

Selective extraction of phospholipids from dairy powders using supercritical fluid extraction

by

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Abstract

In recent years, the interest in functional components such as phospholipids (PLs) is increasing as a result of growing awareness of their health benefits. PLs affect several cell functions, such as growth, molecular transport system, memory processing, stress responses, and central nervous system myelination. Many studies have shown that the neutral lipids can be successfully extracted using supercritical carbon dioxide (SCO₂) from different types of foods such as egg, canola, pumpkin seed, fish and dairy powders. It is an alternative method to avoid the use of large quantities organic solvents. The SCO₂ is a safe, environmentally friendly and economical process to extract edible lipids from a variety of matrices. However, a modifier such as ethanol is needed to fractionate PLs due to limited solubility of PLs in SCO₂. The objectives of this study were to optimize the SFE process parameters and to determine the effect of pressure, temperature, and ethanol concentration on the extraction efficiency of PLs from whey protein phospholipid concentrate (WPPC) and buttermilk powder (BMP). Three different batches of WPPC and BMP were obtained from a commercial manufacturer and followed a unique two-step extraction process to isolate PLs from WPPC and BMP. In Step-1, neat supercritical CO₂ was used to remove all the neutral lipids at 414 bar pressure, 60 °C sample temperature, and 5 L/min CO₂ flow rate. The spent solids, the powder left after the first step extraction, were used to extract PLs in the second step. The Step-2 (SCO₂-Ethanol) process was optimized in terms of pressure (350, 414 and 550 bar), temperature (40 °C and 60 °C) and concentration of ethanol (10%, 15% and 20%) as independent factors. All the lipid fractions were analyzed by high performance lipid chromatography (HPLC) and thin layer chromatography (TLC). For WPPC, only ethanol concentration had significant effect ($P < 0.05$) on the amount of PLs extracted after the Step-2. On the other hand, temperature and ethanol concentration were significantly ($P <$

0.05) affected the efficiency of SFE for BMP. The optimal processing conditions for WPPC and BMP were 350 bar pressure, 60 °C sample temperature and 15% concentration of ethanol, and 550 bar of pressure, 60 °C sample temperature and 15% concentration of ethanol, respectively. This study allowed obtaining PLs from dairy co-products such as WPPC and BMP as a separate ingredient and this could be useful in nutraceutical and infant formulations as well as different food products formulations.

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Chapter 1 - Introduction

Milk fats consist of many types of lipids such as triglycerides, diglycerides, monoglycerides, phospholipids (PLs), free fatty acids, and cholesterol. The lipid fraction in milk is mostly distributed as spherical droplets, the milk fat globule. There is a three-layer membrane around the milk fat globule called milk fat globule membrane (MFGM). The MFGM is a source for most of the PLs present in milk. PLs gained increased interest in the past decade because of their numerous health benefits. They affect cell functions such as growth (Mathiassen et al., 2015), molecular transport system (Lodish et al., 2000), memory processing (Hellhammer et al., 2010), stress responses (Hellhammer et al., 2014), and central nervous system myelination (Helmy and Morris, 2011). Even though there is 29.4 to 40.0 mg PLs /100 g in milk (Rombaut et al., 2006; Rombaut and Dewettinck, 2006; Castro-Gómez et al., 2014), buttermilk powder (BMP) and whey protein phospholipid concentrate (WPPC), two-popular dairy-based ingredients, have relatively higher concentrations of PLs. WPPC is a co-product obtained during the manufacture of whey protein isolates (WPI). The concentration of PLs in WPPC is 12.48 to 38.11 g of PLs/100 g of WPPC (Levin et al., 2016). On the other hand, BMP is a co-product obtained during butter manufacture which contains 3 mg of PLs/100 g of BMP (Contarini and Povolò, 2013). As WPPC and BMP contain relatively a high amount of PLs, they can be used as sources to extract PLs and add values to these two dairy powders.

Supercritical fluid extraction (SFE) using carbon dioxide (CO₂) as a supercritical fluid for extraction of unique compounds from various matrices is popular in the food industry. The SFE is a safe and economical process. The critical temperature of supercritical CO₂ is 31 °C and critical pressure is 74 bar (Sapkale et al., 2010). The principle of SFE is when the temperature and pressure are above the critical points, the supercritical CO₂ can flow through a food matrix

like gas, and act as a solvent to dissolve lipids and other substances. SFE with CO₂ has successfully been used to extract neutral lipids from various foods such as soybeans, canola seed, and salmon (Montanari et al., 1999; Dunford and Temelli, 1995; Tanaka et al., 2004). Polar lipids especially PLs are not soluble in supercritical CO₂ and thereby not extractable. However, few researchers reported that the use of co-solvent such as ethanol in addition to supercritical CO₂ can remove PLs. Due to the polarity of PLs, ethanol was needed in SFE to increase the solubility and disrupt the binding between the protein and polar lipids.

The purpose of this literature review is to describe PLs in milk and their health benefits, and current technologies to concentrate PLs in dairy ingredients. SFE process to extract PLs is also reviewed in detail. Chapters 4 and 5 are focused on optimizing SFE conditions to extract PLs in WPPC and BMP.

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Chapter 2 - Literature Review

According to Code of Federal Regulation, Title 21, Part 131.110 (21 CFR 131.110), milk is defined as “the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows.” Milk is a complex fluid consists of many individual components with wide ranges. Table 2.1 shows the average compositions of bovine milk.

Table 2.1 The composition of bovine milk (Walstra et al., 2006)

| | Component (%w/w) | | | | |
|---------|------------------|---------|---------|-----------|-----------|
| | Protein | Fat | Lactose | water | Mineral |
| Average | 3.25 | 3.4 | 4.6 | 87.1 | 0.7 |
| Range | 2.3-4.4 | 2.5-5.5 | 3.8-5.3 | 85.3-88.7 | 0.57-0.83 |

Milk fat is one of the principle components with nutritional value and physiological importance. Milk fat is mainly present in the form of globules surrounded by a three-layer membrane, the milk fat globule membrane (MFGM) (Haug et al.,2007), and the MFGM contains majority of polar lipids (33% of MFGM) found in milk (Bodson et al., 2006). Polar lipids include sphingolipids and phospholipids (PLs), which has been found to cause many health benefits (Månsson, 2008). The literature review is focused on PLs in milk fat, MFGM, health benefits of PLs, high-phospholipid dairy powders, and lipid extraction methods from various foods.

Milk Fat

Milk fat is not only a source of bioactive lipid components but also an important delivery medium for nutrients, including the fat-soluble vitamins. The lipid fraction is mostly distributed as spherical droplets, named milk fat globules. The major type of lipids in milk called triglycerides, which are approximately 98.3% of lipids. The rest of components are essentially

comprised of diglycerides (0.3%), monoglycerides (0.03%), PLs (0.8%), cholesterol (0.3%), free fatty acids (0.1%), and glycerol ethers (Jack and Smith, 1956; Chandan, 1997). Triglycerides are made from three fatty acids attached to glycerol as a backbone (Drackley, 2011). Because there are many fatty acids with different distribution over the three position on the backbone, the number of triglyceride molecule species can reach to 100,000 (Walstra and Jenness, 1984). Moreover, the composition of milk fat is also highly depended on many factors including species, breed, health, environment, management practices, and diet (Linn, 1988).

Sensory properties of milk are the key for the dairy industry (Chojnicka-Paszun et al., 2012). McGiff, Barbano, and Lawless (1995) studied the effect of fat content (0–2% fat) on the sensory properties, viscosity, and color of milk. They reported that the sensory score for visual appearance and mouth-feel attributes increased with fat content. The color test of milk showed similar results that 2% fat milk was whiter (L-value), less green and blue (a, b-value) than the other low-fat milk samples. The influence of different fat content on the texture and taste of homogenized milk was studied by Chojnicka-Paszun, Jongh, and Kruiff (2012). They showed that when the fat percent was below 1% in homogenized milk, the taste and texture cannot be differentiated by the panelists. However, when fat content was higher than 1%, the attribute of creaminess increased. This results proved that the perceptible creaminess or fat film formation (a fat-like layer in the mouth) is an important attribute in sensory perception of milk (Van Aken et al., 2011). de Wijk, Prinz (2005, 2006), and Malone et al, (2003) also found the creamy taste was not only associated with an increasing fat content, but also decreased friction, which indicates better lubrication properties, mouth-feel, and smooth texture. In a different study, Roland et al. (1999) studied the effect of fat content between 0.1% to 7% on the sensory attributes of ice cream, and found that the ice cream with higher fat content had longer melting time with more

softer and creamier mouth feel compared to the low fat ice cream. In addition, the sweetness and milk powder flavor were less perceivable to the sensory panel.

Neutral lipids improve the sensory attributes in milk and dairy foods, and polar lipids in milk fat globule membrane have more nutrition values and medical values. The following section focuses on the structure and composition of milk fat globule membrane.

Milk Fat Globule Membrane

Over 95% of total milk lipids are in the form of the fat globules (Birdi, 2009). Fat globules are spherical droplets of triglyceride protected by a membrane. The milk fat globule membrane (MFGM) is a thin protective layer, which is largely derived from the apical plasma membrane of the cell (Walstra and Jenness, 1984). MFGM is a three-layer membrane, and the PLs in milk are mainly found in the outer bilayer membrane. The structure of milk fat globule is shown in Figure 2.1. The outside of the bilayer membrane is mainly contained of phosphatidylcholine (PC) and sphingomyelin (SM), and the inner surface of the bilayer is mainly made up of phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Vanderghem et al., 2010). The MFGM helps to stabilize the fat globules and prevents flocculation, coalescence, and enzyme degradation by decreasing the lipid-serum interface (Walstra and Jenness, 1984; Birdi, 2009). The components of MFGM include a complex mixture of proteins, glycoproteins, triglycerides, cholesterol, enzymes, neutral lipids, and polar lipids (Singh, 2006). The PL and protein (butyrophilin, xanthine dehydrogenase, and perilipin-2) composition of MFGM is highly variable and it depends on the size of milk fat globule. Proteins and PLs together account for over 90% of the membrane dry weight (Alberts et al., 2002). The percentage of MFGM proteins in overall protein fractions of milk is around 1-2% (Falus, 2008).

Because of the complex nature of MFGM, the functions of MFGM proteins are not well known (Riccio, 2004). There have been 40 different proteins with molecular mass ranging between 15k to 240k Da are found in the MFGM (Mather, 2000; Ye et al., 2002). According to Spitsberg (2005), MFGM contains butyrophilin (40% of MFGM proteins) and xanthine oxidoreductase (13% of MFGM proteins), which are the most representative MFGM proteins. Lactadherin, adipophilin, fatty acid binding protein, each is less than 5% in MFGM (Contarini and Povolo, 2013). On the other hand, the gross composition of neutral lipids in MFGM, especially triacylglycerol, is the most variable component (0.25-0.88 mg/mg protein). PL content of MFGM is relatively stable with an average of about 0.25 mg/mg protein. Glycosphingolipid content of MFGM is around 13 μ g/mg protein (Mather, 2011).

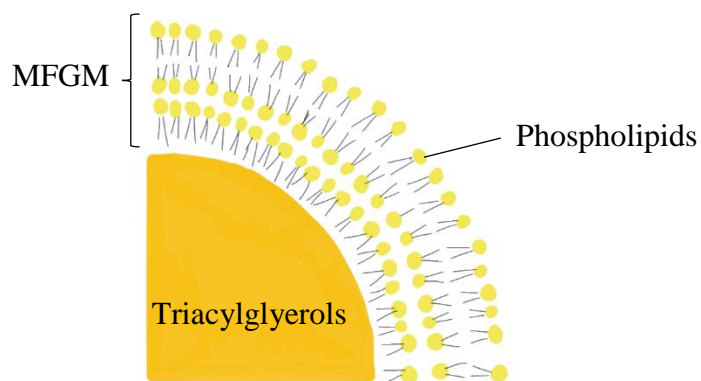


Figure 2.1 The structure of milk fat globule

Phospholipids

Phospholipids (PLs) are amphipathic that contain both polar (phosphate group) and nonpolar (fatty acid chains) properties (Ishihara et al., 1992). PLs are also compound lipids because they are mainly derived from phosphatidic acid attached to various organic heads.

Usually, compound lipids are at neutral pH because they contain both acid and base group. But phosphatidic serine and inositol lipids have net negative charge (Walstra, 2006). Milk contains up to 0.8g of PLs/100 g of total milk fat, and 60%–70% of PLs are mainly present in the MFGM (Fox and McSweeney, 2006). The PLs in membrane is disrupted and detached from the MFGM during dairy processes such as homogenization and churning. The major types of PLs include phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). In Table 2.2 shows the average content of each PLs is shown:

Table 2.2 Phospholipids composition of cow milk (Donato, 2011; Contarini and Povolo, 2013)

| Composition (% of PL in total PLs) | | | | | |
|---|-----------|-----------|-----------|-----------|-----------|
| | PE | PI | PC | SM | PS |
| Average | 38.5 | 6.5 | 25.9 | 21.4 | 7.7 |
| Range | 26.4–72.3 | 1.4–14.1 | 8.0–45.5 | 4.1–29.2 | 2.0–16.1 |

PE=phosphatidylethanolamine, PI=phosphatidylinositol, PC=phosphatidylcholine, SM=sphingomyelin, PS=phosphatidylserine

SM, PE, and PC are the three main PLs found in whole bovine milk, and account for 85 to 90% (Lopez and Ménard, 2011). Since small milk fat globule has large surface area, it had 1.4-fold to 3.5-fold more PLs than in whole milk with large fat globules. However, small fat globule showed lower relative proportions of PC and SM. There are two hypotheses which may explain the situation; first, small fat globules lead to an important change in the curvature of membrane and thereby, lose some membrane materials like SM and PC. Second, since PI, PS and PE are mostly located in the inner surface of the MFGM, and PC and SM are located in the outer layer of the MFGM, SM and PC may be excluded from small fat globules, due to the curvature of surface (Lopez and Ménard, 2011).

In the literature, the reported values of PLs and its compositional variation is due to factors including different extraction and analysis methods, feed methods, and period of lactation (Bitman and Wood, 1990). According to Mesilati-Stahy and Argov-Argaman's study (2014), weight ratio between triglyceride and PLs depends on size of milk fat globule. In a small globule, concentration of PLs is higher and the ratio is lower. One study (Argov-Argaman et al., 2014) found a high PLs concentrate, low-forage cow diet can decrease milk fat globule size. Researchers analyzed the milk produced from cows fed with high concentrate, low-forage diet, and observed all PLs (34%) and cholesterol (65%) yields increased compared to low concentrate, high-forage diet. Lopez and coworkers (2014) noticed that higher amount of phospholipids were produced during spring season due to the fresh pasture based diet for cows, which means the milk contains large proportion of small fat globules that require more PLs attachment to the surface area. In addition, Ferreiro with other researchers (2015) reported conventional milk had more PLs content than organic milk. Briefly, feeding, lactation and genetics are various factors that influence the MFGM composition and PLs content. This indicates that farm methods could be very efficient to change both PL composition, as well as total PL content.

Health Benefits of Phospholipids

The health benefits of milk PLs have gained more attentions recently. Researchers studied the use of PLs to reduce the risk of cardiovascular disease, inflammation, and cancer since the early 1900s (Aoyagi, 2016). Later, PLs were found to be involved in cell functions such as growth, memory processing, stress responses, central nervous system myelination, and cholesterol absorption (Astaire et al., 2003).

According to Schubert and other coworkers (2011), milk PLs have improved and increased the abilities of humans to handle chronic stress, cortisol availability, and weakening

stress-induced memory impairments. A milk beverage with 1% of PLs could efficiently help people who were continually suffering physical and mental health caused by chronic stress. An oral supplementation with 400 mg of phosphatidic acid and 400 mg of phosphatidylserine can normalize the hyper-responsivity of the hypothalamus-pituitary-adrenal axis to an acute stressor (Hellhammer et al., 2014). Compared to placebo-beverages-consumed individuals, PLs-enriched drinks-consumed individuals reacted faster in the working memory task and slow response to psychological stress, even though there was no difference for endocrine stress response between the two treatments (Hellhammer et al., 2010). Polar lipids also have been shown to improve brain functions such as the memory for Alzheimer's patients (Rombaut and Dewettink, 2006).

Dietary PLs from milk fat had been shown to lower Hepatic lipid levels (Chung et al., 2013; Kamili et al., 2010), since milk PLs contains more saturated fatty acids, and the fatty acid chains that are longer than the PLs in eggs and other vegetables. Thus, they are more efficient to suppress absorption of intestinal cholesterol, and reduce plasma and liver lipid levels in animals. A previous study (Noh and Koo, 2004) showed similar results which fed rats with milk SM had a higher efficiency of cholesterol inhibitors and fat absorption than the rats with egg SM. The reason that milk SM is a better cholesterol inhibitor is because the fat acyl groups are highly saturated in SM, which may interfere with micellar formation, hydrolysis of triacylglycerol in the intestinal lumen, and desorption of lipids to the cells on intestine to uptake (Noh and Koo, 2004).

In recent years, PLs have also been found to improve the general condition and barrier functions of skin (Higurashi et al., 2015; Morifuji et al., 2015; Oba et al., 2015). In one of the studies, the hairless mice were given PLs supplemented diet and control diet. The results showed that PLs diet helped adjust epidermal structures, which can inhibit skin inflammation, and

prevent UV-B irradiation to disrupt the skin barrier function. The study also found that PLs enriched diet helped increase the skin hydration and elasticity (Morifuji et al., 2015).

A pilot study reported by Tanaka and coworkers (2013) was about the effect of SM on motor mental and behavioral development of low-birth-weight infants. In the experiment, there were 24 infants randomly assigned to two groups, one group fed with SM-fortified milk, and another fed with control milk. This experiment lasted eight weeks. In the end, researchers found SM-fortified milk improved the neurobehavioural development of those premature infants. However, a long-term study is still required to track the effect of SM on infants. In normal meals, there are 2 to 8 grams of PC and 0.3 to 0.4 grams of SM are ingested per day. Most of the PLs come from milk, eggs, and meat (Küllenberg et al., 2012; Ramprasath et al., 2013). But the amount of PLs for daily consumption has not been clarified.

Those aforementioned results showed more health benefits of PLs that might be of therapeutic value in humans in the future. PLs from milk are better than in plants, it clearly presented that SM in milk fat has better benefits. That's why purifying PLs from milk or dairy products is important. The next sections will introduce two dairy products that are rich in PLs.

Whey Protein Phospholipid Concentrate

As a co-product of cheese-making, whey protein phospholipid concentrate (WPPC), also called pro-cream, is a continued disposal concern for the dairy industry. WPPC is a co-product of whey protein isolate, similar to whey cream, generated during microfiltration of whey. It mainly contains the minimum of whey protein (50 g/100 g dry basis), minimum of lipid (12 g/100 g dry basis), maximum of ash (8 g/100 g dry basis) and the maximum of moisture (6 g/100 g dry basis) (American Dairy Products Institute, 2016). In WPPC, there is 20% of fat (average) and mostly in

the form of PLs (Bund and Hartel, 2013). Levin et al. (2016) studied the composition and functionality of WPPC, and analyzed four WPPC samples from different companies and lots. The data showed the suppliers and lots are important factors that caused high variable WPPC composition. The functionality of WPPC is also affected by suppliers and pH value. Due to the difference in processing, fat or protein content which corresponded. Overall, WPPC has high water holding capacity, heat stability, gelling properties and low foamability. WPPC, as a dairy solid, is added to the final cheese product to make the cheese more firm, improve water binding capacity, melt appearance, and increase the blistering of cooked cheese (Merrill and Singh, 2012).

In a WPPC study, Bund and Hartel (2013) used control ice cream made of nonfat died milk to compare with ice cream made of delactosed whey permeate (DLP) and WPPC blends. The ratios (w/w) of DLP and WPPC used in this study were 30:70, 50:50, and 70:30. There was no significant difference in off-flavor and flavor acceptability between control ice cream and 30:70 blends and 50:50 blends ice cream. Control ice cream had a slightly higher score in both tests. However, when the ratio of delactosed whey permeate increased to 70, the melting rate and low-fat destabilization increased; off-flavor was more noticeable (Bund and Hartel, 2013).

More potential applications of WPPC blends in food were tested by Levin et al. (2016). WPPC was added into ice cream as emulsifier to replace synthetic emulsifiers. A similar result showed that WPPC ice cream increased the dripping (melting) rate. In caramel, DLP and WPPC were added instead of sweetened condensed skim milk and lecithin. They found the stickiness and hardness decreased, while cold flow highly increased. In cake, eggs were replaced by DLP and WPPC. The cake made with DLP and WPPC was compared to control cake (with eggs), there was no change in yield, color, or texture between these two cake samples. Based on the

research, WPPC is a low-cost and good functional ingredient in ice cream and cake, except in caramel candy.

Buttermilk

Buttermilk is in the aqueous phase obtained as a co-product during manufacture of butter, which contains proteins, lipids, lactose, and minerals. Buttermilk is usually dehydrated by evaporating and spray-drying to produce a buttermilk powder (BMP) in order to improve the microbial quality and storage (Shul’kina et al., 1973). Standard dry buttermilk should have maximum of 4% moisture content, minimum of 30% protein and minimum of 4.5% fat content (American Dairy Products Institute, 2014). Compare to PLs in whole milk fat (0.69 g PLs/100 g of fat), the fat in buttermilk contains 4.49 g PLs/100 g of fat, which is 6.5 times more PLs in buttermilk than in milk (Avalli and Contarini, 2005; Fagan and Wijesundera, 2004). Table 2.3 shows ranges of composition in buttermilk powder.

Table 2.3 The range of composition on a buttermilk DM basis (%) (Sodini et al., 2006)

| Sample | Total N | Fats | Phospholipids | Ash | Lactose |
|--------|-----------|----------|---------------|---------|-----------|
| | 14.1-33.1 | 5.7-22.3 | 1.15-1.87 | 6.2-7.6 | 43.7-63.4 |

Table 2.4 Functional properties of buttermilk powder (Sharma et al., 2012; Wong and Kitts, 2003)

| Functional Property | | | | |
|----------------------------|--------------------------------|-----------------------------|---------------------------------------|-----------|
| WHC (g water/g protein) | FC (ml of foam/ml of solution) | FAC (g of oil/g of protein) | EMC (% oil in water) w/ 0.45% protein | Stability |
| 0.75 | 0.5 | 1.2 | 50 | 50 °C |

WHC= Water-holding capacity; FC= Foaming capacity; FAC= Fat-absorption capacity; EMC= Emulsification capacity

During heat treatments in BMP processing, more free sulfhydryl groups were produced, which is a critical factor that influences the properties of BMP. It causes decreasing protein solubility and protein denaturing (Wong and Kitts, 2003). Protein solubility, protein denaturation, surface hydrophobicity, surface charge, and size and flexibility of protein are the factors that affect foaming capacity and stability (Kinsella, 1976).

The most common types of buttermilk include sweet buttermilk obtained from churning sweet cream, sour buttermilk obtained from churning cultured cream, and whey buttermilk from churning whey cream (Sodini et al., 2006). Due to the emulsifying properties of PLs, high content of PLs in BMP. It is used as a functional ingredient (Wong and Kitts, 2003). In the United States, 39% of buttermilk is used by the baking industry to improve the flavor and texture, 33% prepared cake mixes, and 23% is used for dairy industry such as the manufacture of cheese, ice cream, and yogurt (Chandan, 1997; Dairy Research - Madison, 2003; Joshi et al., 1994). Buttermilk is also added to recombined milk due to its increased heat stability by interactions between PLs and protein (Singh and Tokley, 1990). In low-fat cheddar, buttermilk is added to improve texture since PLs have high water holding capacity (Turcot et al., 2001).

Many studies reported the methods to enhance the functional properties of buttermilk and WPPC. Physicochemical treatments including microfiltration, ultrafiltration, and supercritical fluid extraction have been applied to increase the concentration of PLs. The next section focuses on describing the supercritical fluid extraction with its extraction methods.

Overview of Supercritical Fluid Extraction

In the past, the application of organic solvents like hexane has been used for the extraction of fats and oils. With increased regulations and safety concerns in the food industry,

companies are looking for alternatives to hexane (Temelli, 2009). One alternative for conventional extraction methods is supercritical fluid extraction (SFE), a novel extraction technique, which can extract natural products. SFE extraction method is environmentally friendly, requires little or no use of organic solvents with improved efficiency, recovers more of the product, and leads to a product with better quality (Nisha et al., 2012). The main advantages of SFE include the alternative continuous working mode, faster mass transfer, high selectivity, and simple control of process parameters (Pasquali and Bettini, 2008). The efficiency and selectivity of SFE depends on several factors from two aspects, process and product. The factors from process include temperature, pressure, extraction time, flow rate, and solvent-to-feed ratio; product factors include particle size and moisture content of the sample (Temelli, 2009). Hence the extractability of supercritical fluid can be modified as described above by proper controlling of SFE parameters as described above (Khosravi-Darani, 2010).

SFE has been applied to extract functional ingredients and bioactive compounds from many natural sources (Herrero et al., 2006; Khaw et al., 2017). Legumes, spices, aromatic plants, crop, and even fruit beverages have been extracted to obtain natural antioxidant compounds by SFE (Señorans et al., 2001). Nature pigments such as b-carotene, lycopene from tomatoes, and carotenoids from carrots, tocopherols from the olive pomace, caffeine from coffee beans, and phenolic compounds in grape seeds and skins were all isolated by SFE (Murga et al., 2000; Ibanez et al., 2000; Cadoni et al., 1999; Rozzi et al., 2002; Smith and Fish, 2004).

SFE is a simple process, it works by pumping the liquid solvent into a pressurized vessel where it enters the supercritical state. Then, the solvent can interact with the sample, and quickly diffuses into the product to extract the desired compound. In order to release the desired compound, the extraction pressure will be reduced. The compound can be further processed, and

the solvent can be recycled or released into the environment (Temelli, 2009). Figure 4.1 demonstrates a schematic diagram of a supercritical fluid batch extraction. Pressurized supercritical CO₂ is cooled and allowed to pass through the extraction vessel from the bottom of the extraction vessel, the extract then enters a micro metering valve and collected into a collection vessel.

Gases for Supercritical Fluid Extraction

Solvents used for extraction usually include ethanol, propane, ammonia, methanol, isopropanol, and carbon dioxide (CO₂) (Dorf, 2004). Even though ethanol and propane have the highest yield, they are typically not used since they are highly flammable (Temelli, 2009). Ammonia has the disadvantage of dissolving pump seals. Methanol and isopropanol have high critical temperatures, which is hard to maintain (Jinap et al., 2013). In some study cases, nitrous oxide (N₂O) was used as supercritical extraction fluid. Although N₂O has low critical parameters (P_c=7.4 Mpa, T_c=37 °C) which is similar to CO₂ (P_c=4.8 Mpa, T_c=32.4 °C), the gas can cause violent explosions and destruction of extraction vessel especially the sample with high organic content (Raynie, 1993). Thus, it is strongly limiting (Raynie et al., 1990; Pourmortazavi and Hajimirsadeghi, 2007; Capuzzo et al., 2013). CO₂ has the advantage of being inert, nonflammable, less harmful, recyclable, cheap, and easy to recover or remove. Supercritical CO₂ has a surface tension of 0, which allows it to enter quickly and wet the sample (Zhong and Jin, 2008).

Co-solvents for Supercritical Fluid Extraction

Supercritical CO₂ has been documented as a good method to extract neutral compounds since it is relatively non-polar. However, the low polarity index of CO₂ results in poor extraction efficiency for polar analytes. In such case, co-solvents can be used with CO₂ to change the

polarity, improve analyte incompatibility and absorption, and extract the desired compound (Hawthorne, 1990). With co-solvents, an evaporation step is still needed to separate the co-solvent and the desired compound (Temelli, 2009). Co-solvents that can be used include water, methanol, acetone, isopropanol, and ethanol. Acetone and isopropanol are not used as much since they have a rapid desorption. Methanol has lower boiling point and is more polar than ethanol, but due to the risk of toxicity in food application and environment, the use of methanol is decreasing. Water is popular alternative co-solvent in some cases for extracting more polar compounds from aromatic plants (Leal et al., 2008). However, ethanol is used the most. It is a GRAS substance and has been used extensively with soybean, canola, and egg yolk (Montanari et al., 1999; Dunford and Temelli, 1995; Aro et al., 2009).

There are two ways of using co-solvent: the first way is applying the modifier pump in SFE system; the second way is to mix the co-solvent with sample before extraction (Devittori et al., 2000; Temelli, 2009). In most of studies, the first method has been used the most.

Effects of Temperature and Pressure on Extraction

Supercritical CO₂ has low critical temperature and pressure parameters (31.0 °C, 7.38 MPa), and can be easily separated from the extracts. For most of the food applications, SFE pressure between 41.4-68.9 MPa and temperature above 30.6 °C are used. Low pressure is often used for producing high-quality botanicals (Sapkale et al., 2010). Costa et al. (2016) studied the quality of essential oil extracted from two tucumã varieties using supercritical CO₂. In the SFE, pressure was kept at 30 MPa for 180-min extraction time, and the results showed that Amazon Tucumã Oil and Pará Tucumã Oil had higher oil yield when temperature was at 40 °C and 60 °C, respectively. Furthermore, SFE had been applied on many kinds of mixtures to extract lipids and

other lipid-soluble nutrients like vitamin A, D, E and K (Turner et al., 2001; Saldana et al., 2002).

Many studies have been reported to optimize the pressure and temperature in SFE to extract or reduce neutral lipids from different food products. One of the studies focused on determining how temperature and pressure affected the yield of nonpolar lipids in BMP (Spence et al, 2009). In theory, extraction efficiency would linear increase when temperature, pressure or both parameters increased (Spence et al, 2009). The study found that pressure enhanced the extraction efficiency when increased from 15 MPa to 35MPa and temperature was kept constant. However, the yield of nonpolar lipids decreased when temperature increased from 40 to 60 °C and the pressure was constant. In addition, temperature at 40 °C made the results varied and difficult to reproduce. The study showed the optimum combination of pressure and temperature to be 35 MPa and 50 °C. With these settings, protein solubility and browning were not affected (Spence et al, 2009).

Chitra and coworkers (2015) used supercritical CO₂ (20.7 MPa/68 °C) to reduce the cholesterol content in whole milk powder and were successful in removing more than half of the cholesterol from milk powder. Similarly, supercritical CO₂ extraction (31.0-37.9 MPa and 45-55 °C) can approximately extract over 60% of cholesterol and other neutral lipids from dried egg yolks. Supercritical CO₂ conditions of 50–70 °C over a pressure range of 10.9–40.1 MPa were optimized to fractionate buffalo butter oil, results showed that lipids were highly concentrated (88%) at 70 °C/40.1 MPa (Fatouh et al., 2007). High extraction temperature can improve the efficiency of SFE depending on the food matrix. However, high temperature could lead to undesirable lipid hydrolysis or oxidation (King et al., 1993).

Effects of Ethanol Concentration on Extraction

Except reducing the particle size and increasing the temperature or pressure to maximize the efficiency of SFE, adding a co-solvent such as methanol and ethanol for samples containing more polar lipids is another choice. Hydrogen bonding capacity of ethanol with cholesterol is higher compared to other solvent. Shen et al. (2008) compared the recovery of cholesterol extracted from turtle fish egg powder by Soxhlet solvent extraction and SFE with ethanol. Using Soxhlet extraction method, 4.61 mg/g (cholesterol/powder) was recovered. On the other hand, only 70.1% of cholesterol was recovered with the SFE method at 30 MPa/ 65 °C with 1 g ethanol/g of powder. When pressure increased to 35 MPa and ethanol concentration increased to 1.3 g ethanol/g of powder, the recovery of cholesterol statistically improved to 91.5%. Randolph et al. (1988) also reported in his study that addition of 1.5% ethanol as a co-solvent, increased the solubility of cholesterol by over 50% than extract with only neat CO₂.

Co-solvents such as ethanol and methanol is usually used to increase the solubility of polar compounds in foods. Ting and other researchers (1993) studied the relationship between critical points of supercritical CO₂ and co-solvent. They found that the increase in co-solvent concentration increased the critical temperature of supercritical CO₂. Their initial studies indicated that a small amount of ethanol (5%) was not enough to extract the polar lipids, but when ethanol concentration was increased to 10%, total recovery of PLs can be improved considerably. Otherwise, the relative amount of PC, PE, and SM in the resulting extract can be varied using amount of co-solvent greater than 10% (Montanari et al., 1999).

Methods for Enriching Phospholipids in Dairy Powders

To increase the concentration of PLs, filtration techniques such as microfiltration and ultrafiltration coupled with SFE have been used to concentrate this highly valuable lipid into a novel ingredient (Spence et al., 2009). Spence et al. (2009) used buttermilk as a feed material and processed using microfiltration followed by spray-drying. The spray-dried buttermilk powder was treated with SFE to remove neutral lipids. Total fat (neutral lipids) in powder was reduced by 38% to 55%. The concentration of PLs was increased by fivefold, while the ratio of PLs to protein was three times higher in buttermilk powder. It was noted that the concentration of PLs would increase if the extraction process were repeated (Spence et al., 2009).

In another study with buttermilk (Astaire et al., 2003), polar lipids were concentrated using microfiltration and SFE, with a focus on filtration. The researchers studied about whether fresh buttermilk, reconstituted powder, or temperature would affect the concentration of PLs. The SFE with optimal condition (375 bar, 77 °C; 20 g/min CO₂ flow rate; and 75 min extraction time) was used to extract the nonpolar lipids from the filtered and spray dried samples. The temperature did not significantly impact the yield. With SFE, the total fat was reduced by 38%, and they were nonpolar lipids. In reconstituted buttermilk powder, fat was reduced by 30%. It was noticed that particle sizes decreased after the filtration and extraction processes (Astaire et al., 2003).

Costa et al. (2010) reported using ultrafiltration and SFE to obtain a whey buttermilk powder enriched in milk fat globule membrane PLs. The whey buttermilk originally contained 25% of protein, 16% of lipids include 2% of PLs. It was concentrated by ultrafiltration and diafiltration. The retentate was then spray dried and submitted to SFE (350 bar, 50 °C). Most of

lactose and ash were removed by SFE and ultrafiltration, and there were 73% of protein, 21% of lipid including 61% of PLs in dried whey buttermilk.

Some studies used ultrafiltration to concentrate PLs in buttermilk without SFE. Catchpole et al. (2008) ultrafiltrated and spray-dried whey buttermilk. The final powder recovered 95 to 99% of PLs, and 14% of PLs in the powder. In a similar study found by Barry et al. (2017), after ultrafiltration and spray drying, the buttermilk powder contained total PLs in fat increased from 0.79 to 6.16%; lipid content in dry base increased from 6.84 to 43.43%.

Supercritical Fluid Extraction and Phospholipids

Dairy Products

As mentioned in the previous section, co-solvent combined with supercritical CO₂ can extract PLs from food materials such as egg yolk, soy, canola and pumpkin seed. Catchpole (2008) have been successfully extracted nonpolar and polar lipids from “beta-serum.” Beta-serum is a by-product from dairy stream containing approximately 60% fat. It is distinct from buttermilk since it contains more components from MFGM. Dimethyl ether (DME) and CO₂ were both used as solvents. DME can extract both neutral and polar lipids. Spray-dried beta-serum first extracted a majority of neutral lipids by SFE with neat CO₂ under 300 bar and 40 °C. The PLs enriched residual was then re-extracted by SFE with DME under 40 bar and 60 °C. The polar lipid extracts were analyzed and contained 70% of PLs. The study also stated that the spray-dried sample could be subjected to CO₂ and then DME or DME and then CO₂. In a recent study, PLs have been successfully extracted from ultrafiltrated BMP (Barry et al., 2017). The ultrafiltrated BMP with 11.05±0.02% PLs were extracted by SFE and ethanol as co-solvent. The

optimal conditions they used were 40 °C, 300 bar and 20% of ethanol and increased PLs concentration to 56.24±0.07% on the dry base.

Egg Products

Egg products are also rich in PLs and some SFE studies have been conducted on them. These studies typically focused on PC. In Shah and her coworkers' study (2004), they were focused on retaining PC in inedible eggs and then extracting it. A two-step process was used to achieve the extraction. The first step involved CO₂ to concentrate the PC and the second step used CO₂ and ethanol to extract it (Shah et. al 2004).

To concentrate PC, Shah et al. (2004) compared various pressures (13.8, 27.6, and 41.4MPa) , CO₂ flow rates (20 L/min and low 15 L/min), and temperatures (45, 50, and 60 °C) in order to find the optimum conditions for extracting the neutral lipids from spray dried feed grade egg. Extraction time and pressure were the most studied parameters. In the results, a higher pressure (41.4 MPa), longer exposure time (60 min) and lower temperature (45 °C) lead to higher concentration of PC in dried egg. PC in egg residues were 93% of area of HPLC elution. For extracting PC in the second step, it was noticed that more PLs were extracted as the molar fraction of ethanol increased. The optimal conditions to achieve the highest yield of PC (49 g PC/kg of dried egg) were 41.4 MPa, 5 L/min flow rate, 45 °C and 0.015 L/min ethanol for 60 min (Shah et. al 2004). However, the study did not focus on the purity of the PLs in extracts.

A similar study was carried out on using a pilot scale production process to determine the best method to obtain high-quality PLs (Aro et. al 2009). Two-step SFE was used to extract PLs from egg yolk powder. The operating conditions in Step-1 were 70 °C, 45MPa, and flow rate of 0.45L/min for 6 hours. Conditions in Step-2 were 70 °C, 40 MPa with CO₂ and ethanol mass ratio of 12:88. At the end of study, 85 to 95% of PLs recovery was achieved.

Other Food Products

Besides eggs, soybean lecithin is another product rich in PC and studies have been conducted to see if SFE can extract it. The effects of pressure, temperature, and ethanol fraction were evaluated by Teberikler et al (2001). The PLs yield increased as pressure and ethanol fraction increased, and temperature decreased the yield when it was above 60 °C. Teberikler concluded that the best parameters for the sample being exposed for 150 mins were 60 °C and 20.7 MPa with 10% ethanol. These conditions had the highest extraction of PC at 95%.

The previous studies focused on changing factors with the extraction method, but a study from Jinap and other coworkers (2013) was focused on changing the composition of the food to increase yield. SFE was used on ground cocoa nibs with different moisture contents and pH to increase the lipids yield efficiency. The concentration of ethanol in the CO₂/ethanol mixture was 25%, and supercritical CO₂ was maintained at 35MPa, 60 °C at a rate of 2 ml/min. The moisture contents ranged from 1.95% to 17.64% and the pH was between 5 and 7.9. It was noticed that increasing the moisture content up to 9.79% and pH from 7.5 to 7.9 increased the yield (73.70%). This matched a previous study that mentioned drier samples had higher yield than wet samples. However, more work still needs to be conducted to determine and support how exactly characteristics of products such as moisture content, and pH effect extraction.

Analytical Methods for Phospholipids

High-Performance Liquid Chromatography

The basic theory of HPLC involves passing a sample through the system over a stationary phase. The difference in the relative affinities of molecules in the sample for using mobile phase

and stationary phase lead separation of molecules (Kupiec, 2004). Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different individual parts can be separated from each other as liquid mobile phase filters down through a solid stationary phase in the column. The efficient chromatographic separations can be accomplished by using a variety of adsorbent material as stationary phases, including liquids (liquid chromatography), immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), and paper (paper chromatography) (Kupiec, 2004).

HPLC is a common method used for quantitative and qualitative detectors of PLs. An evaporative light scattering detector (ELSD) coupled with HPLC is the standard technique to determine the PLs in the food materials (Rombaut et al., 2007). Compared to the refractive index (RI) detector and ultraviolet (UV) detector, ELSD has a broader range of gradient elution and choices of solvent, the signal is independent of chain length of acyl chain and degree of saturation. Several studies have been reported using ELSD methods with silicon columns to detect PLs in milk lipids and egg lipids (Shah et. al 2004; Avalli and Contarini, 2005; Bodson et al., 2006; Rombaut et al., 2007; Rodriguez-Alcala and Fontecha, 2010; Le et al., 2011). The detector temperature was at 40 to 50 °C, and the size of silica column was from 150 mm x 3.2 mm to 250 mm x 4.6 mm. The mobile phase used in ELSD was usually composed of hexane, iso-propanol, chloroform, methanol, ammonium hydroxide, folic acid, and/or water.

HPLC coupled with mass spectrometry has provided an alternative to perform lipidomics and determine the composition of PLs from total lipids extracts (Lisa et al., 2011). Mass spectrometry can comprehensively identify and quantify specific species profiles of lipid classes (Tang et al., 2012). Although a large number of species cause overlapping because many ion

peaks are composed of isobaric species from different classes of lipid in the mass spectrum, and a low PL content is hard to be detected (Kim and Hoppel, 2013) (Tang et al., 2012), chromatographic separation before mass spectrometry will increase the detection of low abundance PLs.

Some studies also found UV detector with HPLC and simple mobile phase such as deionized water, methanol, isopropyl alcohol and/or acetonitrile, has made the method economic, efficient, and a simple approach for PLs analysis (Jangle et al., 2013; McHowat et al., 1996). The measurements of PL mass in HPLC fractions use total integrated UV absorption and the absorbance response factors to determine the quantitation of individual molecules. In most of the cases, PLs can be detected by UV absorbance at 203 nm (McHowat et al., 1996; Wang et al., 2003).

Thin Layer Chromatography

Thin layer chromatography (TLC) is a simple type of chromatography method for identifying synthesized mixtures by separating the compounds in the samples. Differences in

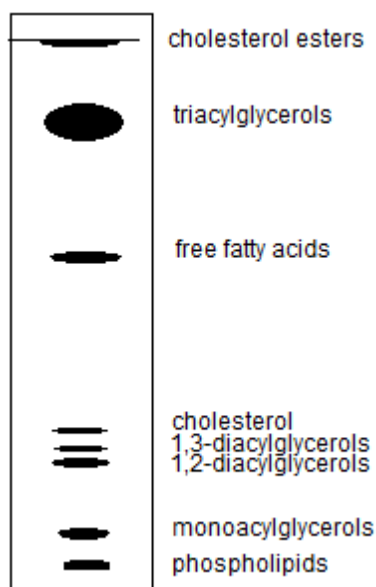


Figure 2.2 Thin layer chromatography separation of simple lipids. (Touchstone, 1995)

analyte affinities include the polarities of TLC plate, development solvent, and compounds in the spot made the balance of intermolecular forces lead solvent to move the solute on the plate.

Figure 2.2 showed non-polar lipids and polar lipids separate on the TLC plate. On one hand, non-polar compounds will be forced to the top of the plate by non-polar solvent, because they do not interact with the polar stationary phase. On the other hand, polar compounds will tend to stick more tightly to the plate (Ilbeigi and Tabrizchi, 2015). This technique has been widely used for determining the identity of compounds, individual components in a sample, and purity of compounds. In food industry, researchers used TLC to identify different neutral lipids and polar lipids. The solvent mixture for detecting PLs compose of chloroform, methanol, and deionised water; hexane, ethyl acetate, acetic acid is the solvent used to detect neutral lipids (Hutchins et al., 2008; Saffon et al., 2015)

Usually, compounds separated on the plate are colorless, visualization methods are needed to observe development. UV light and iodine vapor are the two most common methods. The spots interfere with fluorescence and showing dark color on glowing silica gel plate under the UV light, and mark the spots by pencil to know the locations. For iodine vapors, since most organic compounds will turn to dark color with iodine, spots are easily to be observed after placing the plate into iodine vapors. Visualization reagents such as ferric chloride, potassium permanganate, and ninhydrin. could also make separated spot be visible (Jork et al, 1994). The studies presented later used iodine vaper.

Conclusion

The review carried out on milk PLs showed that these molecules play a key role in both the nutritional and technological field. SFE can be used to extract fats and oils without using

harmful solvents. CO₂ is typically used because it is safe, cheap, and abundant. The SFE-CO₂ process is sensitive to pressure, time, and temperature. Changing these parameters can affect what is extracted and the yield. Increasing pressure and time have been shown to increase yield and components that are extracted. With temperature, yield increases with increasing temperature up to 60 °C, and then yield starts to decrease.

Co-solvents are used to increase selectivity of PLs in SFE. CO₂ typically extracts nonpolar lipids, but in the presence of ethanol, PLs can be extracted. Extraction yield increases as the concentration of ethanol increases. In the dairy industry, iSFE has been predominately used with buttermilk and whey protein powders and has shown to be successful in removing neutral lipids. In the future, SFE may be able to help create new ingredients.

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Chapter 3 - Research Objective

This study focused on developing and evaluating an optimal supercritical fluid extraction treatment for isolating phospholipids(PLs) from buttermilk powder (BMP) and whey protein phospholipid concentrate (WPPC):

1. To determine the optimal supercritical fluid extraction settings including pressure, temperature, and ethanol concentration in order to increase the efficiency of extracting PLs from WPPC, and to quantify and qualify the PLs in the extracts using HPLC-UV.

2. To determine the optimal supercritical fluid extraction settings including pressure, temperature, and ethanol concentration in order to increase the efficiency of extracting PLs from BMP, and to quantify and qualify the PLs in the extracts using HPLC-UV.

Chapter 4 - Selective Extraction of Phospholipids from Whey Protein Phospholipid Concentrate using Supercritical Carbon Dioxide and Ethanol as a Co-solvent

Abstract

The utilization of phospholipids (PLs) as functional ingredients has been increasing recently due to their nutritional and functional benefits in the food industry. Thus, whey protein phospholipid concentrate (WPPC), also called pro-cream, is one of the good sources of PLs. WPPC is a co-product obtained during manufacturing of whey protein isolate. Three batches of WPPC were obtained from one commercial manufacturer and a novel 2-step supercritical fluid extraction (SFE) process was used to separate PLs from WPPC. The objective of the study was to evaluate the use supercritical CO₂ and ethanol as a modifier to extract PLs from WPPC in a 2-step extraction process. In Step-1, 20 grams of WPPC was mixed with glass beads in the ratio 2:1 (w:w) and placed in the extraction vessel. Neat supercritical CO₂ was allowed to pass through the extraction vessel at 414 bar pressure, 60 °C sample temperature, and 5 L/min CO₂ flow rate to remove all the neutral lipids. In Step-2, a 3×3×2 factorial design was used to evaluate the extraction efficiency as pressure (350, 414, and 550 bar), temperature (40 °C and 60 °C) and concentration of ethanol (10%, 15%, and 20%) as independent factors. All the lipid fractions were analyzed by high-performance lipid chromatography (HPLC) and thin layer chromatography (TLC). The amount of PLs extracted in Step-2 was significantly ($P < 0.05$) affected by ethanol concentration. However, pressure and temperature were not found to be

significant ($P > 0.05$). In addition, sphingomyelin, and phosphatidylethanolamine percentage increased in extract with increase in ethanol concentration.

Keywords: supercritical fluid extraction, whey protein phospholipid concentrate, pro-cream

Introduction

Lipids in milk are protected by a membrane called the milk fat globule membrane (MFGM). The MFGM is made up of proteins, phospholipids (PLs), glycoproteins, triglycerides, cholesterol, enzymes, and other minor components (Singh, 2006). In recent years, the interest in functional components such as PLs is increasing as a result of growing awareness of their health benefits. PLs affect several cell functions, such as growth, the molecular transport system, memory processing, stress responses, and central nervous system myelination (Astaire et al., 2003). Dewettinck et al. (2008) reported that polar lipids have become more popular due to their role in improving the health of numerous people through anti-cancer effects. It has been shown that polar lipids have the potential to inhibit the growth of cancer cells in the breast as well as in the colon. Some studies have been reported that the PLs have the ability to lower cholesterol absorption. Polar lipids also have been shown to improve the memory of people who have Alzheimer's Disease (Rombaut and Dewettinck, 2006).

As the interest in PLs increase, especially animal origin PLs, it is important to explore the ways to extract PLs from dairy-based ingredients that are rich in PLs. Consequently, the use of whey protein phospholipid concentrate (WPPC) as a source of PLs is worth exploring. WPPC is a co-product obtained during the manufacture of whey protein isolate. It is rich in whey proteins (63% to 72%), fat (12% to 20%), lactose (2% to 13%), and minerals (3% to 3.5%). Bund and Hartel (2013) studied the applicability of WPPC and delactosed whey permeate (DLP) blends in ice cream formulations and found that the ice cream formulated with WPPC and DLP blends were comparable with control ice cream in terms of mean ice crystal size and showed relatively high melt rate and reduced fat destabilization. In addition, ice creams containing the DLP-WPPC blends were rated less acceptable by panelists than the control ice cream, mostly because of off-

flavors associated with DLP. Therefore, it is important to look for alternative methods to add value to WPPC.

Supercritical fluid extraction (SFE) is a novel extraction technique that can extract naturally occurring components from a variety of complex matrices. Carbon dioxide (CO₂) is the most common solvent in supercritical fluid extraction (SFE) because it is safe, nontoxic, and commercially available. Astaire et al. (2003) used a SFE-CO₂ process to exclusively remove neutral lipids from buttermilk powders. The authors succeeded in removing neutral lipids from microfiltrated buttermilk powder (BMP) and were able to increase the polar lipid content from 31.02 to 83.15 g per 100 g of dry powder. A similar study was also carried out by Spence et al. (2009). However, none of the studies were successful in separating PLs as a pure ingredient from the protein fraction. These studies indicated that it was a challenge to extract polar PLs using SFE due to their limited solubility in SFE-CO₂. The PLs extraction using SFE-CO₂ depends on the degree of association between the PLs and proteins and it is necessary to disrupt the association between the lipid-protein aggregates for a successful extraction (Elst et al., 2003).

Elst et al. (2003) studied the selective extraction of PLs from egg yolk in the presence of ethanol as a modifier and concluded that at least 5 mole% modifier is needed to extract PLs from egg yolk powder. Montanari et al. (1996) proposed a 2-step, sequential scheme using SFE-CO₂ alone, and with ethanol as a modifier to produce oil and PL containing fractions from soybean flakes. They were successful in recovering PL-rich fractions. So far in the dairy industry, only one study has been successful in extracting PLs from ultrafiltrated BMP (Barry et al., 2017). The ultrafiltrated BMP with 11.05±0.02% PLs was extracted by SFE-CO₂ and ethanol as co-solvent. The optimal conditions they used was 40 °C, 300 bar, and 20% ethanol, and PLs concentration increased to 56.24±0.07% in total fat.

Thus, the objectives of this study were to extract PLs from WPPC with supercritical CO₂ and ethanol as co-solvent, and examine the effect of temperature, pressure, and concentration of ethanol on the efficiency of SFE process for WPPC.

Materials and Methods

Experimental Design

Three batches of WPPC were obtained from a commercial manufacturer within the United States. PLs were extracted from WPPC using a unique 2-step process. In the first step, SFE was carried out using only CO₂ to remove neutral lipids, followed by a second extraction using ethanol as a co-solvent along with supercritical CO₂ to extract PLs. In order to optimize the extraction conditions in the second extraction step, pressure, ethanol concentration, and extraction temperature were studied as independent factors.

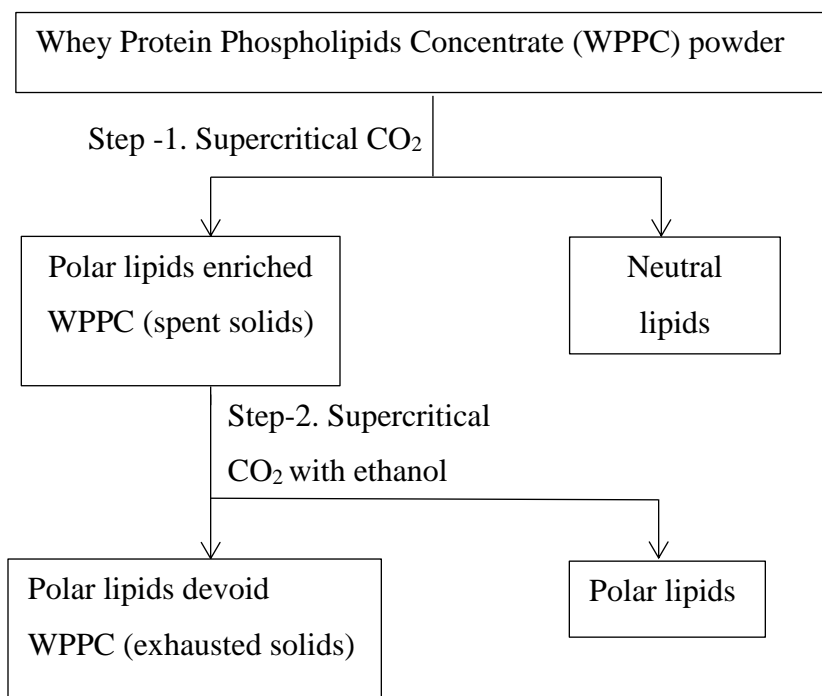


Figure 4.1 Process flow diagram for extraction of phospholipids from whey protein phospholipid concentrate powder

The process flow diagram for extraction of PLs from WPPC is shown in Figure 4.1. Neutral lipids and polar lipids extracted from Step-1 and -2 were analyzed using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Also, functional properties of exhaust solids obtained after Step-2 were examined to compare with control powders.

Materials

Three lots of WPPC powders were obtained from Nutegrity Pure Nutrition (Irvine, CA). All chemical solvents were HPLC grade (Fisher Scientific, Atlanta, GA). Phospholipid standards including phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) were purchased from Sigma-Aldrich (St. Louis, MO). Silica gel thin layer chromatography plates 60 F254 were purchased from EMD Chemicals (Darmstadt, Germany) and the developing tank from Kontes Glass Company (Vineland, NJ).

Solvent Extraction of Polar Lipids

Liquid-liquid extraction of PLs was carried out using the method described by Rombaut et al. (2007) with minor modifications. Extraction process was carried out in duplicate. WPPC (5 g) was diluted with deionized water to make 20 ml slurry and was thoroughly mixed. To the slurry, 80 ml of chloroform:methanol 2:1 (vol:vol) was added and transferred to a separatory funnel. After shaking for 2 min, the mixture was allowed to stand and separate. The clear lower solvent layer was collected. Then 40 ml of 20:1 (vol:vol) chloroform:methanol was added to the upper aqueous phase. Again, the lower phases were collected and this was repeated twice. In the next step, 40 ml of 85:14:1 (vol:vol:vol) chloroform:methanol:water with 1 N HCl was added. The lower phase was collected and washed with a 0.9 % (w/w) of NaCl solution until neutral pH was reached. All of the lower phases were mixed and concentrated by using a rotary vacuum

evaporator (Buchi, Switzerland) at 37 to 39 °C. Finally, 1 mg of extracted lipids were diluted in 1 ml methanol, filtered, transferred into a capped sampler vial and stored at -20 °C until further analysis.

Supercritical CO₂ Extraction

To achieve the objectives of the study, a laboratory-scale SFE system (Applied Separations, Allentown, PA) was fitted with a 32 ml stainless steel extraction vessel (Figure 4.2). In the first extraction step, approximately 20 g of WPPC powder was mixed with glass beads (Fisher Scientific, Atlanta, GA) in the ratio 2:1 and was packed in the extraction vessel as suggested by the manufacturer. Dispensing filler was added to the remaining empty space in the vessel and sealed as per the instructions provided by the manufacturer. Subsequently, the extraction vessel was placed in the temperature-controlled oven set to a target temperature as per the experimental design.

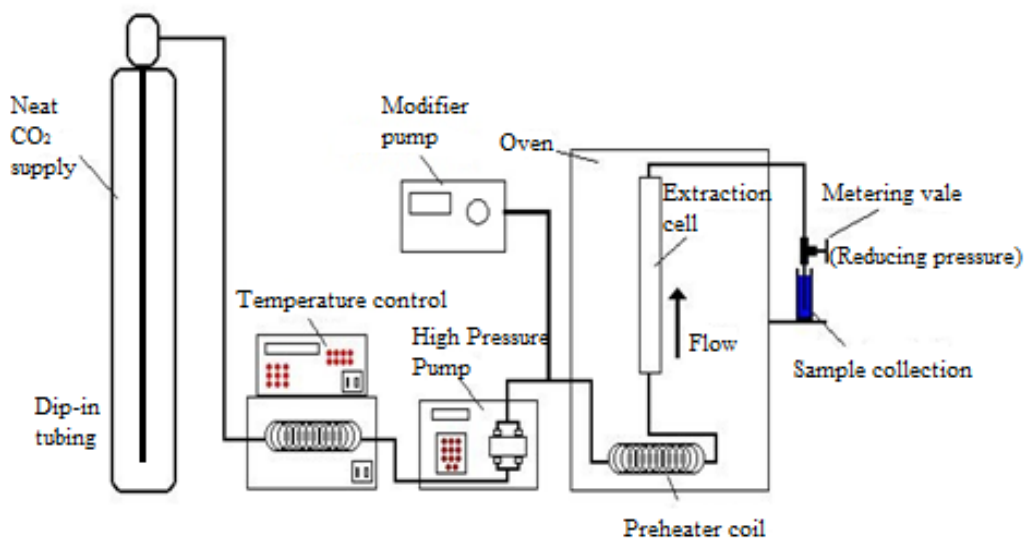


Figure 4.2 Supercritical fluid extraction apparatus.

Step-1 Extraction. The first extraction step using the neat supercritical CO₂ was carried out at an extraction pressure of 414 bar at a constant CO₂ flow rate of 5 L/min for 90 min, including a 30-min soaking time. The soaking time was applied to maximize the solubility of neutral lipids in supercritical CO₂. The extract was collected into a 30 ml collection vial, while CO₂ was released into the environment. The Step-1 extraction process was repeated several times until approximately 180 g of spent solids (WPPC devoid of neutral lipids) were produced. Subsequently, the spent solids were pooled together and used as feed material for the Step-2 extraction process.

Step-2 Extraction. A 3×3×2 factorial design was used to optimize the extraction conditions to maximize the efficiency of polar lipid extraction in Step-2. Pressure, ethanol concentration, and extraction temperature were used as the independent variables. Three levels of pressure (350, 414, and 550 bar), two levels of temperature (40 and 60 °C), and three levels of ethanol concentration (10, 15, and 20%) were incorporated into the experimental design. Each extraction was done in triplicates. Ethanol from polar lipid extract was removed under a gentle stream of air in a hood. The polar lipids extracted from each experiment were weighed, and kept in the freezer at -20 °C for further analysis.

Chemical Analysis Methods

High-performance liquid chromatography (HPLC). The polar lipid extracts (10 mg) from Step-2 were mixed with 1 ml methanol and filtered using a 0.45 µm syringe filter (Alltech, Woodridge, IL). About 20 µL of each extract solution was injected into an Agilent 1050 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm×4.6 mm I.D., 5 µm particle size Phenomenex (Le Pecq Cedex, France) HILIC LC column. The UV detector coupled was set at 35 °C. The mobile phase consisted of deionized water (A) and acetonitrile (B) as binary gradient.

Water was used to mediate the change from 10 to 50% acetonitrile for 3 min. The flow rate of mobile phase was kept at 1 ml/min for a total run time of 25 min. The detection was performed at 203 nm for PC, PE, and SM as described by Wang (2003). Chromatography was recorded and analyzed by ChemStationL or LC 3D (Agilent Technologies, Santa Clara, CA). To identify and quantify the PLs, SM, PC, and PE standards were injected to make calibration curves for comparing retention time and peaks in the extracts ($r^2 > 0.99$).

Thin Layer Chromatography (TLC). TLC method was performed according to the procedure given by Spence et al. (2009). Lipid samples were prepared in chloroform:methanol (1:2, vol:vol) to 10 mg/ml; PLs standards were diluted to 1 mg/ml. Polar lipids were separated using solvent consisted of chloroform:methanol:deionized water (65:25:4 v:v:v). Silica gel plates were loaded with 10 μ g of samples and 5 μ g of standards. Lipids were visualized by exposure to iodine vapor overnight and identified by comparison to standards (Astaire et al., 2003).

SDS-Page. Protein profiling was conducted as described by Liu et al. (2012), with modifications. Approximately 3.5% protein solution was diluted with double deionized water at 1:8 dilution, then mixed with 20 μ L sample solution and 20 μ L sample buffer, which consisted of BioRad Laemmli buffer containing 10% beta-mercaptoethanol. Samples were kept for 8 min at 90 $^{\circ}$ C, and 25 μ l of samples were loaded in 4% to 15% mini-protean TCX gels (Bio-Rad, Hercules, CA) and ran at 120 V for 90 min in 10% tris/glycine/SDS buffer solution (Bio-Rad, Hercules, CA). Seven μ L of Precision Plus Protein Standards, dual color standard (Bio-Rad, Hercules, CA) was the molecular-weight standard. Gels were stained with coomassie brilliant blue R-250 dye (Thermo Fisher Scientific, MA) for 1 hour and washed with double deionized water for 8 hours.

Compositional Analysis

Total nitrogen was analyzed by the LECO system according to method 997.09 (Trumac N, LECO Corp., St. Joseph, MI) (AOAC International, 2002). A conversion factor of 6.38 was used to determine the nitrogen-based protein content. Total solid was determined using the forced air method as described by method 990.20 (AOAC International, 2002). Ash for WPPC was determined using the incineration method as described by method 954.46 (AOAC International, 2002). The quantity of lipids in WPPC was analyzed by CEM Smart Trac Fat System (CEM Corp., Matthews, NC).

Functional Analysis

Water holding capacity and emulsification capacity of exhaust powders (powder obtained after Step-2 extraction) were carried out and compared with the original WPPC powders.

Water Holding Capacity (WHC). The water-holding capacity (WHC) was determined according to the method described by Veith and Reynolds (2004) with some modifications. Briefly, 15% (wt/vol) sample solution was prepared at room temperature and NaOH/HCl was added to the solution until the pH was neutral. The solution was stirred for 10 min, then transferred into 30 ml capped plastic tubes and heated in the water bath at 70 °C for 70 min. The gel was cooled at 20 °C, then cut into 10 mm length slices right before testing. Gel slices were weighed and placed between a folded tissue paper with a cheese screen inside. A plastic plate was put on the top of the tissue and slices were compressed under the plate with 1 kg for 10 min. WHC was present as the percentage of the initial water remaining in the gel after compression.

Emulsification Capacity (EMC). Approximately 5% protein sample dispersions were prepared using deionized water at 40 °C. An overhead stirrer (Caframo, Ontario, Canada) was used to mix the dispersion at 800 rpm for 5 min then at 500 rpm for a total 30 min. Then, the

dispersions were stored in the refrigerator for overnight hydration. The next day, 25 ml of dispersion was placed in a 250-ml beaker and warmed to room temperature. Canola oil was added to dispersion slowly with continuous blending with a homogenizer (PalySalence, Model X120, Niles, IL). A portable waterproof conductivity meter was used to measure the emulsification capacity. Addition of oil was stopped when the meter reading was zero and the amount of oil used was recorded. EMC was expressed as the percentage of canola oil emulsified per 5% protein dispersion until phase inversion.

Statistical Analysis

Data collected were analyzed and compared using SAS 9.4 for Windows. The mean value comparisons were performed by Tukey's test. The P-value was at 0.05.

Results and Discussion

Table 4.1 shows the chemical composition of three lots of WPPC used in the present study. The moisture, fat, protein, and ash were significantly different between the WPPC#1 and WPPC#2 and WPPC#3 ($P < 0.05$). The average composition of three WPPCs was 3.54% moisture, 20.46% fat, 62.15% protein, and 2.39% ash. Levin et al. (2016) analyzed WPPC samples from four different manufactures and found a similar variability, especially in terms of protein and fat concentrations. The authors attributed the variation to differences in fat removal and microfiltration processing conditions. Since, WPPC is a co-product obtained during manufacture of whey protein isolate, the proximate composition of WPPC can vary significantly depending on the process. In the present study, the total fat content from the three batches in this study varied between 18.46% and 24.23%.

Table 4.1 Approximal composition standard deviation of WPPC samples used in the study

| Control | Moisture (%) | Fat (%) | Protein content (%) | Ash (%) |
|---------|-------------------------|--------------------------|--------------------------|-------------------------|
| WPPC#1 | 2.985±0.08 ^a | 24.225±0.02 ^a | 56.638±0.05 ^a | 2.569±0.02 ^a |
| WPPC#2 | 3.605±0.04 ^b | 18.705±0.09 ^b | 64.818±0.12 ^b | 2.315±0.01 ^b |
| WPPC#3 | 4.025±0.05 ^c | 18.4550±01 ^b | 64.994±0.06 ^b | 2.271±0.03 ^b |

^{a,b,c}Means within a column with different superscripts differ (P < 0.05), n=2.

The liquid solvent extraction of PLs obtained from the three WPPCs lots are shown in Table 4.2. The total PLs content varied between 1.78 g to 2.20 g per 100 g of WPPC powder and 11.63 g to 14.57 g per 100 g fat basis. Levin et al. (2016) analyzed free PL content from four different manufacturers and reported between 0.57% and 0.74% of WPPC. The authors also reported that the PL concentration was lower than expected. However, the PL concentrations obtained in the present study were close to what companies advertise for their WPPC.

Table 4.2 The values and standard deviation of dry matter, fat, and polar lipid content of WPPC samples

| Sample | Polar lipid species (g/100 g Fat) | | | Polar lipid species (% of total polar lipids) | | | mg/g WPPC | g/100 g fat |
|--------|-----------------------------------|------------------------|------------------------|---|-------|-------|--------------------|--------------------|
| | SM | PC | PE | SM | PC | PE | | |
| WPPC#1 | 3.58±0.00 ^a | 3.52±0.36 ^a | 4.53±0.05 ^a | 45.64 | 28.72 | 46.74 | 17.80 ^a | 11.63 ^a |
| WPPC#2 | 5.06±0.02 ^b | 3.52±0.00 ^a | 5.98±0.08 ^b | 42.34 | 22.28 | 47.23 | 21.97 ^b | 14.57 ^b |
| WPPC#3 | 4.77±0.83 ^c | 3.75±0.27 ^b | 5.86±0.12 ^c | 41.54 | 24.43 | 46.96 | 22.00 ^c | 14.38 ^c |

^{a,b,c}Means within a column with different superscripts differ (P < 0.05), n=2. PE=phosphatidylethanolamine, PC=phosphatidylcholine, SM=sphingomyelin.

Generally, some companies advertise as approximately 20% of the total lipid fraction is made up of PLs. In addition, the average relative percentage of different PLs include 46.98% of PE, 43.17% of SM, and 25.14% of PC, which are similar to the proportions found in milk (Ferreiro et al., 2015). In comparison to published studies, there are 0.31 g to 0.41 g PLs/100 g fat in human milk, 0.48 g PLs/100 g fat in bovine milk, 1.0 g PLs/100 g fat in buttermilk powder,

and 0.35 g to 0.38 g PLS/100 g fat in bovine cream (Guerra et al., 2015, Le et al., 2011, Lopez and Ménard, 2011, Zou et al., 2015).

Evaluating the 2-step SFE Method for Extraction PLS

Step-1 Extraction. The first step extraction process was carried out using only the supercritical CO₂. The Step-1 is well studied and optimized by several researchers. Costa et al. (2010) were successfully able to remove 72% neutral lipids from buttermilk powder using supercritical CO₂ at 350 bar extraction pressure, and extraction and collection temperature of 50 °C. In a different study, Astaire et al. (2003) were able to reduce from approximate 5% to 1.5% of lipids from buttermilk powders and removed lipids were mostly neutral lipids. In the present study and SFE, neutral lipids were removed using the neat supercritical CO₂ at 414 bar pressure and 60 °C.

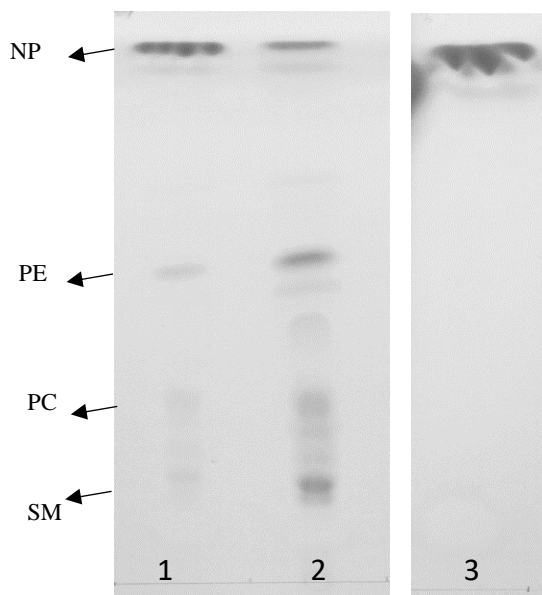


Figure 4.3 Thin layer chromatography of polar lipids profile. Lane 1= WPPC, Lane 2= spent solids obtained after Step-1 extraction, Lane 3= Lipids obtained from Step-1 extraction. NP= neutral lipids; PE=phosphatidylethanolamine, PC=phosphatidylcholine, SM=sphingomyelin

Figure 4.3 shows a picture of the TLC plate of a typical WPPC lot used in the study (lane 1). Lanes 2 and 3 show the remaining lipid fraction extraction from the spent solids and the lipid fraction obtained after Step-1 extraction, respectively. As expected, there were no PLs found and the lipid fraction obtained from the Step-1 process was similar to the results reported by Astaire et al. (2003). Supercritical CO₂ effectively removed the unwanted neutral lipids from WPPC and left the desirable PLs in the spent solids. As can be seen from Figure 4.3, the amount of neutral lipids remaining in the spent solids was lower than the original WPPC powder. Even though the same amount of lipids were loaded in each lane, there was clearly a higher intensity of PLs bands in lane 2 when compared to lane 1, suggesting higher relative concentrations of PLs in the spent solids. It is also important to notice that there were still some neutral lipids left in the spent solids and they were carried onto the Step-2 of the extraction process.

Step-2 Extraction. In the second step of SFE, ethanol as a co-solvent was used along with CO₂ to isolate PLs as a separate fraction. The effect of Step-2 SFE process parameters such as pressure, ethanol concentration, and extraction temperature on the removal of PLs were evaluated using a factorial design. The results obtained from the statistical analysis demonstrated that extraction pressure and temperature were not significantly affecting the extraction efficiency of PLs ($P > 0.05$). However, ethanol concentration was significantly ($P < 0.05$) influencing the recovery of PLs from WPPC. It was observed that the amount of recovered PLs increased when the ethanol concentration increased from 10% to 15%. However, there was no significant ($P > 0.05$) increase in amount of PLs recovered when the ethanol concentration increased from 15% to 20%. Montarini et al. (1996) also observed an increase in PLs extracted as the ethanol concentration increased from 10% to 15% and no further increase in PLs recovery when the ethanol concentration increased to 20%.

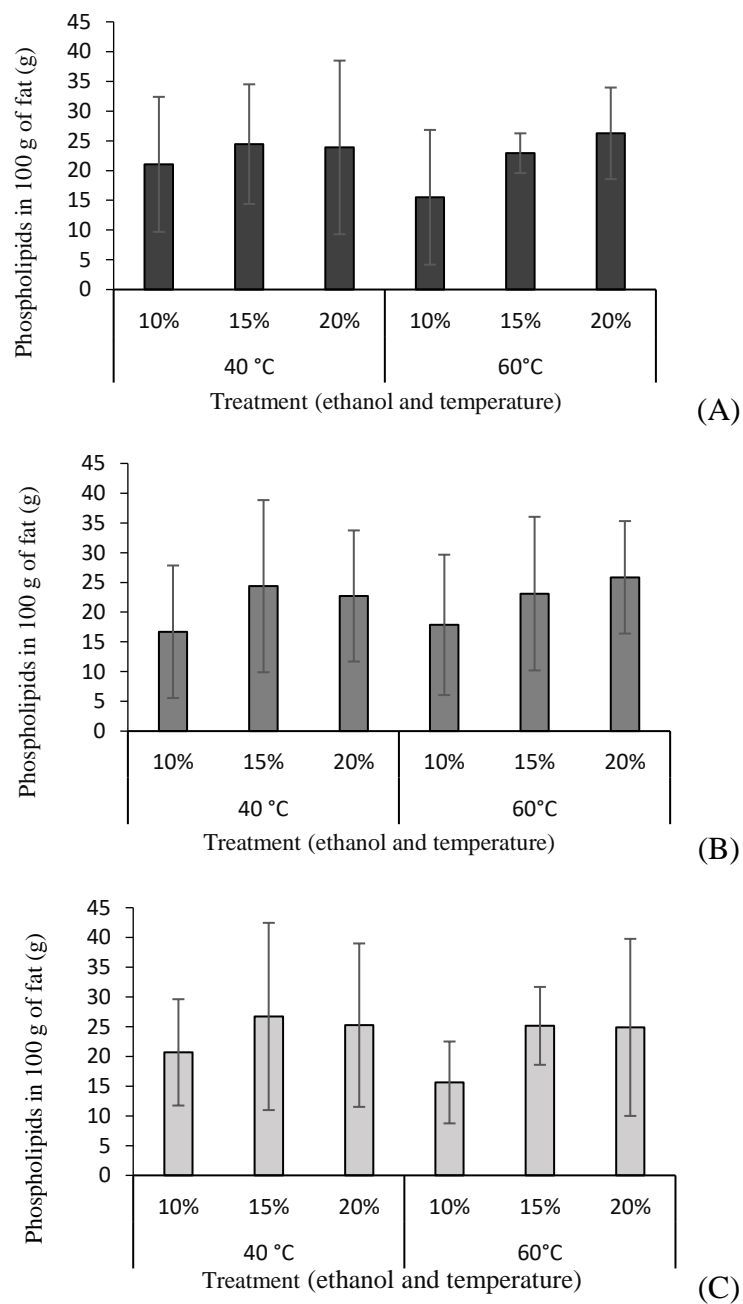


Figure 4.4 Effect of temperature (40 °C and 60 °C), and concentration of ethanol (10%, 15%, and 20%) on the amount of total polar lipids presented in crude lipids from various operating SFE pressure: (A) 350 bar, (B) 414 bar and (C) 550 bar. The average of triplicate samples are used as results, bars indicate standard deviation, n=3

Figure 4.4 (A, B, and C) show the amount of PLs recovered during Step-2 process at 350, 414, and 550 bar, respectively. It was clear to observe that the levels of PLs were similar

under different pressure and temperature. There was 15.5 to 21.0 g of PLs per 100g of fat isolated when 10% ethanol was added as co-solvent. On the other hand, 25.1 and 26.7 g of PLs per 100 g of fat were isolated when ethanol concentration increased from 10% to 15% and 10% to 20%, respectively. From Figure 4.4, it can be seen that the optimal process parameters of 350 bar pressure, 60 °C sample temperature, and 20% ethanol concentration produced the highest PLs recovery in the lipid extract from the Step-2. The extract contained 26.26 g of PLs/100 g of fat, which was more efficient to isolate PLs compared to control WPPC (13.5 g of PLs/100 g of fat). A variation was also found between the triplicates under these treatments.

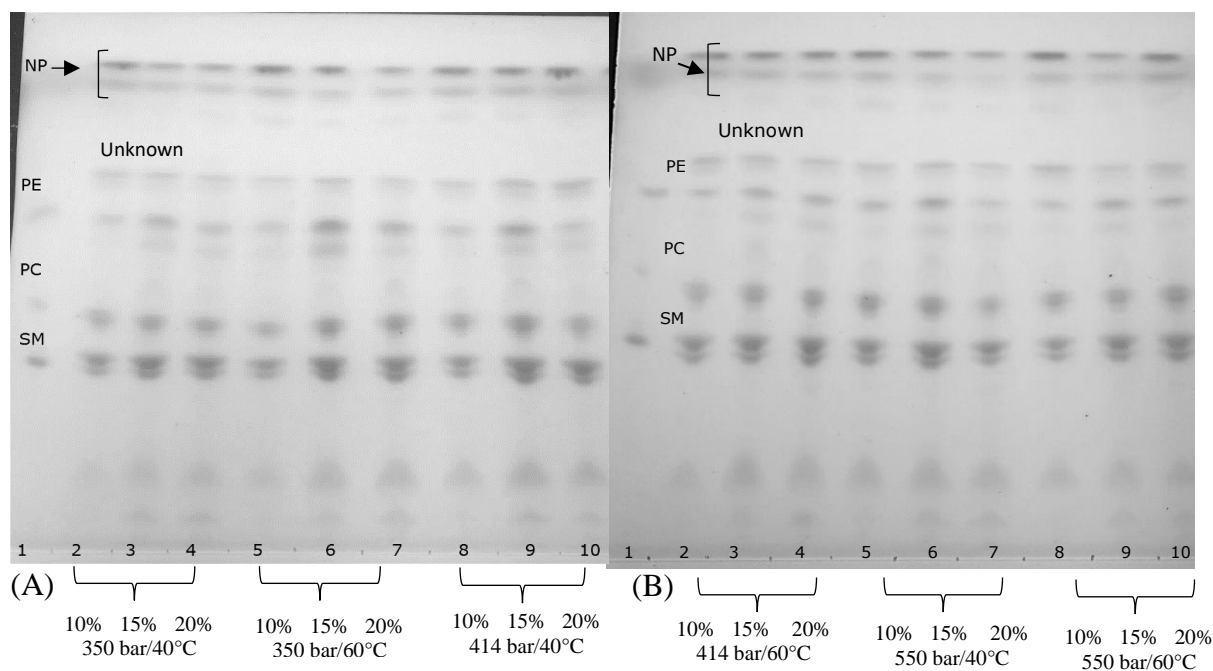


Figure 4.5 Effect of SFE parameters on efficiency of PLs extraction from WPPC. Sample orders in (A) were: lane 1=standard mark; lane 2 to 4 are lipids from SFE treatment: 350 bar, 40 °C, concentration of ethanol at 10%/15%/20%. Lane 5 to 7 are lipids from SFE treatment: 350 bar, 60 °C, concentration of ethanol at 10%/15%/20%. Lane 7 to 10 are lipids from SFE treatment: 414 bar, 40 °C, concentration of ethanol at 10%/15%/20%. Sample orders in (B) were lane 1=standard mark; lane 2 to 4 are lipids from SFE treatment: 414 bar, 60 °C, concentration of ethanol at 10%/15%/20%. Lane 5 to 7 are lipids from SFE treatment: 550 bar, 40 °C, concentration of ethanol at 10%/15%/20%. Lane 7 to 10 are lipids from SFE treatment: 550 bar, 60 °C, concentration of ethanol at 10%/15%/20%. Abbreviations from top to bottom were: NP=nonpolar lipids, PE=phosphatidylethanolamine, PC=phosphatidylcholine, SM=sphingomyelin

TLC was also used as a qualitative check for PLs obtained after Step-2 SFE and TLC plates are provided in Figure 4.5. The intensity of PLs bands was consistently low at 10% ethanol concentration when compared to 15% and 20% ethanol concentrations. The results obtained from TLC confirmed the results from the HPLC analysis of PLs. It was interesting to see neutral lipids bands in all the Step-2 extracts indicating the presence of neutral lipids even after the Step-1 extraction with neat CO₂. Barry et al. (2017) reported similar work and was able to produce PLs-rich fraction (~56%) from buttermilk hydrolysate followed by ultrafiltration and 2-stage supercritical CO₂ extraction process. Barry et al. (2017) produced the PLs-rich fraction using optimal conditions at pressure at 300 bar, 40 °C extraction temperature, and 20% ethanol concentration. Contrary to our findings, Barry et al. (2017) could not recover any PLs at 10% ethanol concentrations from buttermilk hydrolysate.

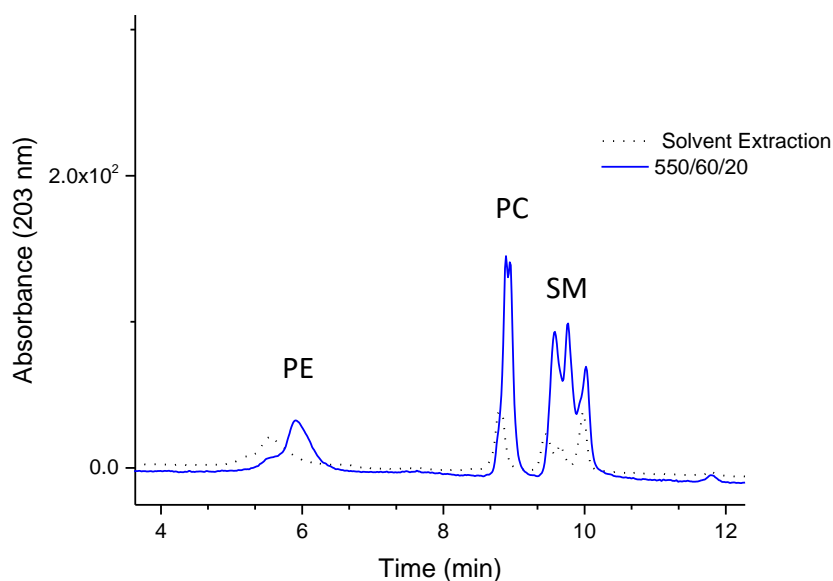


Figure 4.6 HPLC chromatogram of lipid fractions from WPPC with liquid-liquid extraction and SFE at 550 bar, 60 °C and 20% ethanol. PE=phosphatidylethanolamine, PC=phosphatidylcholine, SM=sphingomyelin

To further study each PLs content in the extracts, the lipid fractions were analyzed using HPLC to identify the individual PLs. Figure 4.6 illustrates an example of the HPLC chromatogram that showed both lipid extracts obtained from the solvent extraction and the SFE-Step-2 (550 bar, 60 °C, and 20% ethanol).

The Tukey's test was also used to analyze the results from various parameters to study the effect of SFE treatment on individual PL extraction. According to Shah et al. (2004), the proportion of individual PL in the extract may be vary depending on amounts of ethanol as co-solvent used in SFE. The level of PE and SM were both significantly different ($P < 0.05$) when ethanol increased from 10% to 15% as well as 10% to 20%. However, SM was more sensitive to ethanol changed that a significant difference presented between 15% and 20% ethanol ($P < 0.05$). PC was not affected by any SFE treatment. Figure 4.7 showed when ethanol concentration was at 20%, the highest yield of SM was 11.07 g/100 g of fat. When ethanol concentration at 15%, the highest yield of PE could reach to 7.2 g/100 g of fat and PC was extracted 7.29 g to 10.08 g/100 g of fat.

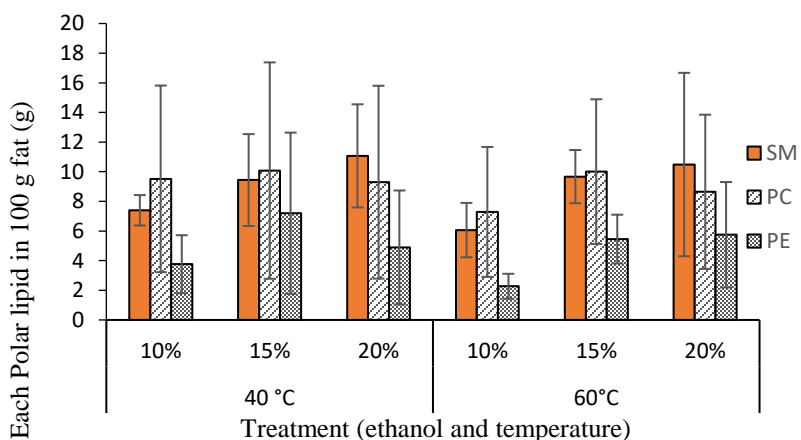


Figure 4.7 Effect of concentration of ethanol (10%, 15%, and 20%) on the amount of each polar lipid (SM, PC, PE) presented in crude lipids from various operating SFE temperature (40/60 °C). The average of triplicate samples were used as results, bars indicate standard deviation. PE= phosphatidylethanolamine, PC=phosphatidylcholine, SM= sphingomyelin

Functional Properties of Protein

Chemical and functional properties were evaluated for the exhaust WPPC powders obtained after the Step-2 extraction (550 bar pressure, 40 °C, 15% ethanol). The total protein content in exhaust WPPC powders increased to approximately 72% because of the removal of lipid fraction from the WPPC powders. Functional characteristics such as EMC and WHC were carried out on WPPC powders only for WPPC #2 and #3 and the results are provided in Table 4.3. As can be seen in Table 4.3, average EMC for the original WPPC powders were 5.1 and 5.7 ml of oil/g of solution. After the removal of PLs from WPPC, the EMC increased to 6.4 and 6.1 ml of oil per g of solution. Approximately 15% average increase in EMC was observed between the original and exhaust WPPC powders.

Table 4.3 The value of emulsification capacity (EMC) and water holding capacity (WHC), and in control WPPC powder, and exhausted powders produced under condition 550 bar/40 °C/15% ethanol

| Sample | EMC (ml oil/g of 5%p solution) | WHC (%) |
|------------------|--------------------------------|-------------------------|
| Control WPPC#2 | 5.72±0.11 ^a | 39.45±1.69 ^a |
| Exhausted WPPC#2 | 6.42±0.14 ^b | 60.32±2.47 ^b |
| Control WPPC#3 | 5.14±0.09 ^c | 41.59±3.93 ^c |
| Exhausted WPPC#3 | 6.08±0.11 ^d | 53.73±5.27 ^d |

^{a,b,c}Means within a column with different superscripts differ (P < 0.05)

Similarly, water-holding capacity of exhaust WPPC powders obtained after the Step-2 extraction (550 bar pressure, 40 °C, 15% ethanol) also increased when compared to original powders. The average water holding capacity of original WPPC was 43% and it was increased to 59.5% for the exhausted WPPC powders. Veith and Reynolds (2004) reported the water holding capacities of 10 commercially available whey protein concentrates and found the water holding

capacities in the range of 3.3 to approximately 10% water release. When compared to whey protein concentrate powders, the WPPC powders in the present study showed higher water release and thereby lower water holding capacities.

Conclusion

The results of two-step SFE process demonstrated that PLs were successfully extracted from WPPCs. SFE with neat CO₂ in the first step removed most of the neutral lipids and left the PLs and protein-enriched spent solids. SM, PC, and PE were isolated by neat CO₂ and ethanol as a modifier in the second SFE step. The amounts of PLs was variable by different optimum treatments. The concentration of ethanol was the main factor to affect the efficiency of PLs extraction. The highest yield of PLs was 26.26 g/100 g of fat under pressure 350 bar, 60 °C and 20% ethanol. This study proved that SFE is a safe and economic method to isolate PLs from WPPC, which can increase the values of the low-cost dairy powders.

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Chapter 5 - Selective Extraction and Characterization of Phospholipids from Buttermilk Powder using Supercritical Carbon Dioxide and Modifier

Abstract

During processing of butter, a large amount of buttermilk is produced every year. However, only a small part has been used in food industries. Thus, buttermilk is considered as a low-value by-product of butter. It is a challenge for researchers to find a more potential use of buttermilk. Since there are plenty of milk fat globule membrane in buttermilk which the phospholipids (PLs) are mainly located at, it is an inexpensive ingredient with high functional and nutritional properties. In addition, PLs are proved in many researches that they have health benefits on the human body such as improved cell functions and providing anti-oxidative properties. Supercritical fluid extraction (SFE), an alternative extraction method, is nontoxic and economical. SFE has been used frequently and successfully in the food industry to remove essential compounds and nonpolar lipids with carbon dioxide. Extracting polar lipids from dairy products is still a challenge. The objective of the study was to optimize SFE condition to selectively isolate PLs from BMP and profile the PLs extracts. Three lots of spray-dried buttermilk were obtained from a commercial manufacturer. PLs rich extracts were successfully isolated by a unique two-stage process. In the first stage, supercritical carbon dioxide (SCO₂) was used to remove most of the neutral lipids under the condition of 414 bar pressure, 60 °C temperature and 5 L/min CO₂ flow rate. In the second stage, PLs were extracted by SCO₂ and ethanol as modifier. To optimize the extraction treatments of the second stage, a 3×3×2 factorial design included pressure, ethanol concentration and temperature sets were investigated.

Triplicates of each extraction were employed to obtain the best concentration of PLs in the lipid extracts. The optimal parameters were found to be 550 bar pressure, 15% of ethanol concentration and 60 °C sample temperature, the recovery of PLs was 16.88 g/100 g of fat. The results demonstrated SFE may have the potential to be used for extracting PLs from BMP thus increasing the value of BMP.

Keywords: supercritical fluid extraction, buttermilk powder, ethanol, modifier

Introduction

In recent years, more different factors with nutritional and medical properties have been found in bovine milk fat globule membrane (MFGM). Phospholipids (PLs) of MFGM are one of the essential components that provide health benefits. Sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the three main PLs in MFGM. Many research studies proved that dietary sphingolipids can lower the hepatic fat, plasma cholesterol, triglycerides, and inhibit absorption of cholesterol. Henríquez and co-workers (2015) noticed attention deficit hyperactivity disorder (ADHD) patients usually had lower level of serum SM. One hypothesis explained that ADHD is associated with brain maturation, however, the role of SM in ADHD pathogenesis is still being studied. Mice fed with PC-rich diet mitigated obese status, non-alcoholic fatty liver disease and cardiovascular disease (Spitsberg, 2005) which were induced by a high-fat diet. Another diet contained either phosphatidylethanolamine and/or lysophosphatidylethanolamine was used on Alzheimer's disease in mice, the results demonstrated PLs could improve the recovery of cognitive function and cellular homeostasis in the mice (Lee et al., 2014).

Buttermilk powder (BMP) is a good source of PLs because it is a low cost dairy powder as well as a highly functional ingredient (Fryksdale, 2001). Buttermilk is a dairy by-product of butter processing which contains large amounts of milk fat globule membranes (MFGM), where most of the PLs are located. In the U.S., the production of butter was about 163.8 million pounds in December 2015, and buttermilk production was 11 million pounds for the same month. In the whole year of 2016, butter production was increased to 1.889 billion pounds while a large amount of buttermilk was also produced (US Cheese Production in 2016 Hit a New Record: 12.074 Billion Pounds, 2017). Buttermilk is spray-dried to make BMP before storage and use.

The benefits of consuming BMP are not only the nutritional values, but also contain moisture, add firmness and flavor, and increase emulsion and whipping properties in food such as ice cream, chocolate, yogurt, toffee, soups, sauces and baked products (Fryksdale, 2001). However, only a small amount of buttermilk has been used in the food industry. In this situation, it is essential to improve the utility value of BMP by developing technologies to isolate or concentrate PLs (Gassi et al., 2016).

Supercritical fluid extraction (SFE) is a common method that has been applied for separating compounds in many foods. Compared to conventional extraction methods, SFE with neat carbon dioxide (CO₂) shows more advantages including high selectivity, purity of extracts, environmental safety, and no organic solvents are used which leave a chemical residue (Zabot and Meireles, 2016). Ethanol is employed as the modifier since it is environment-friendly. The functions of modifiers are to increase the solubility of PLs and to disrupt the association between protein and polar lipids in food. Supercritical carbon dioxide (SCO₂) with ethanol has successfully isolated PLs from eggs, soybean flakes, fish, and seeds (Montarini et al., 1996; Tanaka et al., 2004; Dunford and Temelli, 1995). For dairy products such as BMP, microfiltration, ultrafiltration and/or SCO₂ are usually applied to remove neutral lipids and enrich PLs (Roesch et al., 2004; Morin et al., 2007; Olabi et al., 2015). There is one study has extracted PLs from BMP by SCO₂ and ethanol as modifier (Barry et al., 2017). The BMP was treated with enzymatic hydrolysis and ultrafiltration before SFE. The final PLs recovery in the extract was more than 50%.

However, it is still a difficult task to extract all PLs from BMP without any treatment before SFE since low amounts of lipids (> 10% on dry basis) are usually associated with high amounts of polysaccharides and protein (Gallier et al., 2010). Many chemical extraction methods

have been used to extract PLs in BMP, many hazardous organic solvents were used, and the extracts were not acceptable for human consumption (Gadkowski et al., 2012). Thus, the objectives of this study were to extract PLs from BMP with supercritical CO₂ and an ethanol modifier; and to examine the effect of temperature, pressure, and concentration of ethanol on the SFE process; and to identify different species of PLs in crude lipids from BMP.

Materials and Methods

Experimental Design

A 2-stage SFE sequence was carried out for isolating the PLs fraction from BMP and to study the effect of pressure, temperature and ethanol concentration on extraction. Three lots of BMPS were donated from the commercial manufacturer. In stage-1, neutral lipids were dissolved and extracted by neat SCO₂. In stage-2, ethanol was employed as a modifier to improve the solubility of PLs during the SFE process. Figure 5.1 summarized the 2-stage SFE in the flow diagram. Polar lipid fractions were profiled by HPLC-UV and TLC.

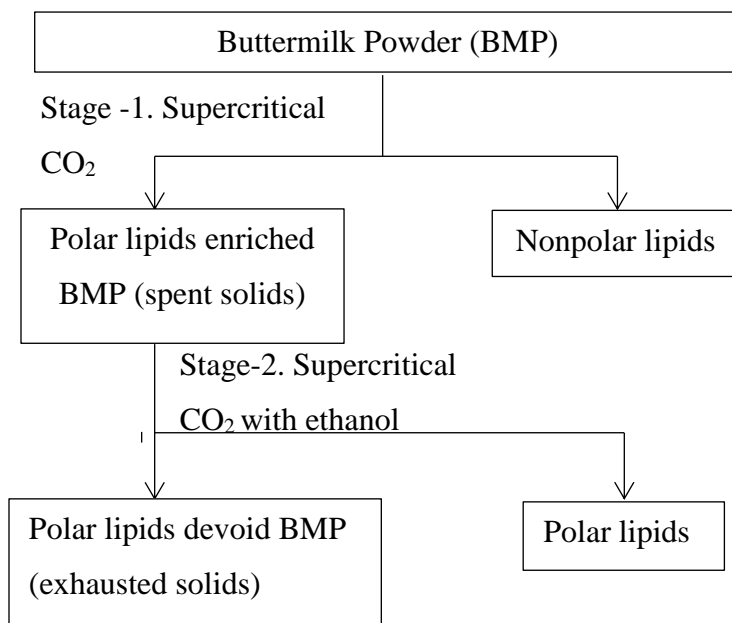


Figure 5.1 A unique two-stage supercritical fluid extraction for isolating polar lipids from BMP

Experimental Setup

A laboratory scale supercritical fluid extraction system used in this study is shown in Figure 5.2, to perform the goals of the study. The extraction system consisted of a SCO_2 cylinder with a dip tube (SFC W/Flet, Matheson, KS), a pump with a maximum capacity of 60 Mpa (spe-ed P/N 7102, 120 VAC, Applied Separations, PA), a temperature controller, a high pressure syringe modifier pump (spe-ed P/N 6870, Applied Separations, PA), and an oven module (spe-ed P/N 7070, 120 VAC, Applied Separations, PA). The extraction unit also consisted of one 32 ml stainless steel cell.

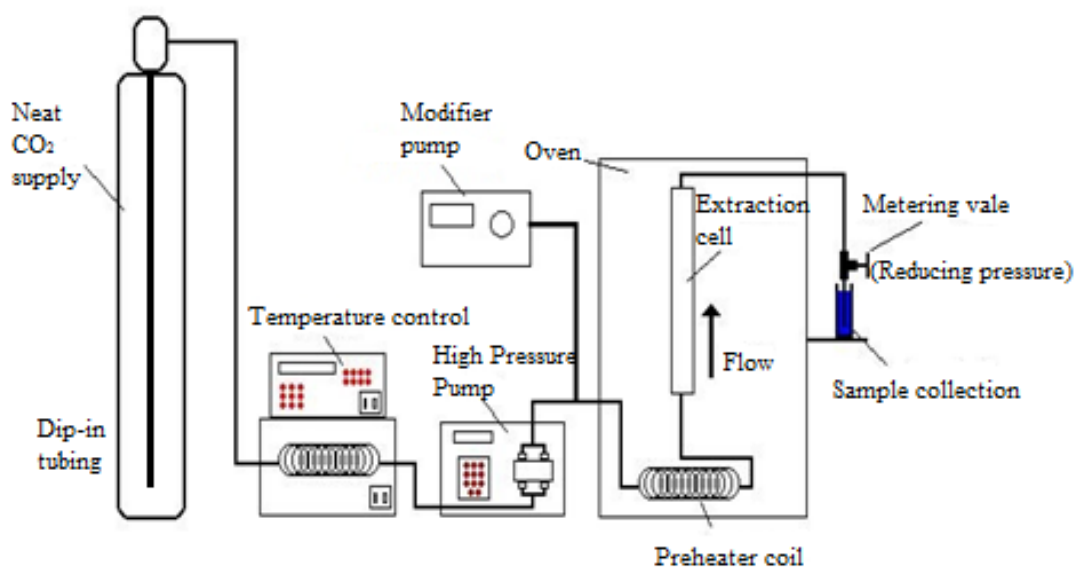


Figure 5.2 Supercritical fluid extraction system

Material

Three lots of BMPs were obtained from Land O' Lakes (Arden Hills, MI). All chemical solvents were HPLC grade (Fisher Scientific, Atlanta, GA). PL standards included phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) were purchased from Sigma (St. Louis, MO). Silica gel thin layer chromatography plates 60 F254

were purchased from EMD Chemicals (Darmstadt, Germany) and the developing tank from Kontes Glass Company (Vineland, NJ).

Supercritical Fluid Extraction

Schematic presentation of a laboratory scale SFE system (Applied Separations, Allentown, USA) fitted with a 32 ml stainless steel extraction vessel is demonstrated in Figure 5.2. About 30 g of BMP was mixed with 3mm glass beads (Fisher Scientific, Atlanta, GA) in the ratio 2:1 and filled into a stainless extraction vessel. The wool put in both sides of vessel to keep the heads from blocking by powder. Glass beads were filled with the rest of the empty space in the vessel as a filler. Finally, the extraction vessel was screwed tight in the temperature-controlled oven, temperature setting was different depending on value per experimental design.

Stage-1 Extraction. In the first stage, neat SCO_2 extraction was applied to carried out at pressure of 414 bar with 60 °C sample temperature in a constant SCO_2 flow rate of 5 L/min for 60 min. A 30 min-soaking process was done before the extraction in order to maximize the solubility of nonpolar lipids in SCO_2 . The extract was separated SCO_2 by reducing the pressure from BMP in the extraction vessel and collected in a 30-ml glass vial. The SCO_2 gas was released into the environment. Around 180 g of spent solids (BMP devoid of neutral lipids) would be needed by repeating stage-1 extraction for several times. The spent solids were mixed thoroughly and used in stage-2 afterwards.

Stage-2 Extraction. To achieved the optimal condition for increasing the efficiency of polar lipid extraction, a $3 \times 3 \times 2$ factorial design was used in stage-2. The three factors included pressure, ethanol concentration, and extraction temperature, which were used as the independent variables. Three levels of pressure (350, 414 and 550 bar), three levels of ethanol concentration (10%, 15% and 20%) and two levels of temperature (40 and 60 °C), were incorporated into the

experimental design. Stage-2 extraction was determined by using three lots of BMP. The ethanolic solution was placed under the gentle stream of air in a hood in order to evaporate ethanol from polar lipids. The polar lipids extracted from each experiment were weighed, and kept in the freezer at -20 °C for further analysis.

Chemical Extraction

An alternative PLs extraction method described by Rombaut (2007) was used to extract PLs from BMP and to compare with the SFE results. Exactly 5.0 g of BMP was diluted with deionized water to 20 ml. The slurry was transferred to a separating funnel after stirring. Then, 80 ml of 2:1 (vol: vol) chloroform: methanol was added and shaken for 2 mins, the slurry was allowed to stand and separate to release and collect the lower phase. The next step was done in replicates that added 40 ml of chloroform: methanol 20:1 (vol: vol) to the upper phase. In the last step, 40 ml of chloroform: methanol: water 86:14:1 (vol:vol:vol) with 1 N HCl was used. The lower phase was washed with a 0.9% NaCl solution until neutral pH was reached. Following, four lower phases were combined, pooled and evaporated, using a rotary vacuum evaporator at 37-39 °C. The crude lipids were weighed and dissolved in 1 mg/ml with methanol, and the solutions were filtered and injected into a capped sampler vial and stored at -20 °C until HPLC analysis.

Lipids Analysis

High-Performance Liquid Chromatography (HPLC). The PL extracts (1 mg) collected from stage-2 were diluted in 1 ml of methanol, filtered with a 0.45 µm*13mm nylon filter (Alltech, IL) and transferred into a 30 ml capped glass bottle. PLs analysis was performed using a Agilent HP-1050 HPLC chromatograph (Palo Alto, CA) coupled with Agilent UV detector (Palo Alto, CA). A 150 mm × 4.6 mm I.D., 5 µm particle size Phenomenex (Le Pecq

Cedex, France) HILIC LC column that was set at 35 °C. The elution program was a gradient of acetonitrile: deionized water 90:10 (vol:vol) at 0 min to 50: 50 (vol:vol) at 5 min. The mobile phase turn back to initial condition at 15 min and the next injection was ready to inject after at 25 min. The flow rate was 1 ml/min constantly cause the backpressure was 45-60 bar. The injection volume was 20 µL. The UV detector was set 203 nm for PC, PE and SM according to Wang and others (2003). Identification of PLs was based on comparing retention time to standards. Quantification was calculated by standard curves equation of three PLs standards generated that plotting peak areas against the concentrations ($r^2 > 0.99$).

Thin Layer Chromatography (TLC). According to Spence (2009), lipid extracts (10 mg) were dilluted in 1 ml with 1:2 (vol:vol) chloroform:methanol solvent; PLs standards (1 mg) were diluted 1 ml solvent. Polar lipids were separated by using 65:25:4 (vol:vol:vol) chloroform:methanol:deionized water. Pipetting 20 µg of samples and 5 µg of standards on silica gel plates. Lipids were visualized by exposure to iodine vapor overnight, and identified by comparison to standards (Astaire et al., 2003).

Protein Analysis

SDS-Page. Protein profiling method described by Liu, Dunstan and Martin (2012) was used with modifications. The 3.5% protein solution was diluted with double deionized water in 1:8 (vol:vol). Then, 20 µL sample buffer which contained 90% Bio-Rad Laemmli buffer and 10% beta mercaptoethanol (Bio-Rad, Hercules, CA) was added to protein sample. The sample was boiled for 8 min at 95 °C, and 25 µl of samples were loaded in 4-15% tri/glycine gel (Mini-Protean TGX Precast gel, Bio-Rad, Hercules, CA), the electrophoresis was ran by Powerease 500 microprocessor (Life Technoligies, Carlsbad, CA) at 120 V, 80 A for 2 hours in 10% concentration of tris-glycine SDS buffer solution (Bio-Rad, Hercules, CA). Precision Plus

Protein All Blue Standards (8 μ L) (Bio-Rad, Hercules, CA) was employed as the molecular-weight standard. Gels were washed in deionized water for 5 min and stained by using coomassie brilliant blue R-250 dye (Thermo Scientific) for half hour and washed with double deionized water for 8 hours.

Compositional Properties

Total nitrogen was analyzed by LECO system (TruMac N, LECO Corp., St. Joseph, MI). The factor for converting percentage of nitrogen in the BMP to the percentage of protein was 6.38. Total solid was analyzed by the forced air method described by method 990.20 (AOAC International, 2002). Ash was analyzed by incineration method described by method 954.46 (AOAC International, 2002). The total lipids in BMP were analyzed by CEM Smart Trac Fat System (CEM Corp., Matthews, NC).

Statistical Analysis

Data collected were analyzed and compared using SAS 9.4 for Windows. The mean value comparisons were performed by Tukey's test. The P-value was at 0.05.

Results and Discussion

Buttermilk Powder

Table 5.1 shows the chemical composition of three lots of BMP used in the present study. The BMP values of moisture, total lipid, protein, and ash had significant differences ($P < 0.05$) between LOT#1, LOT#2, and LOT#3. BMPs typically have maximum 5% of moisture, maximum 4.5% of fat, and minimum 30% of protein, ash is between 6% to 8% (Dry Buttermilk (DBM) Standard) (Sodini et al., 2006; Barry et al., 2016). The average composition of three BMP samples were 3.29% moisture, 3.02% fat, 33.56% protein, and 7.17% ash which met the

BMP standard listed above. The nutritional value of BMP is comparable to skim milk powder, where protein in BMP is in the similar proportion as in skim milk powder, but a higher fat content in BMP was noticed than in skim milk powder (0.74%) (Corredig and Dalgleish, 1997; Morin et al., 2004). In this study, the fat content in BMP samples was varied between 2.51% to 3.6%.

Table 5.1 Percentage and standard deviation of moisture, total fat, total protein, and ash in three lots of BMP powder

| Control | Moisture (%) | Fat (%) | Protein content (%) | Ash (%) |
|---------|------------------------|------------------------|-------------------------|------------------------|
| LOT#1 | 2.58±0.02 ^a | 2.51±0.07 ^a | 33.50±0.07 ^a | 7.22±0.01 ^a |
| LOT#2 | 3.63±0.03 ^b | 2.96±0.08 ^b | 33.16±0.02 ^b | 7.14±0.00 ^b |
| LOT#3 | 3.66±0.14 ^b | 3.6±0.01 ^c | 34.02±0.02 ^c | 7.16±0.05 ^b |

^{a,b,c}Means within a column with different superscripts differ (P < 0.05), n=2

Table 5.2 Average value and standard deviation of dry matter, fat and polar lipid content of three BMPs

| Sample | Polar lipid species (g/100 g Fat) | | | Polar lipid species (% of total polar lipids) | | | DM | Fat |
|--------|-----------------------------------|------------------------|------------------------|---|-------|-------|-------|---------|
| | SM | PC | PE | SM | PC | PE | mg/g | g/100 g |
| LOT#1 | 8.16±0.07 ^a | 6.23±0.82 ^a | 5.01±0.84 ^a | 42.20 | 32.04 | 25.75 | 10.05 | 19.39 |
| LOT#2 | 7.51±0.98 ^b | 5.66±1.16 ^b | 4.80±1.25 ^b | 42.02 | 31.46 | 26.52 | 8.96 | 17.97 |
| LOT#3 | 11.12±0.27 ^c | 6.77±0.02 ^c | 6.30±0.17 ^c | 45.98 | 27.99 | 26.03 | 19.68 | 24.19 |

^{a,b,c}Means within a column with different superscripts differ (P < 0.05) PE=phosphatidylethanolamine, PC=phosphatidylcholine, SM=sphingomyelin, n=2

The PLs results obtained from three BMPs by using liquid solvent extraction are shown in Table 5.2. The PLs in total fat for LOT#1, #2 and #3 were 19.39%, 17.97% and 24.19%, respectively. The average composition of each individual PLs was 43.4% of SM, 30.50% of PC, and 16.1% of PE. Barry and co-workers (2016) reported the amount of PLs in buttermilk was 15 times greater than in whole milk. And there is 35.32% of total milk fat is PLs. Furthermore, PLs consist of 27.62% PE, 31.50% PC, 21.49% SM, 9.63% phosphatidylinositol (PI) and 10.10% phosphatidylserine (PS). In this study, the PLs content in all of the BMP was lower than the previous study reported. The proportion of SM was the biggest, while PE was less than PC. The

compositions of PLs in three lots of BMP were significantly different ($P < 0.05$). The variation of PLs in BMP could be caused by different factors such as farm environment, cow diet, and milk processing (Ferreiro et al., 2016).

Evaluating the 2-stage SFE Method for Extraction PLs

Stage-1 Extraction. In the first stage of extraction process, only SCO_2 was employed. Extraction pressure at 350 bar combined with a temperature of $50\text{ }^\circ\text{C}$ were the optimized parameters for removing nonpolar lipids from BMP in many studies (Spence et al., 2009, Costa et al., 2010, Olabi et al., 2015). On the other hand, Astaire (2003) increased the pressure to 375 and 390 bar in SCO_2 extraction, and the temperature was constant. The results demonstrated that recovery of PLs was higher in BMP with 390 bar, which means higher pressures extract more neutral lipids using SCO_2 . The optimal treatment used to extract neutral lipids in the present study was at 414 bar of extraction pressure and $60\text{ }^\circ\text{C}$ temperature. The optimal condition was considered for the subsequent stage.

Figure 5.3 shows a picture of the TLC plate of lipid fraction obtained from stage-1 (lane 1) and lanes 2 and 3 shows the typical BMP used in the study and the remaining lipid fraction extraction from the spent solids, respectively. No PLs were found in extracts collected from stage-1 as expected, which confirmed what previous studies reported that SCO_2 can remove the unwanted neutral lipids and rich PLs in the BMP (Astaire, 2003). Since the same amount of extract solutions were applied on TLC gel plates, it was noticeable that PLs band had similar intensity in spent solids compared to original BMP. However, TLC plates also indicated the neutral lipids were not completely removed in the first SFE stage from spent solids.

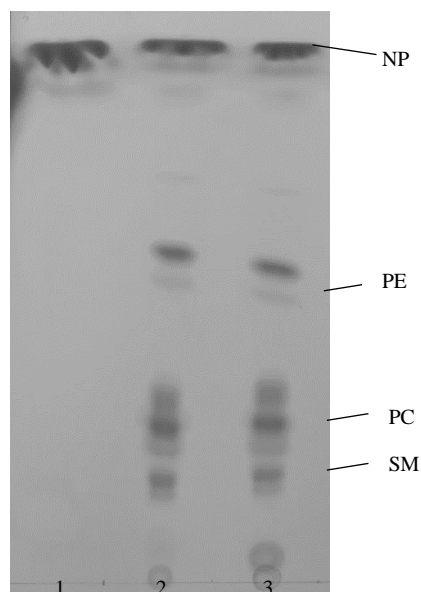


Figure 5.3 Thin layer chromatography of polar lipids profile. Lane 1=lipid extract from SFE stage-1, lane 2= lipids in control BMP, lane 3= lipids in spent solids left from SFE stage-1. NP= Neutral lipids; PE=Phosphatidylethanolamine; PC=Phosphatidylcholine; SM=Sphingomyelin

Stage-2 Extraction. In the second stage of SFE, ethanol as a modifier was employed with CO₂ to selectively isolate polar lipids in BMP. In order to determine the optimal treatment, pressure was selected at 350, 414 and 550 bar, temperature was selected at 40 and 60 °C, ethanol concentration (in CO₂) was set in 10%, 15% and 20%. To understand the effect of different treatments on the efficiency of extracting PLs, the results were analyzed by the Tukey's test. The statistical analysis indicated that sample temperatures and ethanol concentration had a significant effect ($P < 0.05$) on PLs extraction. However, pressure had no significant effects ($P > 0.05$). The results confirmed the SFE theory about how the change of temperature and/or pressure can influence the selectivity of extraction (Sekhon, 2010). On the other hand, the modifier concentration was the most influential factor for isolating the PL moieties (Taylor et al., 2000). The concentration of ethanol as modifier increased from 10% to 20% efficiently increased the

yield of PLs (Montarini et al. 1996). This effect also had been demonstrated experimentally in this study.

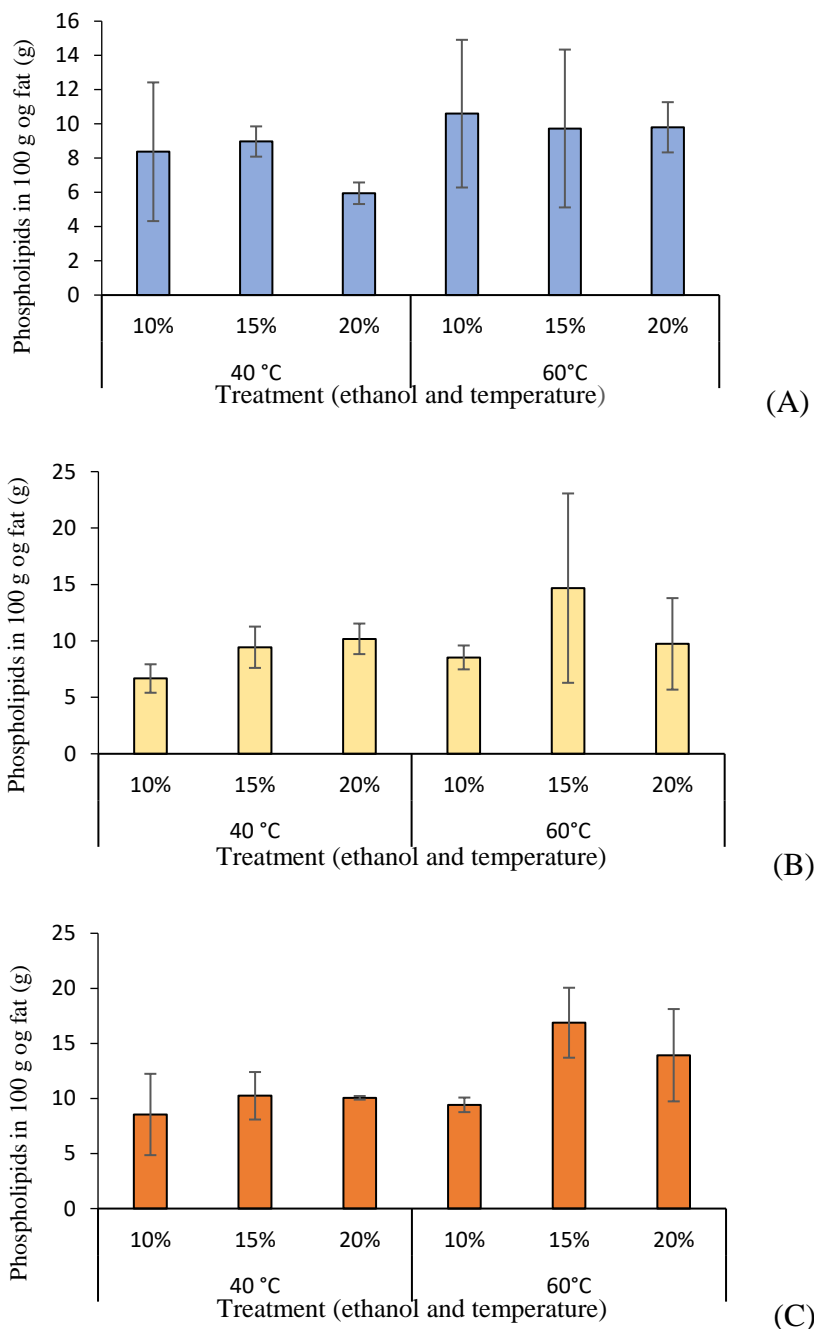


Figure 5.4 Various operating SFE pressure: (A) 350 bar, (B) 414 bar and (C) 550 bar with different temperature (40 and 60 °C), and concentration of ethanol of ethanol (10, 15, and 20%) for PLs extraction from BMP spent solids. The average of triplicate samples are used as results, bars indicate standard deviation

Figures 5.4 (A, B, and C) show the amount of PLs recovered during stage-2 process at 350, 414, and 550 bar, respectively. As observed, when temperature was at 40 °C with 10, 15, and 20% of ethanol, the highest recoveries of PLs were 8.54, 10.24, and 10.18 g PLs/100 g of fat; whereas 60 °C with 10, 15, and 20% of ethanol isolated 10.59, 16.88, and 13.93 g PLs/100 g of fat. The optimal process parameters of 550 bar pressure, 60 °C sample temperature and 15% ethanol concentration extracted 16.88 g of PLs/ 100 g of fat from the SFE stage-2.

The lipid fraction remaining in the exhausted solids is showed in Figure 5.5 (A). The intensity of neutral lipids and PLs was lower compared to original BMP and spent solids, which means part of PLs were still left in exhausted solids. Figure 5.5 (B) and (C) showed the lipid fractions obtained from different SFE treatments. Lower intensity was observed in samples when temperature was at 40 °C. It is interesting to note the neutral lipids present in all the lipid fractions collected from stage-2, and in exhausted solids. The neutral lipids were not completely removed in 2-stage SFE process. This may be due to a polar 3 β -hydroxyl group in cholesterol, it would be isolated with PLs (Yeagle, 1985).

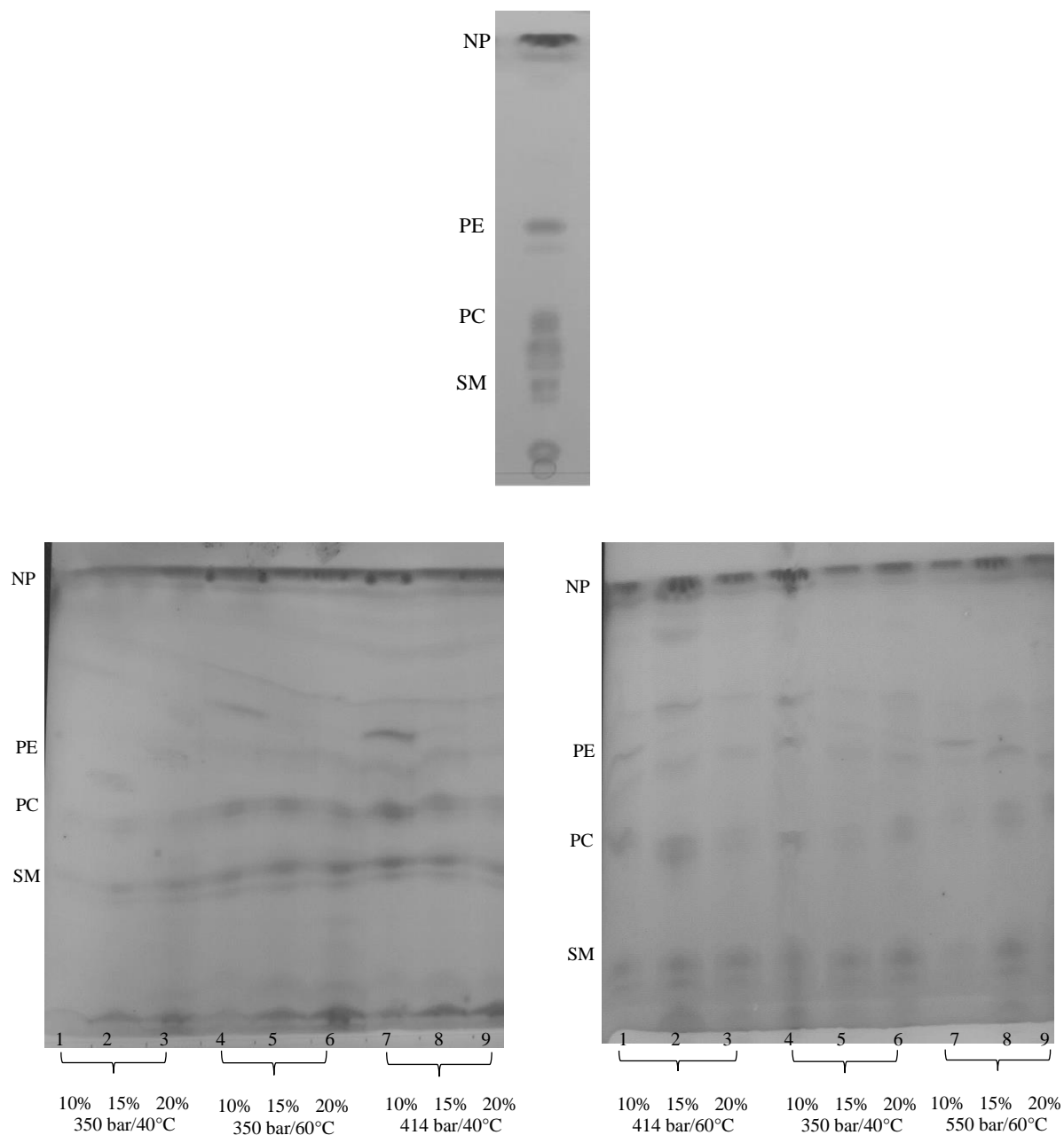


Figure 5.5 Thin layer chromatography using silica gel plate, 10 μg of polar lipids from different conditions of SFE and 5 μg of standards was applied on plate. (a) lipids in exhausted solids obtained from treatment 550 bar, 60 $^{\circ}\text{C}$, 15% ethanol concentration. Sample orders in (b) lane 1 to 3 are lipids from SFE treatment: 350 bar, 40 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%, lane 4 to 6 are lipids from SFE treatment: 350 bar, 60 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%, lane 7 to 9 are lipids from SFE treatment: 414 bar, 40 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%. Sample orders in (c) were: lane 1 to 3 are lipids from SFE treatment: 414 bar, 60 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%, lane 4 to 6 are lipids from SFE treatment: 550 bar, 40 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%, lane 7 to 10 are lipids from SFE treatment: 550 bar, 60 $^{\circ}\text{C}$, ethanol concentration at 10/ 15/ 20%. Abbreviations from top to bottom were: NP-nonpolar lipids, PE-phosphatidylethanolamine, PC-phosphatidylcholine, SM-sphingomyelin

To identify the PLs content in the extracts, HPLC was employed to analyze the lipid fractions obtained from stage-2. Figure 5.6 shows the HPLC chromatogram that compared extracts obtained from the solvent extraction and the optimal SFE stage-2 (550 bar, 60 °C, and 15% ethanol). All three individual PLs were presented in both fractions. The optimal stage-2 isolated more SM and PE than liquid solvent extraction based on peak area, but PC was not efficiently isolated.

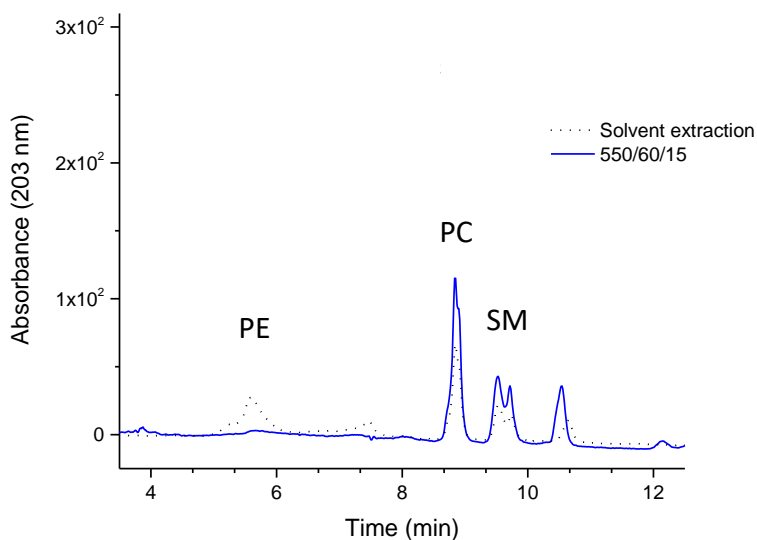


Figure 5.6 HPLC-DAD elution of lipid fractions from liquid extraction and SFE treatment at 550 bar, 60 °C and 15% ethanol

Tukey's test also was used to further analyze whether the various parameters affected individual PLs extraction. The statistical results demonstrated ethanol concentration and sample temperature had significant effects ($P < 0.05$) on SM and PC. The individual PL can be obtained by varying the modifier concentration in SFE, with pressure and temperature being kept constant (Yip et al., 2008). It was proved in the study that there was a big difference between 10% and 15% ethanol for SM. However, 15% to 20% had no difference on SM. PC was more influenced

by temperature, it had higher recovery when temperature was at 60 °C. There was no effect on PE observed from graphs.

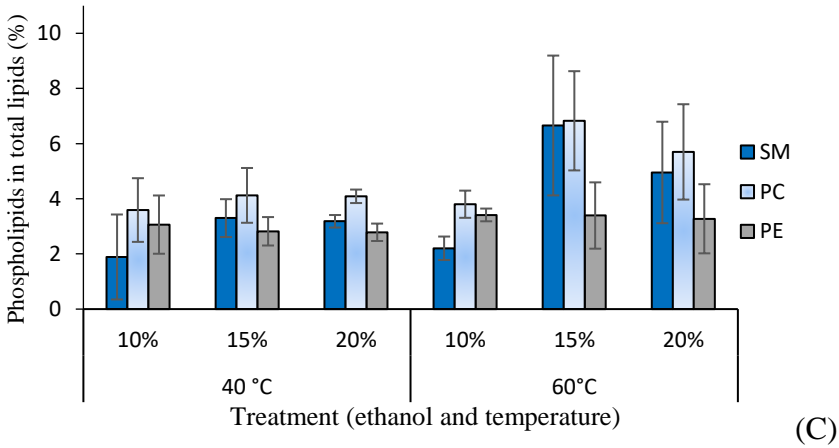
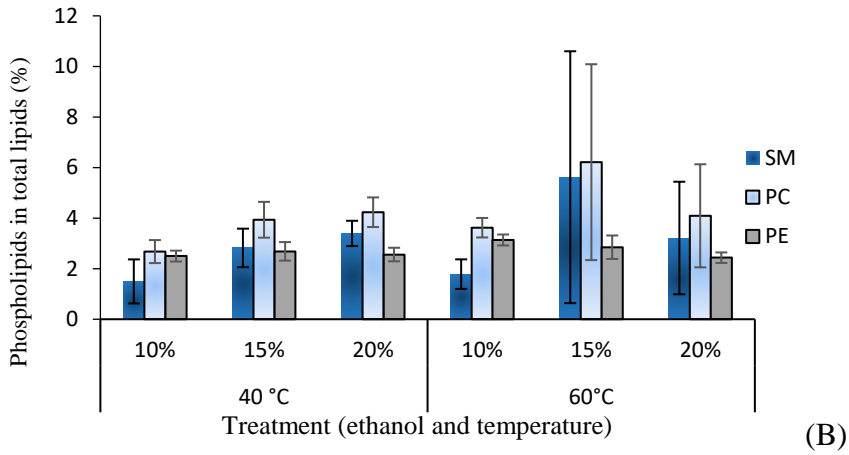
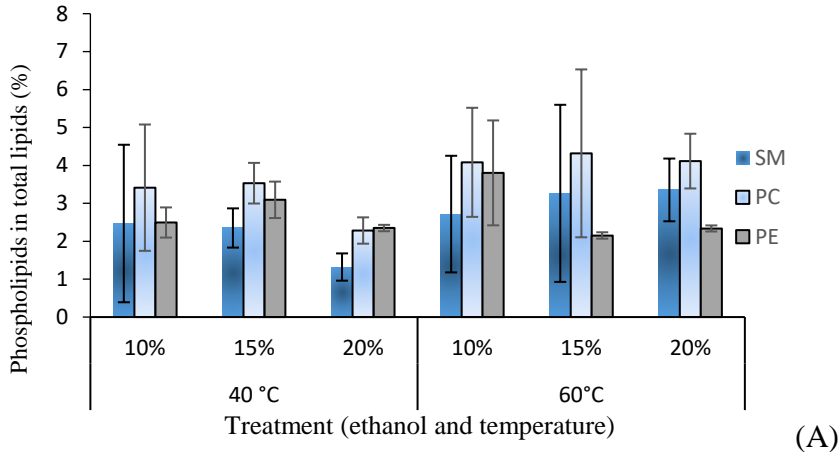


Figure 5.7 SM, PC and PE extracted from different concentration of ethanol of ethanol (10%, 15%, and 20%) and temperature (40/60 °C) at various operating SFE pressure: (A) 350 bar, (B) 414 bar and (C) 550 bar. The average of triplicate samples are used as results, bars indicate standard deviation

From Figure 5.7, large variations in 15% ethanol concentration was noticed, the possible reasons could be the variation of PLs extraction between three lots of BMP and/or the loss of ethanol solution with dissolved lipids during collecting process. The optimized condition for extracting SM and PC was at 550 bar pressures, temperature of 60 °C and 15% of ethanol, 6.66 g of SM/100 g of fat and 6.83 g of PC/100 g of fat was isolated; PE had a higher recovery (3.80 g/100 g of fat) under condition of 350 bar pressure, 60 °C sample temperature and 10% ethanol.

Protein Profiling by SDS-page

In order to compare the proteins in control BMP and exhausted solids from optimal extraction condition (550 bar/60 °C/15% ethanol), reducing polyacrylamide gel electrophoresis was carried out to observe if polymerization happened to protein during SFE treatments (Figure 5.8).

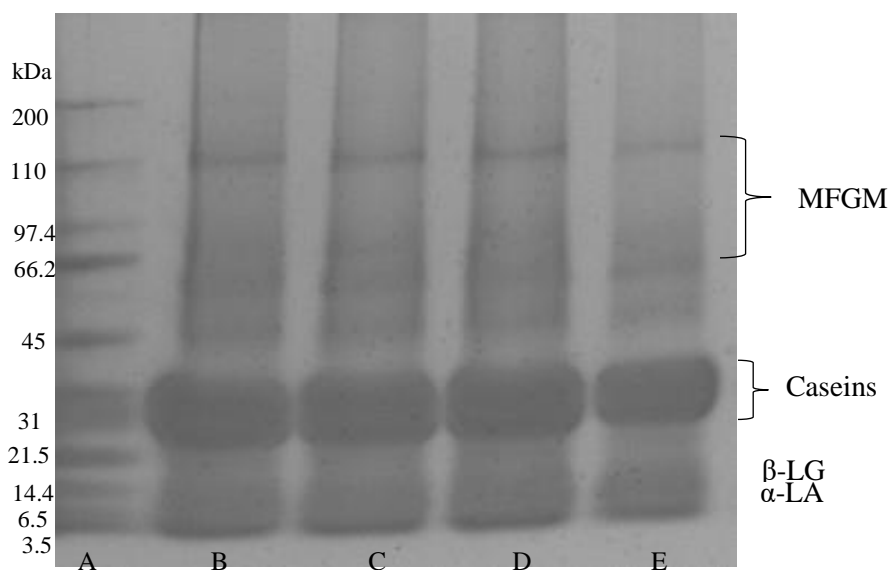


Figure 5.8 Polyacrylamide gel electrophoresis using a 4-15% tri/glycine gel; 25 µl BMP protein samples was applied to each well. A= standard; B = LOT#2 exhausted solids from treatment 550

bar/60 °C/15% ethanol; C= control LOT#2; D = LOT#3 exhausted solids from treatment 550 bar/60 °C /15% ethanol; E= control LOT#3. MFGM= milk fat globule membrane

Protein fractions were observed and had no influence by SFE pressure and temperature in this study. However, pressure and temperature had significant effect ($P < 0.05$) on the yield of protein in exhausted solids.

Conclusion

In conclusion, two stage SFE process could be used to isolate PL fractions as well as enriched protein in the exhausted solids or lipids devoid BMP. In this study, temperature is the most effective factor to influence the recovery of total PLs extract. There was 16.88 mg PLs/100 mg fat extracted by using optimal conditions of 550 bar pressure, 60 °C sample temperature and 15% ethanol concentration. Ethanol and temperature had more effect on extracting SM and PC. The results show SCO_2 with ethanol as modifier is useful and efficient method to selective isolate PLs in BMP. In the future study, higher amount of ethanol will be the subjected if the yield of total polar lipids includes individual PL increase.

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Chapter 6 - Conclusion

Phospholipids (PLs) have been approved as an essential and functional ingredient and have many nutritional and medical benefits for human body. Whey protein phospholipid concentrate (WPPC) and buttermilk powder (BMP) are two low-value dairy powders which contain large amounts of milk fat globules where PLs are in their membranes. Supercritical fluid extraction (SFE) with neat carbon dioxide and ethanol as modifier is alternative method to isolate neutral lipids and PLs from food. It is economical, safe, and environmental without using any hazard organic chemicals.

To develop the SFE method, the pressure, sample temperature, and ethanol concentration were optimized for both WPPC and BMP samples. The treatment of pressure at 350 bar, 60 °C temperature, and 20% ethanol concentration was optimal condition to extract PLs in WPPC, 26.26 mg of PLs/100 mg of fat or 715.86 mg of PLs/100 g of spent solids was isolated. Compared to pressure and temperature, ethanol concentration had significant effect on efficiency of PLs extraction in WPPC. The optimal treatment of pressure at 550 bar, 60 °C temperature, and 15% ethanol concentration extracted 16.88 mg of PLs/100 mg of fat from BMP. In this case, temperature significantly affected the extraction of PLs in BMP. In additional, neutral lipids and PLs were still found in exhausted solids for WPPC and BMP. Extracts from Step-2 also contained neutral lipids.

Overall, SFE with neat CO₂ and ethanol as modifier was able to extract PLs from dairy powder. Increased temperature and ethanol concentration may increase the solubility of PLs. However, pressure had no influence to PLs extraction from WPPC and BMP. Future research with SFE can focus on increasing ethanol concentration and adjusting extraction time.

Appendix A-SFE Process

SFC experiment steps:

1. Turn on the water bath, choose program#2, press ok. Wait for about 45 min until the temperature drop from room temperature (23 °C) to -3 °C. (figure A-1)
2. Weight certain amount of sample on the scale as need, mix with glass beads or matrix.
3. Remove one of the vessel end fittings, put some wool in the vessel and use tamping rod to push the wool to the bottom (figure A-2, 3, 4).
4. Pull sample mixture into the extraction vessel, tamp and pack the sample bed firmly
5. Add another wool layer on the sample bed. Fill the rest of space with glass beads or matrix.
6. Add wool layer at the top of glass beads or matrix, place the seal rings and tighten the end fitting.
7. Invert the vessel, secure inside the SFC oven, make sure that ALL the outlet, inlet and oven tubing should keep the same line (ie. #1 outlet match #1 inlet) (figure A-5).
8. Weight and record empty collection vessel, place the vessel under the micro metering valve, stab the two vent needles one connected to micro-metering vale, one connected to the flowmeter (both needle could take off) (figure A-9).
9. Turn on the power switch of oven and pump.
10. Adjust oven temperature, metering valve at 140C.
11. Open the inlet valve, house air valve, CO₂ cylinder valve in order (figure A-6,7,8).
12. Wait for 30 mins until the sample in the vessel reaches to the target temperature.
13. Set the pressure at 100 bar, and press the green button.
14. Slowly open the outlet valve, and adjust the metering valve, make the CO₂ flow rate at 5L/min (figure A-9).
15. Keep increasing pressure until reach target number, and CO₂ flow rate should be monitored at 5L/min.
16. Extracting for one hour.
17. After no lipids come out from metering valve, turn off the pump and close the inlet valve.
18. When all of the CO₂ is released from extraction vessel (watch the CO₂ flowmeter to 0L/min), turn off all the equipment, close CO₂ cylinder and house air.
19. Remove the sample collection vial.
20. Take off the vessel from oven, and inject 2-3ml of ethanol into the top tubing. The ethanol will wash the residual in the tubing and come out from metering valve. (adjust metering vale if no ethanol wash out)
21. Weigh and record the collection vessel, storage at -20 °C.
22. Save the spent sample (as needed) and clean the extraction vessel.
23. When extracting with neat CO₂ and ethanol, turn on the modifier pump, set the flow rate, (figure A-10).
24. Press 'RUN' while pump is on. (Make sure there are no bubbles in the tubing, if there are bubbles, attached syringe to the pump, pull the syringe until the bubbles are sucked out).
25. Repeat step#15 to 20.

26. Vapor all the ethanol in hood and record the sample weight.

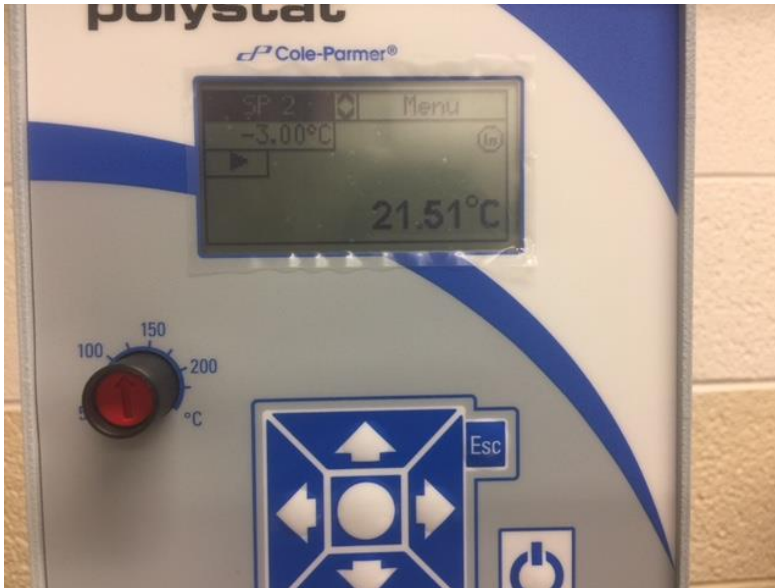


Figure A-1 Water bath setting



Figure A-2 Put tip on one side of vessel

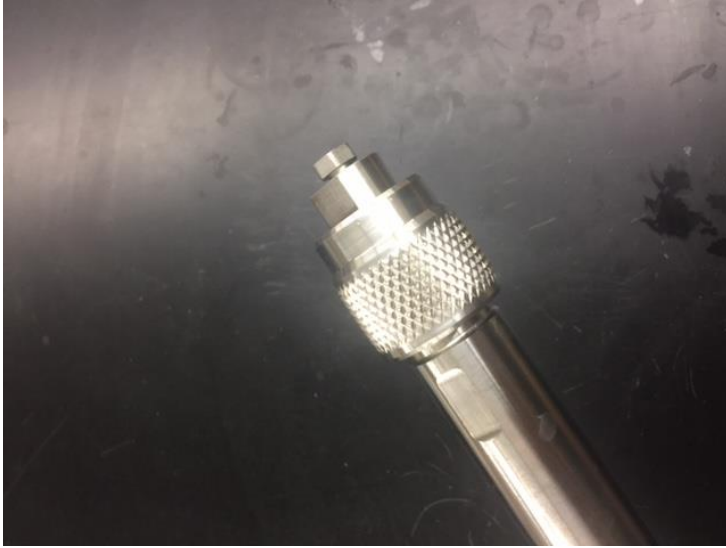


Figure A-3 Screw tight one side of fitting



Figure A-4 Tamping wool, sample, and glass beads or matrix in vessel with rod



Figure A-5 SFE oven



Figure A-6 CO₂ cylinder switch on

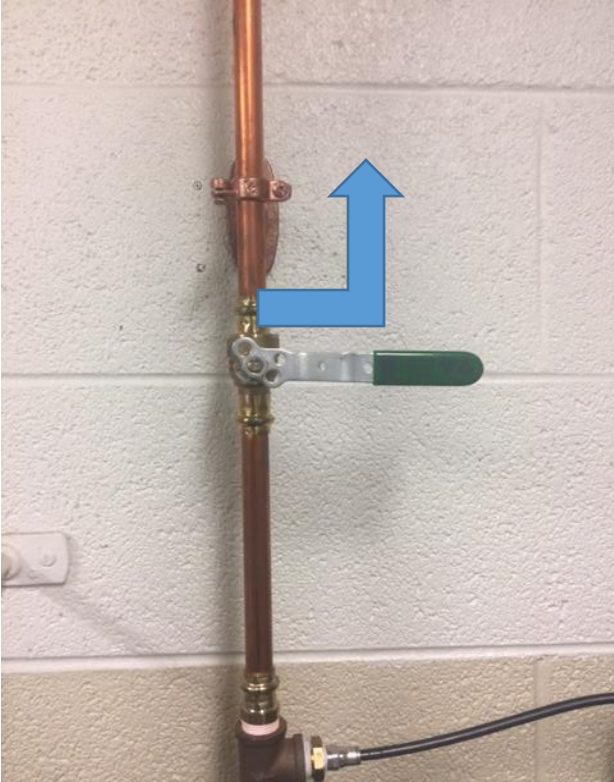


Figure A-7 Compressed air on



Figure A-8 The location of inlets switch

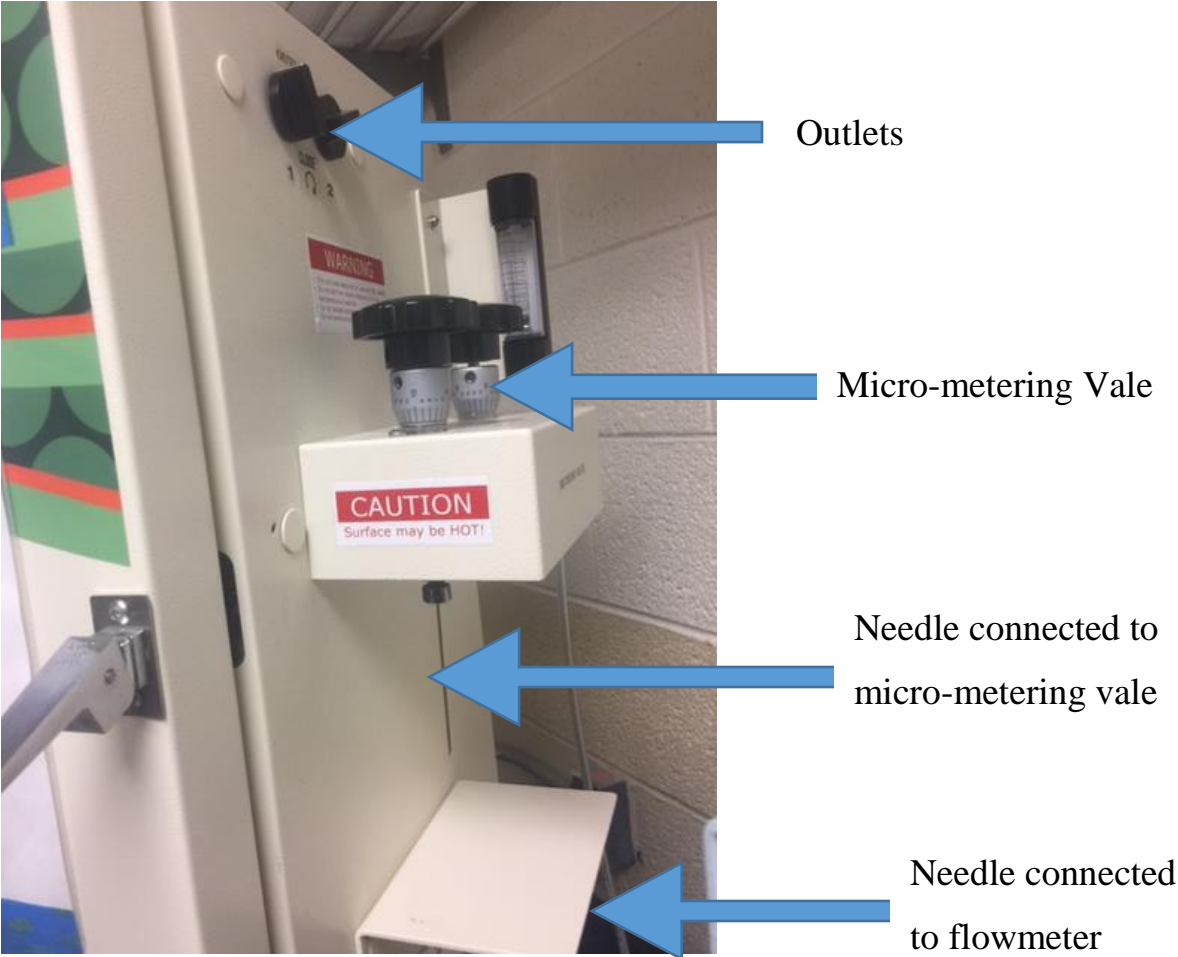


Figure A-9 The location of outlets, micro-metering vale and needles

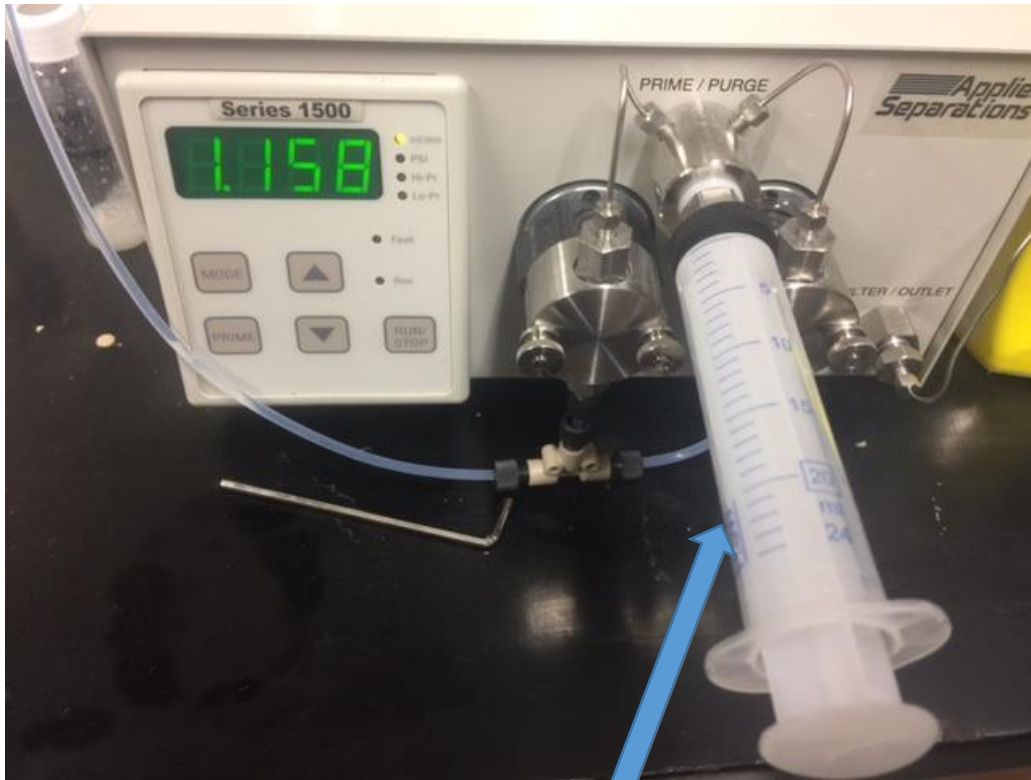


Figure A-10 Modifier pump system

Collaborate the pump
if any bubbles are in
the tubing

Appendix B-SAS Code

SAS code for WPPC analysis: row#1: 18 runs of each WPPC (total 3 lots of WPPC);
row#2-4: independent factors include three SFE parameters, pressure, temperature, and ethanol
concentration; row#5-9: dependent factors include total protein left in the exhausted WPPC
powders, individual PLs, and total PLs in SFE Step-2 extracts.

```
dm "output;clear;log;clear;";
options linesize=95 pagesize=65 nodate pageno=1;

data RCBD;
input Sample      PRESS      TEMP  ETOH  Totalprotein      SM PC PE
TotalPLs;
1      350      40      10      67.46 0.06218      0.0469      0.0180      0.1271
1      350      40      15      65.64 0.07413      0.0456      0.0314      0.1511
1      350      40      20      67.69 0.06228      0.0300      0.0206      0.1130
1      350      60      10      67.66 0.02794      0.0303      0.0089      0.0671
1      350      60      15      63.94 0.09302      0.0578      0.0500      0.2009
1      350      60      20      68.45 0.00918      0.0062      0.0052      0.2064
1      414      40      10      67.37 0.03477      0.0219      0.0074      0.0640
1      414      40      15      66.87 0.05993      0.0312      0.0144      0.1055
1      414      40      20      68.06 0.08124      0.0470      0.0335      0.1618
1      414      60      10      68.01 0.02550      0.0256      0.0083      0.0594
1      414      60      15      67.38 0.04816      0.0338      0.0157      0.0976
1      414      60      20      67.79 0.07822      0.0525      0.0423      0.1731
1      550      40      10      66.05 0.06239      0.0428      0.0153      0.1205
1      550      40      15      66.65 0.06230      0.0388      0.0191      0.1201
1      550      40      20      68.87 0.09064      0.0609      0.0392      0.1907
1      550      60      10      68.26 0.04053      0.0358      0.0132      0.0896
1      550      60      15      68.11 0.08337      0.0638      0.0387      0.1858
1      550      60      20      68.69 0.05027      0.0445      0.0333      0.1281
2      350      40      10      72.21 0.09996      0.1502      0.0895      0.3397
2      350      40      15      72.98 0.10167      0.1505      0.0988      0.3510
2      350      40      20      73.21 0.16136      0.1685      0.0690      0.3989
2      350      60      10      72.32 0.08598      0.1328      0.0640      0.2829
2      350      60      15      73.07 0.08310      0.1202      0.0627      0.2660
2      350      60      20      72.70 0.12990      0.1277      0.0928      0.3503
2      414      40      10      71.97 0.08215      0.1502      0.0529      0.2853
2      414      40      15      71.59 0.10444      0.1658      0.1238      0.3941
2      414      40      20      72.53 0.14033      0.1510      0.0631      0.3544
2      414      60      10      72.47 0.08195      0.1511      0.0623      0.2953
2      414      60      15      72.96 0.10366      0.1538      0.0978      0.3553
2      414      60      20      73.35 0.14866      0.1340      0.0775      0.3601
2      550      40      10      72.34 0.08183      0.1650      0.0522      0.2991
2      550      40      15      73.99 0.12421      0.1812      0.1277      0.4332
2      550      40      20      71.79 0.15088      0.1678      0.0913      0.4100
2      550      60      10      72.65 0.07651      0.1212      0.0293      0.2270
2      550      60      15      73.44 0.08963      0.1556      0.0716      0.3168
2      550      60      20      72.34 0.17205      0.1447      0.0984      0.4151
3      350      40      10      71.71 0.07303      0.0681      0.0235      0.1646
3      350      40      15      72.87 0.09454      0.0741      0.0624      0.2310
```

| | | | | | | | | |
|---|-----|----|----|-------|---------|--------|--------|--------|
| 3 | 350 | 40 | 20 | 70.55 | 0.09751 | 0.0764 | 0.0309 | 0.2048 |
| 3 | 350 | 60 | 10 | 72.22 | 0.05485 | 0.0437 | 0.0165 | 0.1151 |
| 3 | 350 | 60 | 15 | 71.58 | 0.08864 | 0.0741 | 0.0579 | 0.2206 |
| 3 | 350 | 60 | 20 | 73.30 | 0.10322 | 0.0861 | 0.0417 | 0.2311 |
| 3 | 414 | 40 | 10 | 70.93 | 0.07498 | 0.0725 | 0.0043 | 0.1518 |
| 3 | 414 | 40 | 15 | 69.84 | 0.09129 | 0.0821 | 0.0578 | 0.2313 |
| 3 | 414 | 40 | 20 | 73.31 | 0.08462 | 0.0584 | 0.0224 | 0.1654 |
| 3 | 414 | 60 | 10 | 72.17 | 0.07777 | 0.0708 | 0.0326 | 0.1811 |
| 3 | 414 | 60 | 15 | 72.71 | 0.09162 | 0.0843 | 0.0647 | 0.2406 |
| 3 | 414 | 60 | 20 | 74.10 | 0.10755 | 0.0812 | 0.0537 | 0.2424 |
| 3 | 550 | 40 | 10 | 72.43 | 0.07774 | 0.0777 | 0.0453 | 0.2007 |
| 3 | 550 | 40 | 15 | 71.01 | 0.09662 | 0.0823 | 0.0692 | 0.2482 |
| 3 | 550 | 40 | 20 | 72.51 | 0.09056 | 0.0501 | 0.0163 | 0.1569 |
| 3 | 550 | 60 | 10 | 72.52 | 0.06480 | 0.0616 | 0.0257 | 0.1520 |
| 3 | 550 | 60 | 15 | 72.73 | 0.11712 | 0.0808 | 0.0533 | 0.2513 |
| 3 | 550 | 60 | 20 | 72.30 | 0.09220 | 0.0702 | 0.0407 | 0.2032 |

```

;
title "total protein";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model Totalprotein = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans temp/pdiff;
lsmeans etoh/pdiff adjust=tukey;
run;

title "SM";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model SM = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans etoh/pdiff adjust=tukey;
run;

title "PC";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model PC = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
run;

title "PE";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model PE = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans etoh/pdiff adjust=tukey;
run;

title "TotalPLs";
*** TotalPLs showed to be right-skewed;
proc univariate data=RCBD;
histogram TotalPLs;
run;
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model TotalPLs = PRESS|TEMP|ETOH/dist=gamma;

```



```
random sample; *** random blocking factor;  
lsmeans temp/pdiff ilink;  
run;
```

SAS code for BMP analysis: row#1: 18 runs of each BMP (total 3 lots of BMP); row#2-

4: independent factors include three SFE parameters, pressure, temperature, and ethanol

concentration; row#5-9: dependent factors include total protein left in the exhausted BMP

powders, individual PLs, and total PLs in SFE Step-2 extracts.

```
dm "output;clear;log;clear;";
options linesize=95 pagesize=65 nodate pageno=1;

data RCBD;
input Sample      PRESS      TEMP  ETOH  Totalprotein      SM PC PE
TotalPLs;
cards;
1      350      40      10      67.46 0.00145      0.0150      0.0206      0.0370
1      350      40      15      65.64 0.01934      0.0320      0.0364      0.0878
1      350      40      20      67.69 0.01145      0.0225      0.0242      0.0582
1      350      60      10      67.66 0.03141      0.0431      0.0456      0.1201
1      350      60      15      63.94 0.02710      0.0338      0.0216      0.0825
1      350      60      20      68.45 0.02498      0.0330      0.0243      0.0822
1      414      40      10      67.37 0.01144      0.0225      0.0231      0.0571
1      414      40      15      66.87 0.02763      0.0374      0.0259      0.0909
1      414      40      20      68.06 0.03732      0.0467      0.0277      0.1117
1      414      60      10      68.01 0.01505      0.0336      0.0330      0.0817
1      414      60      15      67.38 0.11312      0.1066      0.0231      0.2429
1      414      60      20      67.79 0.01454      0.0271      0.0263      0.0680
1      550      40      10      66.05 0.01967      0.0329      0.0262      0.0787
1      550      40      15      66.65 0.03248      0.0363      0.0279      0.0967
1      550      40      20      68.87 0.03053      0.0395      0.0293      0.0993
1      550      60      10      68.26 0.02280      0.0358      0.0351      0.0937
1      550      60      15      68.11 0.08690      0.0846      0.0265      0.1980
1      550      60      20      68.69 0.02852      0.0374      0.0265      0.0925
2      350      40      10      72.21 0.04147      0.0421      0.0258      0.1093
2      350      40      15      72.98 0.02928      0.0415      0.0286      0.0994
2      350      40      20      73.21 0.01079      0.0195      0.0236      0.0539
2      350      60      10      72.32 0.03995      0.0539      0.0464      0.1403
2      350      60      15      73.07 0.05824      0.0685      0.0223      0.1490
2      350      60      20      72.70 0.04150      0.0467      0.0231      0.1112
2      414      40      10      71.97 0.02487      0.0316      0.0245      0.0810
2      414      40      15      71.59 0.03610      0.0472      0.0309      0.1142
2      414      40      20      72.53 0.02819      0.0357      0.0226      0.0864
2      414      60      10      72.47 0.02457      0.0406      0.0321      0.0973
2      414      60      15      72.96 0.03477      0.0439      0.0307      0.1094
2      414      60      20      73.35 0.02469      0.0313      0.0246      0.0805
2      550      40      10      72.34 0.00312      0.0262      0.0230      0.0524
2      550      40      15      73.99 0.02640      0.0347      0.0232      0.0843
2      550      40      20      71.79 0.03053      0.0395      0.0300      0.1001
2      550      60      10      72.65 0.02589      0.0437      0.0315      0.1011
2      550      60      15      73.44 0.07465      0.0712      0.0275      0.1734
2      550      60      20      72.34 0.05738      0.0701      0.0245      0.1521
3      350      40      10      71.71 0.03109      0.0453      0.0284      0.1048
3      350      40      15      72.87 0.02184      0.0325      0.0277      0.0820
3      350      40      20      70.55 0.01732      0.0264      0.0226      0.0663
3      350      60      10      72.22 0.01009      0.0254      0.0221      0.0575
```

| | | | | | | | | |
|---|-----|----|----|-------|---------|--------|--------|--------|
| 3 | 350 | 60 | 15 | 71.58 | 0.01253 | 0.0273 | 0.0206 | 0.0604 |
| 3 | 350 | 60 | 20 | 73.30 | 0.03408 | 0.0437 | 0.0228 | 0.1006 |
| 3 | 414 | 40 | 10 | 70.93 | 0.00858 | 0.0262 | 0.0273 | 0.0621 |
| 3 | 414 | 40 | 15 | 69.84 | 0.02088 | 0.0334 | 0.0238 | 0.0781 |
| 3 | 414 | 40 | 20 | 73.31 | 0.03630 | 0.0446 | 0.0265 | 0.1074 |
| 3 | 414 | 60 | 10 | 72.17 | 0.01390 | 0.0344 | 0.0289 | 0.0772 |
| 3 | 414 | 60 | 15 | 72.71 | 0.02064 | 0.0358 | 0.0316 | 0.0881 |
| 3 | 414 | 60 | 20 | 74.10 | 0.05716 | 0.0643 | 0.0222 | 0.1437 |
| 3 | 550 | 40 | 10 | 72.43 | 0.03390 | 0.0487 | 0.0427 | 0.1253 |
| 3 | 550 | 40 | 15 | 71.01 | 0.04009 | 0.0527 | 0.0335 | 0.1263 |
| 3 | 550 | 40 | 20 | 72.51 | 0.03446 | 0.0437 | 0.0242 | 0.1024 |
| 3 | 550 | 60 | 10 | 72.52 | 0.01747 | 0.0346 | 0.0358 | 0.0879 |
| 3 | 550 | 60 | 15 | 72.73 | 0.03817 | 0.0490 | 0.0478 | 0.1350 |
| 3 | 550 | 60 | 20 | 72.30 | 0.06272 | 0.0635 | 0.0472 | 0.1733 |

```

;

title "total protein";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model Totalprotein = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans temp/pdiff;
lsmeans etoh/pdiff adjust=tukey;
run;

title "SM";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model SM = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans etoh/pdiff adjust=tukey;
run;

title "PC";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model PC = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
run;

title "PE";
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model PE = PRESS|TEMP|ETOH;
random sample; *** random blocking factor;
lsmeans etoh/pdiff adjust=tukey;
run;

title "TotalPLs";
*** TotalPLs showed to be right-skewed;
proc univariate data=RCBD;
histogram TotalPLs;
run;
proc glimmix data=RCBD plots=studentpanel;
class sample PRESS      TEMP  ETOH;
model TotalPLs = PRESS|TEMP|ETOH/dist=gamma;
random sample; *** random blocking factor;

```

```
lsmeans temp/pdiff ilink;  
run;
```