability of phospholipids (PLs), which are generally referred to as lecithin in the food industry. PLs are amphiphilic molecules making up complex classes of polar lipids (Contarini & Povolo, 2013; Donato et al., 2011). They are excellent emulsifiers and desired food ingredients that can be used in a wide variety of food applications. This property also exposes PLs to interfaces where lipid oxidation typically occurs.

The PLs content in raw milk represents only 0.5-1.0% of total milk lipids (Fong et al., 2007), but they are of interest due to their nutritional value. Dairy PLs contain higher concentrations of sphingomyelin (SM; 4.1-29.2% of total PLs), and phosphatidylserine (PS; 2.0-16.1% of total PLs) compared to other lecithin sources such as soy lecithin and egg lecithin (Burling & Graverholt, 2008; Contarini & Povolo, 2013; Nejrup et al., 2017). The nutritional benefits associated with these PLs classes are cell regulation, tumor suppression, and improving cognitive performance, which is especially significant for Alzheimer's treatments (Contarini & Povolo, 2013; Dewettinck et al., 2008; Rombaut et al., 2006; Burling et al., 2008; Pepeu et al., 1996; Rombaut & Dewettinck, 2006). These health benefits of dairy PLs have the potential to increase the value and utilization of dairy by-products that have high PLs concentrations. But before that, the oxidative stability of such ingredients must be understood.

Whey protein phospholipid concentrate (WPPC) is a by-product that is microfiltered from cheese whey during the production of whey protein isolate. This low value dairy by-

product contains a high concentration of PLs (30% of the lipid fraction). Whey powders can develop a stale oxidized off-flavor and brown discoloration during storage due to a series of complex and inter-related chemical reactions that include lipid oxidation and Maillard browning due to the components associated with PLs (Morr and Ha, 1991). These off flavors then can limit the use of whey based products in the food industry (Carunchia Whetstine et al., 2005). The use of WPPC in human food applications is limited and this product is currently underutilized, as it is used as an animal feed or spread on fields (Levin et al., 2016). A reason for its underutilization is the lipids found in whey based products are believed to be more prone to oxidation or are more oxidized compared to other dairy products (Jensen et al., 2011). Thus an investigation to better understand the oxidative stability of the lipids found in WPPC and how they compare to other commercially available lecithins was conducted. We hypothesized that an isolated WPPC lipid fraction can be oxidized under accelerated conditions, and it would have a higher oxidative stability than egg lecithin and soy lecithin. Additionally, primary and secondary antioxidants will extend the shelf-life of powdered WPPC and prevent lipid oxidation. The information gained in this study will provide some understanding of the oxidative stability of the lipids found in WPPC and provide insight for further studies.

Materials and Methods

WPPC powder was obtained from Leprino Foods (Denver, CO) and liquid WPPC was obtained from Bongards Creamery (Perham, MN). Crude soy gum (a by-product from soybean oil refining, the degumming step) was obtained from KemXGlobal (Boone, IA) and eggs were purchased from WalMart (Ames, IA). Reagent-grade solvents and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO).

Storage stability of WPPC powder

The storage stability of WPPC powder was conducted in an oven at 50°C for 39 days. Two different antioxidants were used to determine if the oxidative stability of WPPC could be extended with the use of 700 ppm alpha-tocopherol and 500 ppm ethylenediaminetetraacetic acid (EDTA). The antioxidants were compared to the control WPPC powder with no antioxidant addition. The treatment samples were prepared by dispersing the antioxidant in a 10% dispersion (w/w) of WPPC powder and deionized water. The antioxidant and WPPC components were allowed to interact for 3 h under 450 rpm agitation at ambient temperature. The dispersion was then freeze dried (Virtis Genesis 25LE) until a powder was formed. This powder was then placed in a one-gallon plastic bucket with an aluminum foil cover with punctured pinholes. The buckets were placed in the oven and samples were taken periodically throughout the study. The total lipid from the sampled powder was extracted using 2:1 (v/v) chloroform: methanol (Rodriguez-Alcala & Fontecha, 2010; Folch and Stanley, 1957), and analyzed for peroxide value (PV; AOCS Cd 8b-90) and anisidine value (AV; AOCS Cd 18-90) to determine the degree of oxidation.

Oxidative stability of WPPC lipid, soy gum lipid, and egg lipid

The oxidative stability of egg lipid, soy gum lipid, and liquid WPPC were investigated. To obtain egg lipid, the eggs were cracked and the yolk was removed from the egg white. The total lipid from egg yolk or soy gum was extracted using 2:1 (v/v) chloroform: methanol (Rodriguez-Alcala & Fontecha, 2010; Folch and Stanley, 1957). Once the isolated lipid fractions were obtained the PLs content and PLs class composition were determined by using ^{31}P NMR, following a method reported by Price et al. (2018) and Wang et al. (2014). The lipid was then dissolved in a hexane with a final concentration of 90 mg/mL. Then 1 mL of this solution was dispersed into a labeled 20 mL glass vial with 30 glass beads with a diameter of 5 mm, as

described by Polacios and Wang (2005). The vials with lipid were then rotated to ensure that glass beads were evenly coated with lipid, while the hexane was then removed with a stream of nitrogen. The vials were then placed on racks and placed horizontally into shaker ovens (Thermo Scientific MaxQ 4450) at 50, 60, and 70°C under consistent agitation of 150 rpm. The racks were covered with aluminum foil to prevent photo-oxidation. Samples were taken periodically and analyzed for PV and AV through the time periods. To measure the PV of the oxidized lipid fraction, a ferric thiocyanate colorimetric method was used following a previously established procedure (Wang & Wang, 2010). The AV was determined using AOCS method Cd 18-90 (AOCS Cd 18-90). Additionally, the initial lipid fraction was analyzed by pressure differential scanning calorimetry (PDSC) as described by Sharma and Stipanovic (2003).

GC analysis to determine fatty acid profile

Fatty acid methyl esters (FAME) were made using acid catalysis; 5 mL of 3% sulfuric acid in methanol (v/v) was added to 100 mg of the initial lipid fraction (Christie, 1993). The glass vial was then capped and wrapped in Teflon tape and placed in an oven at 80°C for 8 h. The reaction was stopped using 2 mL of deionized water, and the FAME was extracted with 1 mL of hexane. The samples were vortex mixed and then centrifuged at 3,500 rpm for 5 min. The top hexane layer was collected and placed into a gas chromatography (GC) vial to determine the fatty acid composition. FAME samples were analyzed by using a HP 5890 Series II GC (Hewlett-Packard, Palo Alto, CA, USA) with a flame-ionization detector (FID). A SP 2330 (Supelco, Bellefonte, PA, USA) column was used for FAME analysis with helium as the carrier gas. The injector and detector were set at 250°C, with an oven temperature of 160°C. The temperature of the oven was increased at 5°C/min to 210°C after 1 min at 160°C.

Chromatographic peaks were identified by comparing the retention time with FAME standard mixture (17A; Nu-Chek-Prep, Elysian, MN).

Statistical analysis

Data analyses were done by using Statistical Analysis System program (SAS 9.1; Cary, NC). The difference in treatment means and significant differences were evaluated at P = 0.05.

Results and Discussions

WPPC composition

The composition of WPPC powder was 68.1% protein, 11.1% fat, 11.1% lactose, 5.0% ash, and 4.7% moisture. The PLs percentage of the total lipid fraction was 38.3% with the major PLs classes being phosphatidylethanolamine (PE; 38.9%), phosphatidylcholine (PC; 28.2%), and sphingomyelin (SM; 17.9%). The minor PLs classes were phosphatidylserine (PS; 11.9%), and phosphatidylinositol (PI; 3.1%).

The composition of the Bongards liquid WPPC, which was utilized for total lipid extraction, was 20.2% total solids, with a total lipid content of 5.5% (27.0% on a dry basis), and a PLs content of 1.8% (33.3% of the total lipid fraction). The protein content of Bongards WPPC was approximately 65% on a dry basis, which equates to 13.1% as-is, with the remainder of the product being ash and lactose. The PLs class composition can be seen in Table 1, with the major classes being PE (44.8%) and PC (28.8%). The minor PLs classes were PS (10.1%), SM (9.4%), and PI (6.9%).

Storage stability of WPPC powder

The WPPC powder was stored in an oven for 39 days at 50°C and two different antioxidant systems (EDTA and alpha-tocopherol) were compared to the control WPPC powder. The primary oxidation product measured as PV is an indication of the early stages of oxidation

and hydroperoxide formation. All the treatments and sampling periods had lipid with zero PV, while the secondary oxidation product, AV, increased throughout the study (Figure 1). There was no significant difference between the EDTA, alpha tocopherol, and control treatments ($P>0.05$). Alpha tocopherol is a primary antioxidant that is free radical scavenger, and is EDTA a secondary antioxidant that is a metal chelator. Neither of these antioxidants were effective in reducing AV in the milk powder system. The AV increased from day 1 until day 39, indicating that lipid oxidation was occurring throughout the study for all the treatments. AV measures lipid hydroperoxide breakdown products of aldehyde and ketone (Talbot, 2016). The final sampling period (day 39) had an AV ranging from 61.4 to 65.5 for all the treatments. An AV greater than 10 can lead to off-flavors in the final product (Zajdenwerg et al., 2011). With these high AV values, the lipid would give a strong oxidized flavor in food systems.

The results from this experiment show that the primary oxidation products, lipid hydroperoxides, were broken down quickly in the powder system. This, along with the initial high AV indicates that the WPPC powder's lipid fraction had undergone lipid oxidation during manufacturing or storage of the dairy powder. Multiple processing steps are required to produce WPPC. These steps include heating (cheese making, pasteurizations, and spray drying) and membrane filtrations steps (ultrafiltration and microfiltration). The pressure changes that occur during membrane filtration can lead to lipid cleavage and degradation (Barbano et al., 1982). This along with heating has the potential to lead to the oxidation of WPPC lipids and PV breakdown. Therefore, only secondary oxidation products were present in the initial WPPC powder. These results also indicate lipid hydroperoxides maybe unstable in the powder. They broke into secondary products as soon as they are formed, and the binding of these volatile and

rancid-odored compound with the protein powder can cause flavor problems in food applications.

Oxidative stability of WPPC lipid, soy gum lipid, and egg yolk lipid

When the WPPC powder was analyzed for oxidative stability, there was a possibility that the non-lipid components, such as proteins, were interfering with oxidative stability analysis. WPPC is a whey based product, and whey contains several antioxidants that could limit lipid oxidation (Colbert & Wrieden, 1991; Allen & Decker, 1981). In order to determine if the lipids in WPPC have a similar oxidative stability compared to commercial products, an experiment was designed to limit the matrix interference. The lipid in the WPPC was extracted using a total lipid extraction prior to oxidative stability analysis. This pure lipid fraction was then compared to egg and soy gum lipids at various storage temperatures (50, 60, and 70°C). A liquid WPPC was utilized in an attempt to obtain a less processed source of WPPC. The PLs content and PLs class composition of each lipid fraction are shown in Table 1. The lipid fraction of soy gum contained significantly higher ($P<0.05$) PLs content (49.3%) than egg (30.5%) and WPPC (33.3%). The major PLs classes found in egg lipid and WPPC were PC and PE, while soy gum lipid's major classes were PE, PC, and lysophophosphatidylcholine (LPC).

These lipid fractions were analyzed for PV, AV, and PDSC to monitor the state of lipid oxidation. The PV results under the various storage temperatures are shown in Figures 2. A similar trend was observed under the 50 and 60°C storage temperatures, with WPPC having a PV peak prior to egg and soy gum lipids. The 70°C storage temperature displayed a similar trend for egg and soy gum lipids but WPPC did not display a PV peak. The elevated temperature of 70°C broke the hydroperoxides prior to the second sampling period for WPPC (Figure 2), while the 50 and 60°C storage temperatures showed WPPC PV peaks near 45.0 meq peroxide/kg.

Lipid hydroperoxides are known to break faster at elevated temperature (Christie and Han, 2010), agreeing with our observations. There was no significant difference in the PV results between the soy gum and egg lipids ($P>0.05$), which indicates they had similar oxidative stability. Under all the storage temperatures, the WPPC exhibited a significantly higher rate of PV breakdown compared to soy gum and egg lipids ($P<0.05$).

As seen in the previous study with WPPC powder, the liquid WPPC's lipid fraction may have undergone oxidation during manufacturing. The initial WPPC had a significantly higher PV, of 17.3 meq peroxide/kg, compared to soy gum and egg lipids, 5.2 and 7.6 respectively ($P<0.05$). Peroxides were identified in the liquid WPPC, which indicates that this lipid fraction had undergone less lipid hydroperoxide degradation compared to the powdered WPPC. The spray drying process may have resulted in lipid degradation as this process uses extensive heat. Further studies are needed to determine which WPPC processing steps create the highest degree of lipid oxidation. The shape of PV curve observed in this study is very similar to that reported by Palacios and Wang (2005) that the lecithin hydroperoxides reach a peak value between 30 to 45 meq/kg lipid then the break-down of these molecules are more predominant then their formation.

The AV results are shown in Figures 3. As the lipid fractions were stored for longer time periods, the AV increased under each storage temperature. The initial AV for each lipid fraction was 3.0 for soy gum lipid, 3.2 for egg lipid, and 7.1 for WPPC. All the storage temperature treatments displayed a similar trend, with WPPC having significantly higher AV compared to soy gum and egg lipids ($P<0.05$). The rate of AV formation was highest during the 70°C storage, which was significantly higher than the 50°C treatment ($P<0.05$), showing the rate of lipid oxidation increased at elevated storage temperatures. Due to the extensive oxidation that

WPPC endured prior to receiving the samples, the examination of less processed dairy PLs fraction is necessary to make a comparable comparison between soy gum lipid and egg lipid and to more accurately evaluate the oxidative stability of WPPC lipid.

These results were compared to the PDSC data, which is able to detect the end point of lipid oxidation after the lipid has been fully degraded and used to confirm the PV and AV results. A lower PDSC temperature indicates that the lipid has a lower oxidative stability. The induction end point determined by PDSC for WPPC was 174.4°C, egg lipid 187.6°C, and soy gum lipid 188.5°C. These results indicate that WPPC had significantly lower oxidative stability than egg and soy gum lipids ($P<0.05$). These results correspond with the PV results obtained and indicate that the WPPC lipid fraction was oxidized at a faster rate compared to soy gum and egg lipids.

Fatty acid profile of bulk samples

The fatty acid composition of each initial lipid fraction is shown in Table 2. Soy gum lipid contained significantly higher levels of linoleic acid (18:2), linolenic acid (18:3), and total unsaturated fatty acids compared to egg lipid and WPPC ($P<0.05$). The rate of lipid oxidation for polyunsaturated fatty acids is much higher than monounsaturated fatty acids (Wang & Wang, 2008; Kolakowska, 2003; Fatemi and Hammond, 1980). Studies have shown that fatty acid composition is the dominant factor affecting lipid oxidative stability of oils and the reaction rate of linoleic acid was 20 to 40 times faster than oleic acid (Wang & Wang, 2008; Kolakowska, 2003; Fatemi and Hammond, 1980). Palacios and Wang (2003) showed that soy lecithin is much more oxidizable than yolk lecithin using a similar oxidation evaluation method mainly due to the fatty acid composition. Based on this general rule, the WPPC should have had higher oxidative stability when compared to lipid of egg and soy gum, because WPPC contained significantly higher amounts of saturated fatty acids compared to soy gum and egg lipids ($P<0.05$). The most

prevalent fatty acids found in WPPC were palmitic acid (16:0; 39.6%) and oleic acid (18:1; 28.1%). However, natural antioxidants such as tocopherols and Maillard reaction products were most likely present in the crude soy gum, as this product was not refined (Judde et al., 2003). The soy gum lipid had a darker color indicating the presence of Maillard browning reaction products that are known antioxidants (Namiki, 1998). More parallel comparison should be made in the future when all the initial lipids are more native, i.e., not pre-oxidized, and the other compositional factors, particularly the anti- and pro-oxidant content, are considered.

Dairy processors may consider the incorporation of antioxidants into WPPC during manufacturing to slow lipid oxidation, and more research is required to evaluate the effectiveness of antioxidants in preventing flavor change. Also, it will be beneficial to determine which manufacturing steps are the main culprit for the lipid degradation in WPPC and understand the oxidative stability of other PLs rich dairy by-products. This additional research will ensure the quality of a PLs rich dairy by-products and increase their utilization in food products.

In conclusion, this preliminary work suggests that powdered and liquid form of industrial WPPC was oxidized as indicated by the high initial AV, and any lipid hydroperoxides formed were broken down quickly. Much more work is needed to study the oxidative stability of dairy by-products and identify which manufacturing steps have induced lipid oxidation. Then the best prevention strategy for lipid oxidation can be designed.

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Figures and Tables

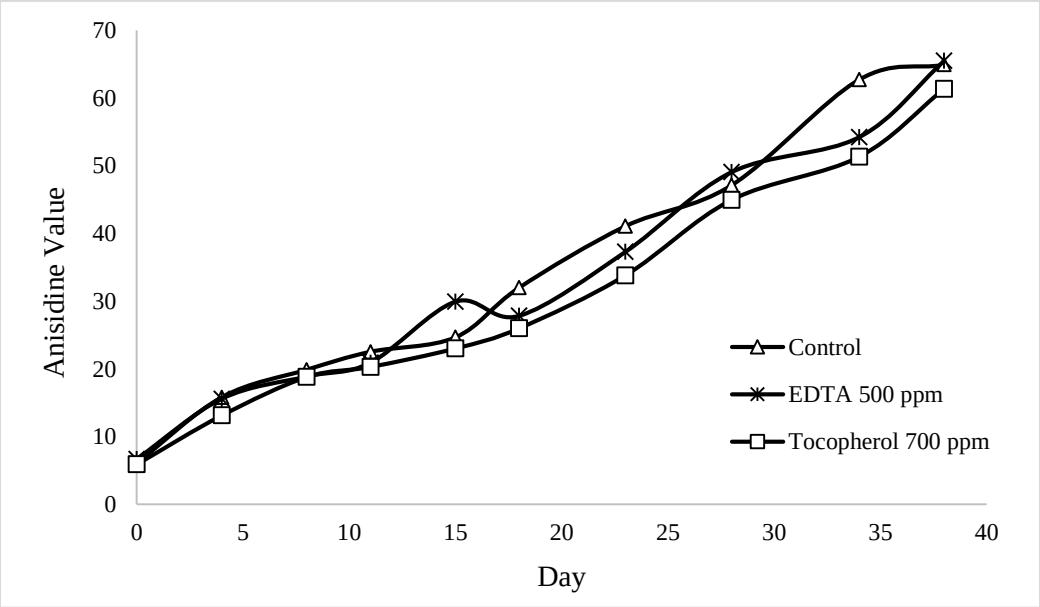
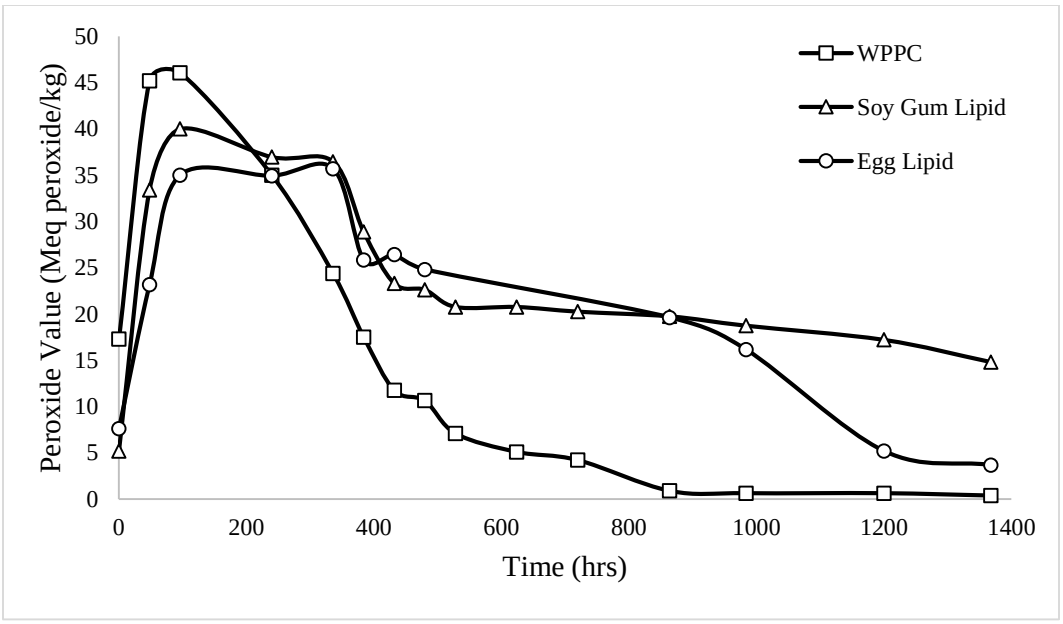
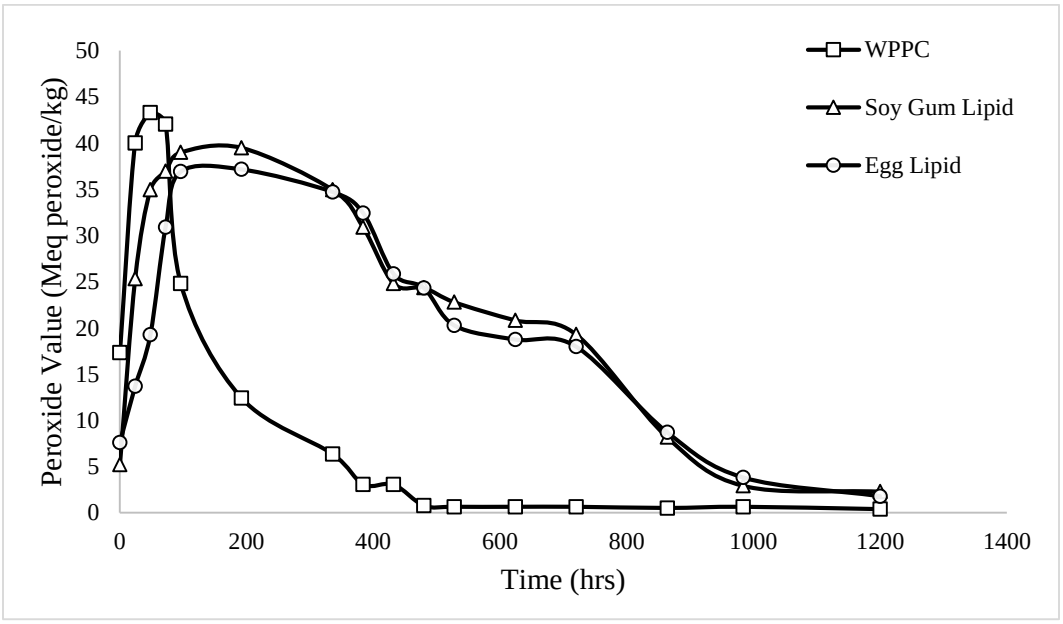


Figure 1. Secondary oxidation products (AV) produced from whey protein phospholipid concentrate (WPPC) during 50°C storage.

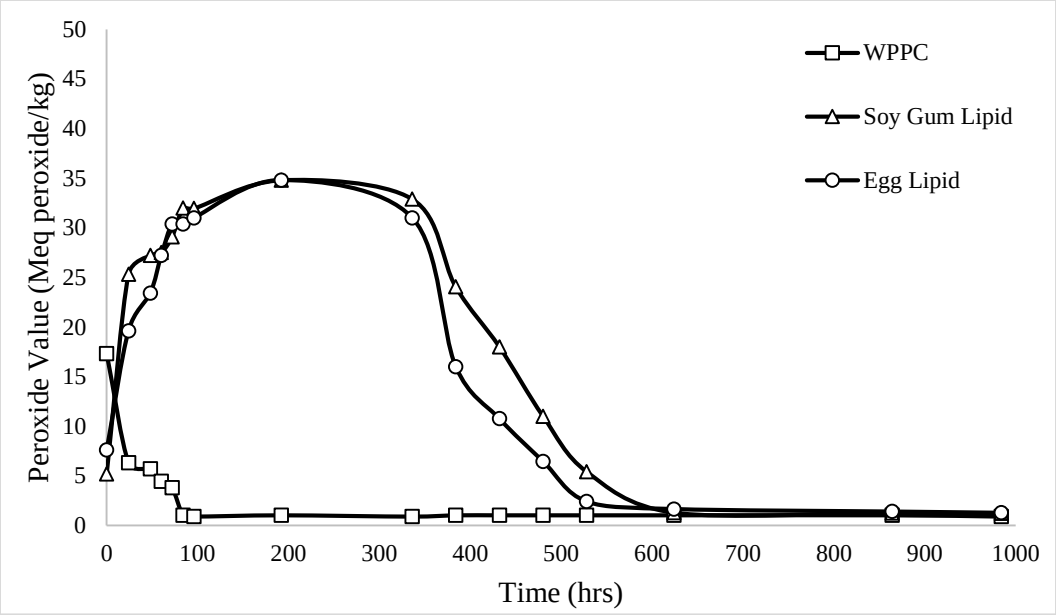


(a)



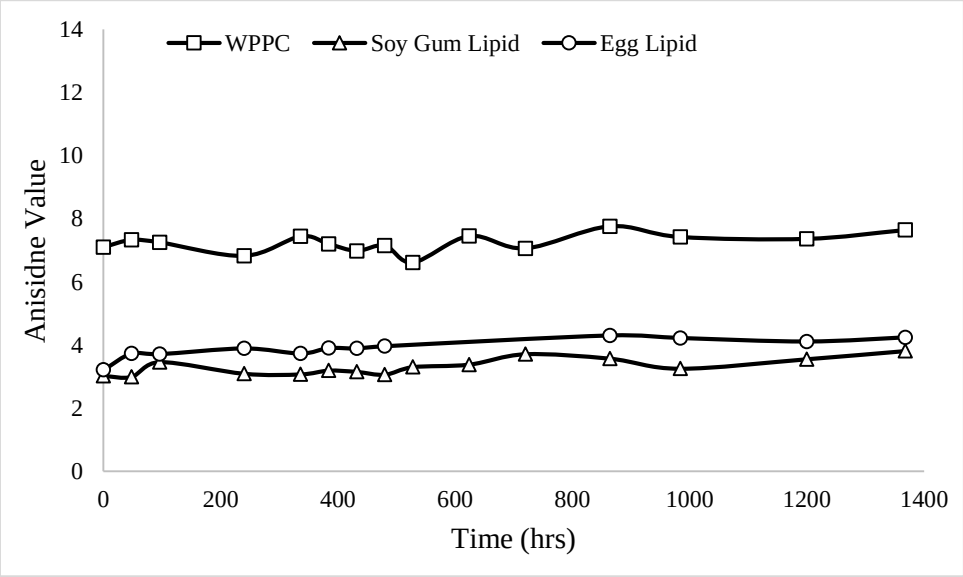
(b)

Figures 2. PV at a storage temperature of 50°C (a), 60°C (b), and 70°C (c) for WPPC, soy gum lipid, and egg yolk lipid.



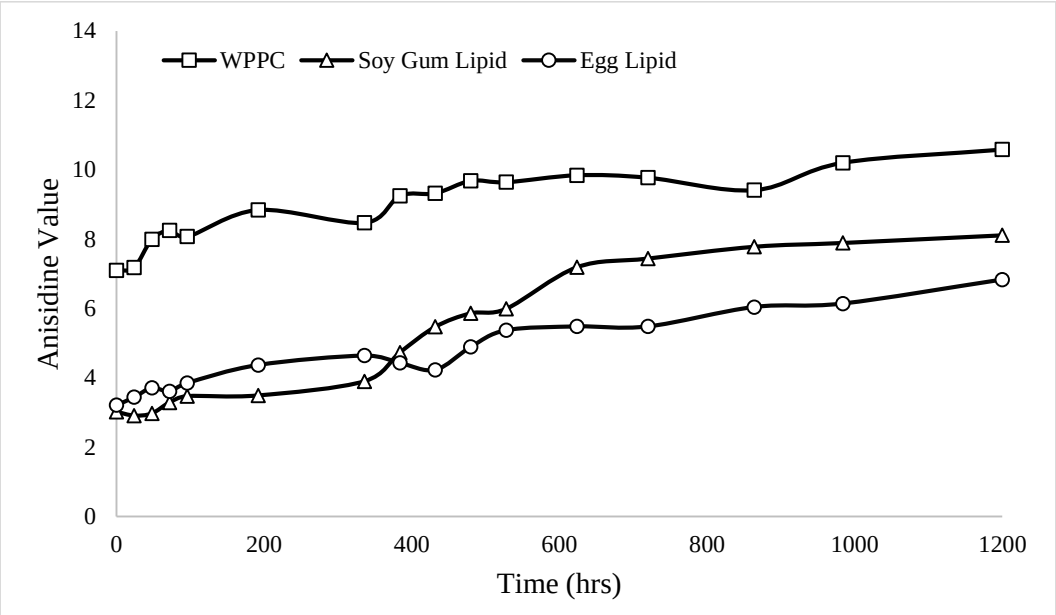
(c)

Figures 2. (continued)



(a)

Figure 3. AV at a storage temperature of 50°C (a), 60°C (b), and 70°C (c) for WPPC, soy gum lipid, and egg yolk lipid.



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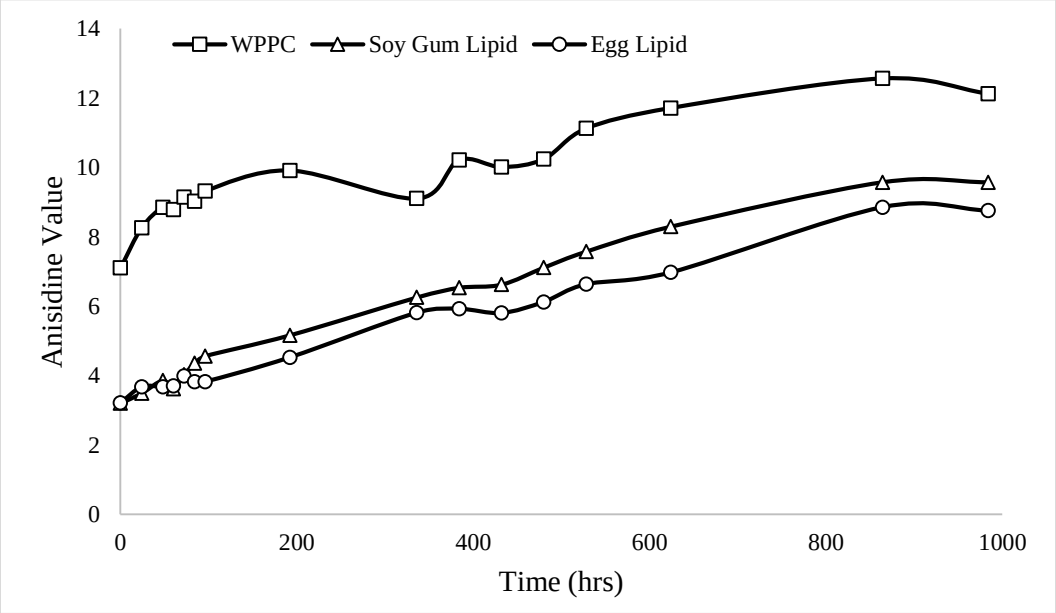

(c)

Figure 3. (continued)

Table 1. The PLs content and PLs class composition of liquid WPPC, egg lipid, and soy gum lipid.

Sample	Total PLs (% total Lipid)	PA	SM	PE	PS	PI	PC	LPC	PG	APE	DPG
WPPC	33.3 ± 1.7	-	9.4 ± 1.1	44.8 ± 1.7	10.1 ± 1.8	6.9 ± 1.6	28.8 ± 2.9	-	-	-	-
Egg lipid	30.5 ± 0.7	1.6 ± 0.4	1.6 ± 0.4	21.7 ± 0.7	2.6 ± 1.3	0.2 ± 0.1	72.3 ± 1.5	-	-	-	-
Soy gum lipid	49.3 ± 0.6	5.5 ± 0.2	-	36.7 ± 0.7	0.8 ± 0.2	14.2 ± 0.5	19.9 ± 0.1	15.8 ± 0.3	1.8 ± 0.2	2.9 ± 0.1	2.4 ± 0.2

*PA: phosphatidic acid; SM: sphingomyelin; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; PC: phosphatidylcholine; LPC: lysophosphatidylcholine; PG: phosphatidylglyercol; APE: N-acylphosphatidylethanolamine; DPG: diphosphatidyglycerol

Table 2. The initial fatty acid composition of soy gum lipid, egg lipid, and liquid WPPC prior to oxidative stability analysis.

Fatty Acid	Soy gum lipid (%)	Egg lipid (%)	WPPC (%)
16:0	15.5 ± 0.2	28.3 ± 0.1	39.6 ± 0.1
16:1	0.3 ± 0.1	0.7 ± 05	9.1 ± 0.2
18:0	3.7 ± 0.1	9.6 ± 0.1	13.5 ± 0.2
18:1	11.8 ± 0.1	38.1 ± 0.8	28.1 ± 0.2
18:2	55.5 ± 0.7	22.6 ± 0.2	7.1 ± 0.1
18:3	13.2 ± 0.2	0.7 ± 0.4	2.6 ± 0.1
Total Saturated	19.2	37.9	53.1
Total Unsaturated	80.8	62.1	46.9

CHAPTER 7: GENERAL CONCLUSIONS

The isolation and concentration of dairy phospholipids (PLs) from dairy by-products was successfully achieved with the use of solvents. Ethanol was able to effectively remove dairy lipids from dairy by-products while acetone was able to further concentrate PLs; dairy lecithin was obtained with a PLs concentration greater than 50%. The technologies utilized in these studies will provide a means for the dairy industry to utilize dairy by-products more effectively and provide methodologies appropriate for industry scale-up.

The STEP method, modified from the method used for egg yolk, which utilized ethanol, was shown to be an effective and efficient way to produce a dairy PLs concentrate from whey protein phospholipid concentrate. Higher ethanol concentration led to higher total lipid recovery, while lower ethanol concentration had a greater affinity for PLs. The optimum processing condition was found to depend on the desired PL content and recovery yield. The highest total lipid recovery was achieved with 90% ethanol at 70 and 80°C, and lipid fraction with highest PL content was obtained using 70% ethanol at 70°C. Overall, a dairy PL concentrate comparable to the commercial lecithin product can be produced with an ethanol extraction.

The use of food grade surfactants was shown to be effective in improving the PLs distribution to buttermilk during cream churning and the optimum hydrophilic-lipophilic value was determined to be 12.9. Surfactant concentration and churning temperature significantly impacted the PLs content of the resulting buttermilk. The surfactant concentration of 750 ppm led to the highest PLs content buttermilk when combined with the churning temperature of 10°C. Although the process did not provide a dramatic enrichment of PLs as initially hypothesized, this

work was a proof of concept, and the experiments may provide insights for future work with practical or theoretical importance.

Both zinc acetate and calcium acetate were effective for precipitating the total lipid and PLs found in the beta stream. The lowest effective salt concentration treatment for zinc acetate was 25 mM, at pH above 6.5 at 30°C; for calcium acetate, the effective conditions were salt concentration of 75-200 mM, pH above 6.5 at 60°C. An aqueous ethanol extraction was an effective way to remove the lipid from the precipitated pellet, and PLs recovery of as high as 98% can be achieved, though high ash content was observed in the ethanol extracted lipid fraction. This work demonstrates that zinc acetate and calcium acetate precipitation along with an ethanol extraction has a great potential for producing valuable lipid fractions from the beta stream or other similar dairy processing by-products.

The use of solvents was shown to be an effective way to concentrate PLs from the lipid fraction isolated from the beta stream. The solvent type and fractionation temperature were significant factors for PLs concentration. The use of acetone resulted in a precipitated lipid fraction with a high PLs content (70.3%), while 95% ethanol was able to solubilize PLs into the supernatant. Ethanol had an affinity for SM, especially in the sequential fractionations, where a PLs fraction with elevated SM content was obtained (56.2-57.8%). Solvent fractionation provided an effective means to concentrate PLs and produce a dairy lecithin.

A preliminary investigation into the oxidative stability of whey protein phospholipid concentrate (WPPC) lipid fraction suggests that powdered and liquid forms of industrial sources of WPPC were oxidized. This was indicated by the high initial AV, and any lipid hydroperoxides that formed were broken down quickly. Much more work is needed to study the oxidative

stability of dairy by-products and identify which manufacturing steps have induced lipid oxidation. Then the best prevention strategy for lipid oxidation can be designed.

All five studies provided insight into PLs isolation and concentration methods, along with giving an understanding of the oxidative stability of dairy by-products lipid fractions. The information gained from these studies can be utilized for further optimization to concentrate valuable PLs classes, such as sphingomyelin and phosphatidylserine, allowing for gaining a better understanding of the oxidative stability of dairy by-products lipid fractions. Additionally, understanding the economics associated with each technologies and the costs associated with each methods will provide insight for industrial scale-up.