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The Effects of Milk Phospholipids on Functional Properties of Anhydrous Milk Fat and Oil Structured Gel Systems

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THE EFFECTS OF MILK PHOSPHOLIPIDS ON FUNCTIONAL PROPERTIES OF ANHYDROUS MILK FAT AND OIL STRUCTURED GEL SYSTEMS

by

Zachary Cooper

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ABSTRACT

The Effects of Milk Phospholipids on Functional Properties of Anhydrous Milk Fat and Oil Structured Gel Systems

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Zachary Cooper, Master of Science

Utah State University, 2020

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Phospholipids (PL) were isolated from phospholipid concentrate 700 (PC700, Fonterra) using a water and ethanol extraction. Water was first used at a water:PC700 ratio of 3:1 to suspend the phospholipids (PL) in the form of an emulsion and then ethanol was used at an emulsion:ethanol ratio of 1:2 to "precipitate" out the PL. After this step the PL were filtered and purified with hexane and centrifugation to eliminate minerals and obtain a phospholipid isolate (PLI). The PLI was analyzed using thin layer chromatography (TLC) to identify lipid classes contained in PLI in which pixel software and P^{31} NMR was used to identify and quantify the various PL species in the PLI. No significant differences were observed between the methods used for the PL identification ($P<0.05$). The effects of the PLI on crystallization behavior of anhydrous milk fat (AMF) at concentrations of 0%, 0.01%, and 0.1% PL and crystallized at 24, 26, and 28 °C was evaluated. The Avrami equation was used to fit to the solid fat content (SFC) which was measured as a function of time, and the crystal morphology, melting point, viscoelastic

properties, and thermal behavior were measured at 90 min. All measurements were repeated along with hardness after being stored at 48 h at 5 °C. The addition of PL changed crystal morphology, resulting in larger crystal formation at 90 min and in a delay in crystallization at high temperatures (26 and 28 °C) when compared to the AMF samples. The elastic modulus showed a decrease at 90 min in the 0.01% PL sample at 24 and 26 °C, with the 0.1% PL being more than 0.01% PL and less than AMF. Samples containing 0.01% PL were harder when crystallized at 26 °C sample but softer at 24 °C compared to the AMF samples. The melting enthalpy decreased while PL concentration increased suggesting a decrease in crystallization by PL which was supported by the crystal morphology.

PLI was also used to formulate gels at concentration of 15, 30, and 45% PL and the melting behavior, morphology, solid fat content, visual stability, oil binding capacity and viscosity were evaluated. Visual stability was observed in samples with 45% PL. Samples showed an increase in solid fat content, enthalpy, and oil binding capacity with the increase in PLI. The viscosity at low and intermediate shear levels (0.01% and 0.1% shear) was significantly less than the one obtained for the 45% PL, the 30% PL, and 15% PL, but at high shear rate (1.0%) a significant difference was seen between the 15% PL sample and the other samples, 30% PL and 45% PL. Overall, isolating PL from dairy waste streams may be an effective use for a new ingredient which can be used to tailor the crystallization and structuring of fat and oil products.

(150 pages)

PUBLIC ABSTRACT

The Effects of Milk Phospholipids on Functional Properties of Anhydrous Milk Fat and Oil Structured Gel Systems

Zachary Cooper

Lecithin is an ingredient commonly used in foods composed of high concentration of phospholipids. Phospholipids are a type of fat that can mix with both water and oil. In the current food industry, most lecithins come from sunflower, egg, and soybean sources. An additional source of phospholipids can be the waste streams from the dairy industry, such as the whey protein and butter making processes, since these contain a high concentration of phospholipids. Dairy phospholipids have been isolated in the past, but their traits as a functional ingredient are still unknown. In addition, most isolated dairy phospholipids still contain protein, lactose, minerals, or other types of non-fat soluble molecules. In the food, cosmetic, and pharmaceutical industry the crystallization of fats and functionality of phospholipids is very important. Crystallization gives structure to food, cosmetic products, and some pharmaceutical products as well. The ways in which the molecules align and organize themselves into structures is referred to as crystallization. In this thesis it will be discussed how to isolate dairy phospholipids from a commercially available phospholipid concentrate into an oil soluble isolate and how the phospholipids influence the crystallization of anhydrous milk fat (concentrated butter containing less than 0.01% water) and the structuring of soybean oil. For the anhydrous milk fat, low concentrations of phospholipid was added (0%, 0.01%, and 0.1%) and crystallized at 24, 26, and 28 °C in anhydrous milk fat where the amount of solids (solid fat content), type of crystals (polymorphism), viscoelastic properties (the way crystals react under shear stress),

hardness, melting behavior (the way in which the samples melt as temperature increases), and crystal shapes (crystal morphology) were measured. Results showed that PL can be used to tailor the crystals in anhydrous milk fat. In addition, three different mixtures were made using soybean oil and various concentration of PL (15%, 30%, and 45%). The amount of solids, oil binding capacity (ability to entrap liquid oil), viscosity (ability for samples to flow), melting behavior (the way in which the samples melt as temperature increases), and crystal shapes (crystal morphology) were measured. It was observed that PL could be used to form a semi-solid structure and tailor the crystal formations that are formed due to the fatty acid composition of the lipid components of the PLI.

ACKNOWLEDGMENTS

I would like to acknowledge Silvana Martini as a personal mentor in this research, my committee Robert Ward and Luis Bastarrachea for their time and suggestions in improving this research, Fonterra for the donation of PC700, funding from BUILD Dairy Program, the Utah Agricultural Experiment Station at Utah State University, The NSF MRI-1429195 in funding the Bruker 500 MHz NMR, Casey Simons in aiding in the P^{31} NMR analysis, and the Ward lab for guidance on thin layer chromatography and gas chromatography.

DEDICATION

I dedicate this work to my mother Dana Cooper, father Randall Cooper, siblings, friends, and mentors. These are the people who have supported me throughout my life and made me who I am.

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SYMBOLS, NOTATIONS, & DEFINITIONS

2LPC	Lysophosphatidylcholine
2LPE	Lysophophatidylethanolamine
^{31}PNMR	Nuclear Magnetic Resonance ^{31}P
AMF	Anhydrous Milk Fat
CE	Cholesterol Esters
DAG	Diacylglycerols
DSC	Differential Scanning Calorimeter
EtOH	Ethanol
FA	Fatty Acid
FFA	Free Fatty Acids
GC	Gas Chromatography
KF	Karl Fischer
Lac-PE	Lactosylatedphosphtidylethanolamine
MAG	Monoacylglycerols
NMR	Nuclear Magnetic Resonance
PA	Phosphatidic Acid
PC	Phosphatidylcholine

PC700	Phospholipid Concentrate 700
PE	Phosphatidylethanolamine
PHO	Partial Hydrogenation of Oil
PI	Phosphatidylinositol
PL	Phospholipids
PLA	Phospholipid Agglomerate
PLI	PL Isolate
PLM	Polarized Light Microscopy
PS	Phosphatidylserine
SAS	Statistical Analytical Software
SFC	Solid Fat Content
SM	Sphingomyelin
TAG	Triacylglycerols
T_c	Crystallization Temperature
TLC	Thin Layer Chromatography
T_{on}	Onset Temperature
T_p	Peak Temperature
XRD	X-Ray Diffraction

ΔH	Enthalpy

CHAPTER I
INTRODUCTION

Lecithin is a common ingredient in the food industry. In the U.S. Code of Federal Regulation Title 1 Volume 3 lecithin is defined as "a naturally occurring mixture of the phosphatides of choline, ethanolamine, and inositol, with smaller amounts of other lipids." Another definition is given to lecithin by the International Lecithin and Phospholipid Society (Watkins et al., 1990) that "lecithin is a mixture of glycerophospholipids obtained from an animal, vegetable, or microbial sources, containing a variety of substances, such as sphingosylphospholipids, triglycerides, fatty acids, and glycolipids."

Lecithin can be found in various sources such as plants including soybean, corn, sunflower seed, rapeseed, and peanuts. Animal lecithins are commonly obtained from eggs and bovine brain. Commercially the common lecithin sources are soybean or eggs and are commonly used as wetting or emulsifying agents. In the food industry lecithin can be found in foods, beverages, animal feed and dietary products. Usually lecithin is added to items such as shortening, margarines, baked goods, chocolate, compound confectionary coating, peanut butter, powder mixes and dietary foods. Besides being used as an emulsifying agent lecithin is also used to promote and control stabilization, antioxidation, crystallization, and spattering (Wendel, 2014).

Bovine milk contains a low percentage (0.23-0.32% d.b.) (Rombaut et al., 2006a) of phospholipids (PL), but some by-products from milk processing have a higher

concentration of PL (11.54% in butter serum) (Rombaut et al., 2006b). Butter serum, a low profit by-product from butter and butter oil manufacturing, and the isolated fat portion from the manufacturing of whey protein isolate are dairy sources containing the highest concentration of PL(Ahmad & Xu, 2015). Some of these by-products such as buttermilk can be turned into valued products such as powdered butter milk, but for other by-products industry either tries to rework them into other dairy products, discard them, or use them as low profit products such as animal feed.

Several ingredients from dairy sources that are high in PL are currently available in the market. Phospholipid concentrate 700 (PC700) is a complex milk lipid ingredient from Fonterra and is an example of such product containing 85.0% total lipids with 59.2% of phospholipid. According to the specification sheet of this product the phospholipid fraction of PC700 is composed of 3.0% phosphatidylserine (PS), 31.0% phosphatidylcholine (PC), 8.7% phosphatidylethanolamine (PE), and 16.5% sphingomyelin (SM). Other components found in PC700 include 2.5% moisture, 6.6% lactose and a maximum content of 12.0% ash. Another ingredient from Fonterra is Phospholac 600, which contains more than 70% phospholipids. Arla Foods Ingredients also produces polar lipid milk products known as Lacprodan-20, which contains 20% phospholipids, and Lacprodan-75, which contains 75% phospholipids.

Milk polar lipids have a different composition than other sources of polar lipids that are currently used in the industry. Literature shows that the polar lipid composition in soy lecithin is 32 % PC, 23 % PE, 21 % phosphatidylinositol (Pl) and 9 % phosphatidic acid (PA). In sunflower lecithin the polar lipid composition is 34% PC, 17% PE, 30% Pl, and 6% PA (van Nieuwenhuyzen, 2008). In literature SM is not reported in soy or

sunflower lecithins due to the low concentration and it is usually only found in animal-based lecithins. It is also important to note that PS is also found in low concentrations (1-2%) and is often times not reported in vegetable-based lecithins (Wendel, 2014).

Animal lecithins and polar lipids tend to have a higher variance in terms of PL composition compared to vegetable lecithins because of the many variables that affect their composition such as breed or individual (Graves et al., 2007), stage of lactation (Tsioulpas et al., 2007), and feeding (Chilliard et al., 2001). In eggs polar lipid composition is 78.9% PC, 16.6% PE, 0% PI, 0.3% PA, 1.7% SM, and 0% PS (Sotirhos et al., 1986). In milk the observed composition is 26.8% PC, 25.8% PE, 14% PI, 0.5% PA, 26.8% SM, and 1.5% PS (Murgia et al., 2003.)

Obtaining a PL isolate (PLI) that is soluble in oil would be important to understand the effects that milk PL would have on a food system. Due to the polar and non-polar portions of PL, it can be difficult to isolate PL. Dairy streams that have been utilized to isolate PL have been butter milk powder (Barry et al., 2016), whey protein PL concentrate (Price et al., 2018), and whey buttermilk (Costa et al., 2010), which is a by-product of making butter with whey protein PL concentrate (Sodini et al., 2006). The methods that have been used to isolate or concentrate dairy PL include hydrolysis of buttermilk powder protein and passing it through an ultrafiltration system and introducing a super critical fluid treatment to the fat concentrated retentate (Barry et al., 2016) (Costa et al., 2010), a hydrolyzing process and then a ultrafiltration and diafiltration process to obtain a PL retentate (Konrad et al., 2013), an ethanol extraction method (Price et al., 2018), fractionation using heptane and ethanol (Boyd et al., 1999), and a microfiltration process paired with supercritical fluid extraction (Spence et al., 2009). Although these

methods were successful at producing a PL concentrate they still had some minor components such as ash or proteins.

There are three reasons why there is interest in using bovine milk PL as a food ingredient. One, to increase value of waste or by-products obtained from the dairy industry as described earlier. Second, to eliminate soy or egg allergen claims from foods that already contain dairy. Third, milk-based PL have nutritional value including benefits in brain development, cell membrane repair, as well as aiding in cell messaging, signaling, and protein synthesis in humans (Blusztajn, 1998).

PL are soluble in organic solvents and the incorporation of PL into food products would involve the dissolution of these materials into the fat phase. Lipid present in foods may be in the liquid or semi-solid form. When in the semi-solid state lipid crystallization becomes an important phenomenon in the food industry and is dependent on the composition of the fat and processing conditions (Foubert et al., 2002). Different conditions, such as introducing lecithin or PL to a fat, can alter crystallization characteristics. Past literature on this topic shows varying results. In some instances the addition of PL may not affect the nucleation of the fat even as concentrations of PL are increased (Lončarević et al., 2013) while in other studies PL may cause a delay in crystallization by acting as a retardant with an increasing effect as PL concentration increases (Vanhoutte et al., 2002). Moreover, some research shows evidence that specific PL, such as PC, PI, PE, and PA, can induce crystallization serving as nuclei prior to triacylglycerol (TAG) nucleation (Jeffery, 1991). The effects of milk PL on a fat system have currently not been identified and due to the difference between milk PL composition and other lecithins on the market it makes it difficult to anticipate any specific results.

In the food industry, structured fats are a needed commodity since the ban on partially hydrogenated oils that occurred in the US in 2015. Some structured fats that are being studied are oleogels. Oleogels use liquid oil as a fluid phase consisting of 90% or more by weight and an oil gelator that creates a 3-D structure that has gel like properties and encloses pockets of liquid oil (Sato, 2018). In the early development of oleogels the definition was less clear and focused more on the ability that a compound had to form a gel like structure in an organic solvent. Due to the clarity in definition over the years the gels that do not qualify as an oleogel, due to the oleogelator concentration exceeding 10%, will be referred to as structured lipids or phospholipid gels.

Common oleogelators can be identified within one of the following categories: (a) anisotropic crystalline particles, (b) self-assembled structures such as micelles, (c) agglomerates of non-crystalline particles, or (d) polymer-based networks (Sato, 2018). Oleogels have been created in the presence of lecithin when mixed with other compounds such as soribitan tristearate (Pernetti et al., 2007), alpha tocopherol (Nikiforidis & Scholten, 2014), β-sitosterol and γ-oryzanol (Okuro et al., 2018), sucrose esters (Sintang et al., 2017), or water to create a structured gel (Scartazzini & Luis, 1988). Lecithin based structures have been identified to form anisotropic crystalline particles and self-assembling structures, dependent on the system. Some results show that the lecithin can hinder the strength of the gel system (Okuro et al., 2018) while other studies show that when lecithin oleogelators are made, using ratios of a compound and lecithin, low concentrations such as 8:2, 7:3, and 6:4 can have a synergistic effect to support a structured gel system which neither of the compounds have the ability to do on their own (Sintang et al., 2017). The structured gel of interest will be using water and PL. The

formation of a structured gel with lecithin is through the formation of cylindrical tubular micelles that form upon the addition of water (Kumar & Katare, 2004). Although the water is contained within the tubular micelles it can still flow freely (Capitani et al., 1996). No study has been performed using milk PL to structure lipids. Although there is a high potential for a structured fat to be used in the food system, limited edible applications have been discovered due to processing or USDA GRAS status (Okuro et al., 2018).

Hypothesis

Milk polar lipids can be isolated from a commercial source and can be used to tailor the crystallization characteristics of lipids and to produce a structured lipid.

Objective 1

Isolate an oil soluble PLI from a milk-based PL concentrate available from the industry.

Objective 2

Identify the effects that milk PLI has on the crystallization behavior of anhydrous milk fat.

Objective 3

Produce a structured lipid using a milk-based PLI, water, and oil.

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CHAPTER II

LITERATURE REVIEW

Phospholipids

PL are a category of lipids that have both a polar and nonpolar section in their molecule. They are made of glycerol backbones with two large nonpolar fatty acid (FA) chains and one polar group which includes the negatively charged phosphate group with either an ethanolamine, inositol, serine, choline, or just the phosphate in the form of an acid (Figure 2-1). The other PL found in milk, SM, has a slightly different structure (Figure 2-1). Rather than having a glycerol backbone, SM has a sphingosine backbone with a phosphate group attached, where other compounds such as choline can bond, and can also contain a FA attached to the amine group Figure 2-1).

Health Benefits of Phospholipids

PL are important for human health, in which specific PL species play different roles in the human body and cells (Blusztajn, 1998). Thus, different PL species play different roles in health functions. PC, SM, and PS have been associated with brain

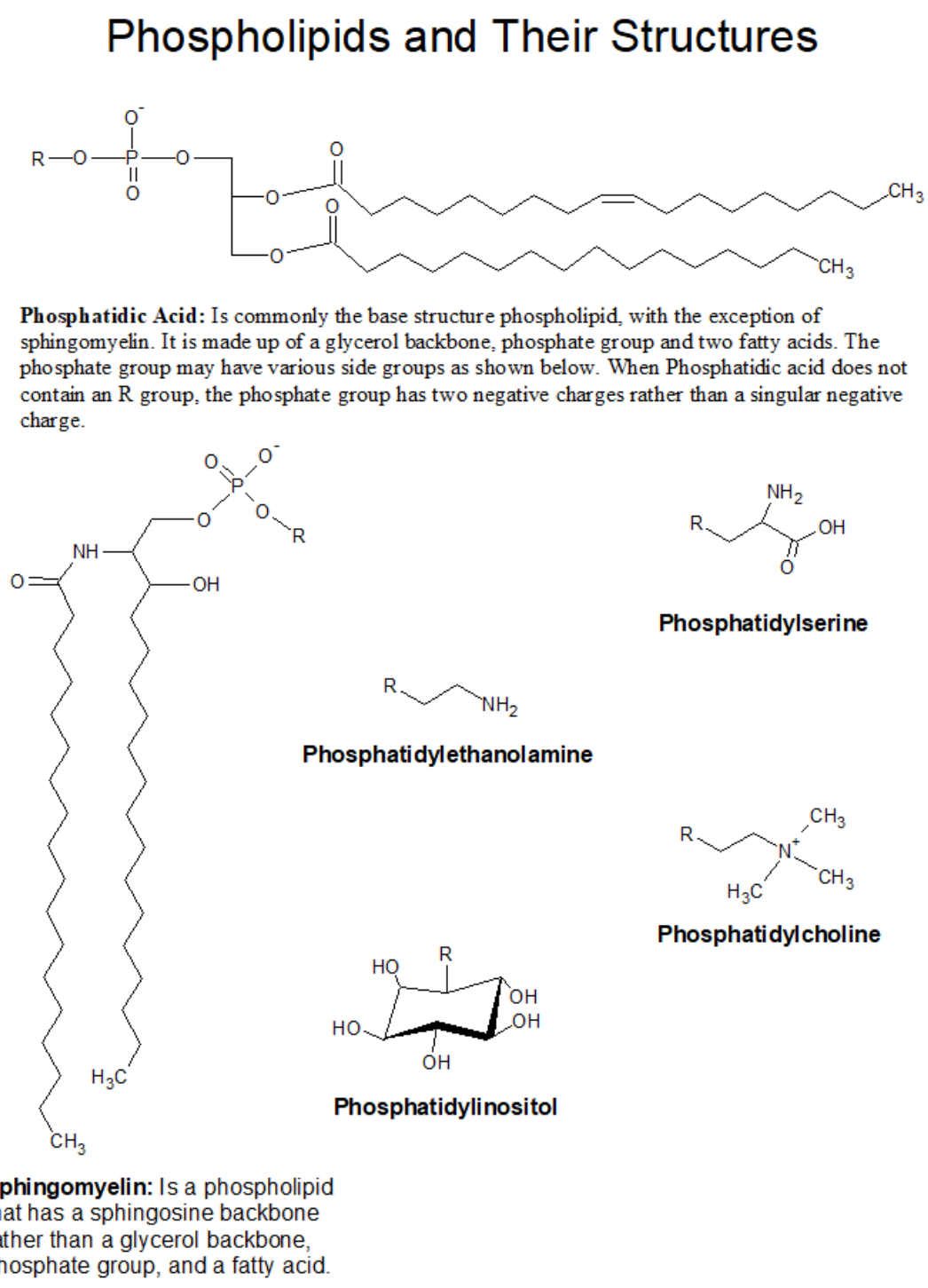

Figure 2-1: The phospholipid base and side groups are shown for each of the PL found in milk. SM contains the same choline R group as PC. Each of these species vary in functionality and polarity.

development, function, and prevention of cognitive and attention disorders in humans (Blusztajn, 1998; Pepeu et al., 1996; Kim et al., 2014; Hirayama et al., 2006; Vaisman et al., 2008; Kidd, 2000). PC and SM have shown to contribute to the functionality and repair of the nervous system (Jager et al., 2007; Oshida et al., 2003). PC and SM aid in liver health (Niederau et al, 1998; Duivenvoorden et al., 2006; Watanabe et al., 2011), as well as aiding in the reduction of toxicity and inflammatory stress on the colon (Ehehalt et al., 2010; Lichtenberger et al., 2009; Stremmel et al., 2010; Hartman et al., 2009; Tökés et al., 2011; Treede et al., 2009; Treede et al., 2007). SM reduces cholesterol absorption (Eckhardt et al., 2002; Nyberg et al., 2000) in which a dairy SM is more effective than SM obtained from egg (Noh & Koo, 2004) and can help prevent colon cancer (Berra et al., 2002; Dillehay et al., 1994; Schmelz et al., 1996; Snow et al., 2010). SM also serves as a bactericidal agent against *Listeria monocytogenes* and *Campylobacter jejuni* (Sprong et al., 2002). PS helps to control membrane fluidity and regulates membrane activities (Jager et al. 2007) specifically in the brain, lungs, heart, liver, and skeletal muscle (Pepeu et al., 1996) and can aid in the recovery of exercise induced stress (Starks et al., 2008; Jager et al., 2007) as well as serving as an endogenous regulator for immune systems (Carr et al., 1992; Gaitonde et al., 2011; Ponzin et al., 1989; Yamazaki et al., 1997).

Unlike the other PL that show unique nutritional benefits PI and PE show benefits through their unsaturated fatty acid tails (Fong et al., 2007; Lopez et al., 2008). PI contain at least 37.9-44.6% unsaturated fatty acids while PE contains at least 57.7-76.1% unsaturated fatty acids (Fong et al., 2007). Polyunsaturated fatty acids, which can be found in PI and PE, are prevalent in brain cells and may reduce risk of certain diseases

such as colon, breast or prostate cancer and inflammatory conditions such as asthma or Crohn's. Polyunsaturated fatty acids have been associated with health benefits related to cardiovascular disease, diabetes, cancer, depression, mental illnesses, cognitive decline, periodontal disease, and rheumatoid arthritis although there are some controversies on the effects of ω-3 polyunsaturated acids and the health effects they have on strokes, diabetes, cancer, visual development, and neurological development (Shahidi & Ambigaipalan, 2018).

Functionality of Phospholipids

PL play many functions in food systems due to the unique structures they can create. Hartel (2001) describes the four different structures (Figure 2-2) that PL can form in a system. At low concentrations a micellar structure forms, where PL create small micelles with two FA hydrophobic tails on the inside with phosphate groups facing the solvent. With a slight increase in concentration hexagonal formations occur where the PL align themselves similar to the alignment found in micelles, but rather than creating small micelles long tubular strands are formed. As PL concentration increases a lamellar formation occurs where layered sheets of PL form bilayers with the hydrophobic tails on the inside and the hydrophilic phosphate groups on the outside of the bilayer interacting with the solvent. As PL concentration increases to very high concentrations a reverse hexagonal structure occurs where tubular structures are formed similar to the ones found in the hexagonal formation where the solvent and phosphate groups that are towards the inside of the tubular strands.

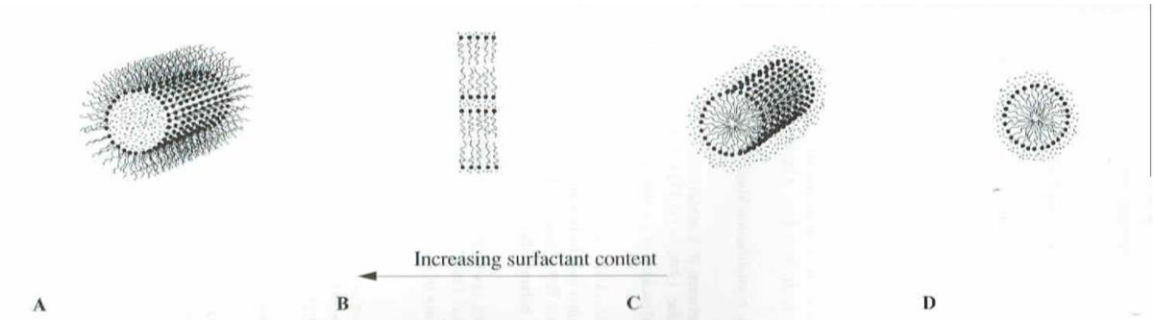

Figure 2-2: Different structures of PL in solution. Reverse hexagonal structure where fatty acid tails are on the exterior of the structure (A), lamellar where the phospholipids create layers with polar heads interacting with one another while non-polar tails interact with one another (B), hexagonal where the polar heads are on the exterior structure (C), and micellar where the PL forms a spherical globe with the polar head groups on the exterior (D) (HARTEL, R.W. CRYSTALLIZATION IN FOODS, published 2001 Springer reproduced with permission of SNCSC).

PL in general are used on an industrial level for their ingredient functionality as well as processing aids. Some of the current processing aids that PL can assist in are the elongation of natural hog casings to improve sausage production (Santos et al., 2008) and the decrease in apparent viscosity of fluid chocolate to improve the transfer of chocolate through equipment and piping (Afoakwa et al., 2009). The ingredient functionality of PL will be discussed using current research performed using dairy-based PL specifically. Dairy PL assist in the formation of emulsions (Zhu & Damodaran, 2013) and encapsulation of nutrients through liposomes (Thompson & Singh, 2006). PL can assist in forming oil-in-water, water-in-oil emulsions, and foams. However, PL can destabilize foams when mixed with milk proteins (Wilde et al., 2004; Dickenson, 2010). PC was the

determining factor in emulsion formation when creating emulsions with PL (Miura et al., 2004; Miura et al., 2006). Miura et al. (2006) showed a more homogeneous particle size in emulsions produced by dairy PL rather than soy PL. Zhu and Damodaran (2013) showed the importance of concentration of dairy PL with results suggesting that an emulsion with less than 2% dairy PL was unstable while an emulsion with 4% or more was stable for more than 60 days at room temperature. It has also been shown that some isolated PL-based ingredients such as Lactoprrodan-20 and Lacprodan-75 (Arla Foods Ingredients Amba, Denmark), have better emulsifying properties at a lower PL concentration (20%) than a higher concentration (75%) (Horn et al., 2011). PL are also used to encapsulate compounds such as polyphenolic compounds (Gùlseren & Corredig, 2013), ascorbic acid (Farhang et al., 2012), and sodium chloride/sodium azide solution (Thompson & Singh, 2006). Liposomes made using milk PL rather than soy PL are smaller and are more efficient at encapsulating compounds. Milk PL liposomes also showed more stability in a variety of pH ranges, temperatures, and in the presence of cations (Thompson et al., 2006; Thompson et al., 2007; Gùlseren & Corredig, 2013). Encapsulation can aid in biological delivery of nutrients, drug target delivery, and to encapsulate volatile or unstable compounds such as aroma, flavor, and natural coloring compounds used in foods (Lu et al., 2011). One of the last functional traits of PL that will be discussed is the ability they have to crystallize and how they influence crystallization.

Crystallization

Crystallization is the process of the organization of molecules into a hardened state that restricts the movement of molecules. There are 4 steps to crystallization: 1) the

generation of a driving force through either supersaturation or supercooling, in fats supercooling is the difference between the melting point and the temperature in which a sample is introduced, 2) nucleation, which is the initiation of crystallization and can occur either heterogeneously, which is the most common type of nucleation, or homogenously, which is usually only observed in emulsions, 3) crystal growth, the point at which molecules begin to attach themselves to a nuclei and expand the size of the crystal, and 4) recrystallization, which is when a crystal undergoes a change in size, shape, number, orientation, or polymorphic form (Hartel, 2001). In fats, the driving force of crystallization is supercooling. Triacylglycerols (TAG) are common fats that have the ability to crystalize. TAG molecules can be broken down into two parts, the glycerol back bone and three FA (Figure 2-3). FA can be further broken down into two classes, saturated and unsaturated FA. The difference between the saturated and unsaturated FA is that saturated FA have carbon chain FA that are completely hydrogenated while unsaturated FA chains have one or more double bonds (Shier et al., 2007). Unsaturated FA can be broken down into further groups as *cis* or *trans* FA. In nature, *cis* unsaturations are more commonly observed. *Cis* FA have a double bond which contains the carbons of the aliphatic chain on the same side, while *trans* FA have them on opposite sides (Figure 3). The number of carbons and the presence and type of unsaturation affects the melting point of the FA with *cis* being the least likely to crystallize having a melting point lower than a saturated FA while a *trans* FA would have a melting point lower than a saturated FA but higher than an unsaturated fatty acid with the same number of carbons (Damodaran et al., 2008).

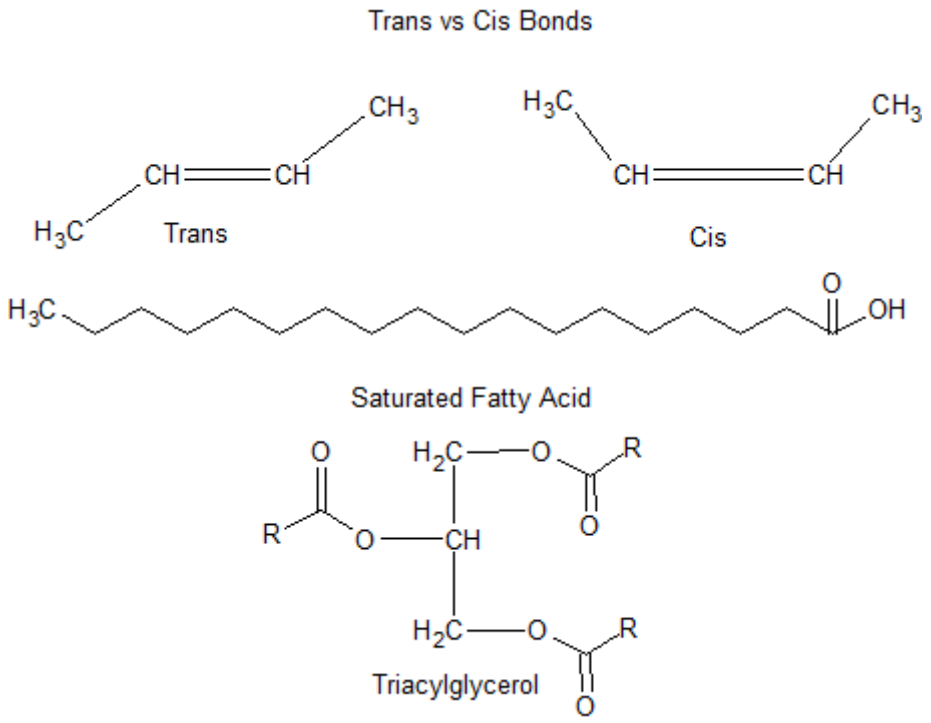

Figure 2-3: A display of a *trans* bond and a *cis* bond, a saturated fatty acid, and a triacylglycerol molecule. R=a hydrocarbon chain.

In the food industry there is a need for fats with stable crystalline structures to aid in shelf stability and consumer sensorial acceptance. The presence of stable crystalline structures is dependent on the crystallization of TAG and their FA composition. When crystallization occurs, different types of polymorphs can be obtained that influence the stability of fat crystals. The different polymorphs found in fats listed from least stable to most stable are ɤ, α, β', and β. The ɤ polymorphism is extremely unstable and is very difficult to obtain. The α polymorphism creates a hexagonal structure, while the β' polymorphism forms an orthorhombic perpendicular structure and β forms a triclinic parallel structure. In chair stacking confirmations α is only capable of double stacking,

while β’ and β are capable of double and triple stacking (Sato, 2001; Sato & Ueno, 2011)(Figure 2-4).

Crystallization in lipids can be dependent on several factors including fat composition, temperature, cooling rate, additives, and agitation (Hartel, 2001). Crystallization influences the melting behavior, solid fat content (SFC), hardness and viscoelastic properties (Hartel, 2001) and it is important to note that mechanical properties of lipids are related to their SFC and microstructure (Herrera & Hartel, 2000).

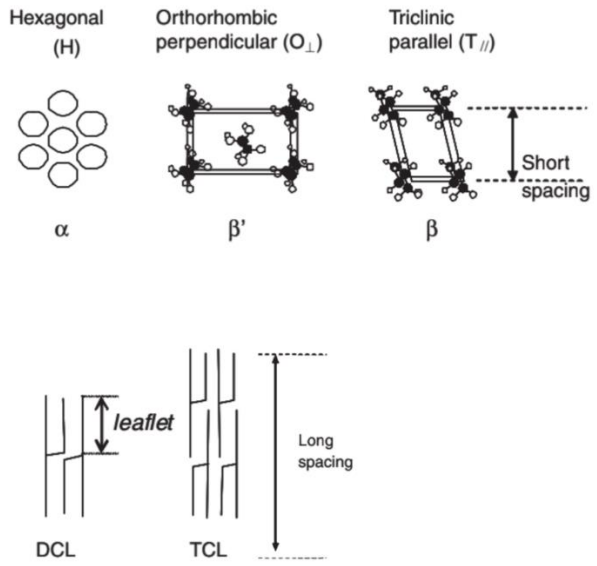

Figure 2-4: Organization of triacylglycerols in short and long spacing. Long spacing displays double (DCL) and triple (TCL) chair stacking. Reprinted from Current Opinion in Colloid & Interface Science, Vol. 16, Sato, K., Ueno, S., Crystallization, transformation and microstructure of polymorphic fats in colloidal dispersion states, Pages 384-390, Copyright 2011, with permission from Elsevier.

Solid Fat Content:

Solid fat content (SFC) is measured using low resolution nuclear magnetic resonance (NMR). The NMR aligns the protons in the sample using a magnetic field, then sends a cross radio frequency pulse through the sample (Sato, 2018). By doing so it causes a tilt to occur in the proton's alignment and the NMR measures the relaxation time it takes molecules to realign with the original magnetic field. The protons in liquid and in solid state have different relaxation times allowing for a percentage to be calculated of how much solid fat is in the sample. An increase in SFC is an indicator of an increase in crystallization and can be measured over a course of time to better understand the crystallization process of a sample. The SFC can be fit to the Avrami or Gompertz equation to evaluate the crystallization kinetics of the sample (Marangoni, 2012; Lee et al., 2018). Cooling rate has shown to have a decreasing trend in the SFC as cooling rate increases, but no significant difference is seen in static conditions (Martínez et al., 2007). In other conditions SFC has been shown to increase with an increase in cooling rate (Campos et al., 2002; Herrera & Hartel, 2000; Martini et al., 2002). Agitation has also been shown to produce an increase in crystallization thus indicating an increase in SFC (Sato et al., 2013), but with current techniques SFC cannot be measured over time with the effects of agitation being recorded in-situ by p-NMR (Sato, 2018). Some methods have been shown where agitation is applied in a crystallization cell and the sample is then transferred to an NMR tube, but there are some techniques that are currently being studied such as combining an NMR with a rheometer (rheo-NMR) to be able to measure SFC during agitation (Mazzanti et al., 2008; Martini et al., 2005a, b, and c). These techniques still need further advances in order to be applied in research and industry

(Sato, 2018). Higher supercoolings result in higher SFC when measured at specific time points (Herrera & Hartel, 2000). The chemical composition also plays a role in the ability for solid material to form. Unsaturated FA produce less solid material, while TAG that contain a high percentage of FA that are similar in length and are saturated show higher SFC (Pereira et al., 2019).

Crystal Morphology

The morphology of the crystals obtained during crystallization can affect the functionality of the fat. Crystal morphology can be observed through polarized light microscopy (PLM). PLM can be used to observe crystal size, shape, and structure. PLM can also give a visual of the type of crystal that is growing such as needle, disc, or spherulite (Marangoni, 2012). Shape and structure of crystals are often dependent on the type of sample and any additives that have been added, while size often is dependent on the induction time of crystallization (Martini et al., 2002). The induction time is the time it takes for a sample to begin crystallizing from the melt. The longer it takes a sample to crystallize the larger the crystals are (Martini et al., 2002). Cooling rate has been shown to have an effect on crystal morphology, as the cooling rate decreases the size of the crystals also increases (Martínez et al., 2007). When introducing agitation, the main effect that it has on morphology is the crystal size and count. As agitation increases the size of the crystals decrease while the count of the crystals increases (Herrera & Hartel, 2000). Although Herrera & Hartel (2000) visually observed differences it was noted that the average crystal size did not change, but rather the distribution and surface density were what was notably different. It was also observed that although crystals did not differ much in size with a change in crystallization temperature, the size of the crystals showed

an increasing trend as temperature increased. Fat composition also showed an influence on the morphology of fat crystals as high melting fractions increase the size of crystals decreased (Herrera & Hartel, 2000).

Viscoelasticity

Viscoelasticity is commonly used to characterize the physical properties of fats. The viscoelastic properties are measured with a rheometer and help to describe the liquid-like and solid-like properties of the sample. G' is the elastic modulus while G" is the viscous modulus. As shear is applied to the sample, the moduli are measured and correspond to how much the sample is deformed. In terms of fat crystallization an increase in G' is observed as crystallization increases and crystallization can be increased by increasing the supercooling or agitation (Martínez et al., 2007). Cooling rate has shown not to have any effect on the viscoelastic properties (Martínez et al., 2007). Observing the effects that rheology has during crystallization is very time consuming and difficult and so it is rarely done, thus rheology is usually measured at specific time points after crystallization has occurred (Sato, 2018). Often experiments apply agitation prior to crystallization and is discontinued to measure rheology (Sato, 2018).

Melting Behavior

The melting behavior of fats provides valuable information to understand how fast or slow the fat melts and to evaluate fats functional properties. The melting behavior of fat can be identified using a differential scanning calorimeter (DSC). In the DSC the sample is compared to a reference cell. Crystallization is an exothermic reaction and so the DSC measures how much energy is required to obtain the same temperature as the

reference cell. DSC is sensitive and so is useful to understand the thermal behavior of a sample even when no crystals can be visually observed. The DSC can also be used to measure the melting point of the sample. Marangoni et al. (2006) showed that fat composition influences the melting behavior and characteristics of fats. As cooling rate increased a bell-shaped trend was seen in the melting behavior where at low cooling rates (0.5 °C/min) the melting behavior began at a lower temperature than at a mid-range cooling rate (2-3 °C/min) and the melting behavior would return to a lower temperature at higher cooling rates (5 °C/min) often times being lower than at 0.5 °C/min. DSC measures crystals that are forming or melting. Therefore, the presence of crystals, whether induced through temperature, agitation or chemical composition, are needed in order for the melting behavior to be identified (Sato, 2018).

The hardness of a sample is measured using a texture analyzer. Smaller crystals tend to create a smaller tight knit network resulting in a harder sample, while large crystals have a harder time organizing themselves into a compact manner which results in a softer sample (Herrera & Hartel, 2000). The polymorphism also influences the hardness of a sample. Since β is the most stable polymorphic form, it is the hardest and the hardness decreases as stability decreases in polymorphic forms (Sato, 2018). Campos et al. (2002) showed that as crystallization and cooling rate slows, a softer fat is observed. As supercooling decreases hardness does as well, therefore SFC is often highly correlated with the hardness of a sample, but the hardness cannot be contributed to one single factor (Campos et al., 2002). Hardness, due to current methods, is often confounded because sample preparation requires a solid to have formed. The way in which this is often accomplished is by placing it at a low temperature (5 °C). This means that a high

supercooling is introduced if a large portion of the sample is still liquid oil at the time of storage. This results in a tight knit crystalline structure displaying harder results (Lee & Martini, 2018). This means the results for samples with a low supercooling or a low melting point can often be confounded due to secondary crystallization that occurs during storage.

Additives such as free FA (FFA), monoacylglycerols (MAG), diacylglycerols (DAG), PL, specific classes of TAG at low concentrations, minerals, metal ions, proteins, and foreign material have been added to fat systems to understand the effects on crystallization (Smith et al., 2011). Lončarević et al. (2013) showed that there was no effect on the crystallization characteristics of cocoa spread creams and fat fillings using sunflower, rapeseed, and soy lecithin. Vanhoutte et al. (2002) observed a retardation in AMF with the addition of soy lecithin and Smith (2000) also observed a retardation of the crystallization of palm oil with the addition of PL. Miyasaki et al. (2016) showed an induction of crystallization in cocoa butter with the addition of modified soy lecithins. The theories for the induction of crystallization is due to PL serving as nuclei before TAG nuclei can form (Jeffery, 1991) and for the retardation of crystallization it is attributed to the interference of the hydrophobic ends of PL attaching to crystal growth sites, therefore inhibiting other TAG from being able to interact and grow on crystal growth sites (Vanhoutte et al., 2002).

Health Benefits of Lipids

In the human body lipids have important function as constituents of cell membranes, source of energy, and hormones (Shier et al., 2007). Unsaturated FA,

specifically polyunsaturated FA containing more than one double bond, have many health benefits to them which have been discussed under the *Health Benefits of Phospholipids* such as being associated with improving cardiovascular disease, diabetes, cancer, depression, mental illnesses, cognitive decline, periodontal disease, and rheumatoid arthritis (Shahidi & Ambigaipalan, 2018). Due to the double bonds found in unsaturated FA, lipid oxidation occurs much quicker than compared to saturated FA, alongside the lower melting point. High consumption of *trans* fats have shown to cause adverse health effects such as increasing low-density-lipoprotein cholesterol and decreasing high-density-lipoprotein cholesterol (Mensink & Katan, 1990; Zock & Katan, 1992; Judd et al., 1994; Bowen et al., 2019), coronary disease (Ascherio & Willett, 1997; Sacks et al., 2017), and death (Willet & Ascherio, 1994; Kummerow, 2009). Saturated fats also have high crystallization tendencies that can be used in the food industry and more recent studies have shown the necessities for saturated fat consumption, but it is still recommended that saturated fats remain as a lower percentage of fat consumption (Lorgeril & Salen, 2012). High intake of saturated fatty acids has shown to have adverse side effects such as cognitive decline (Morris et al., 2006), increasing low-density-lipoprotein cholesterol (Mensink & Katan, 1990), and influence the obesity associated gene (Corella et al., 2011).

Structured Gels in Oils

The challenge the food industry faces is finding a mechanism that allows for an unsaturated fat to be structured, due to the benefits that unsaturated fats have. *Trans* fats, commonly made through a process known as the partial hydrogenation of oil (PHO),

form stable crystalline structures and therefore more ideal when attempting to create a structured food system. In 2015 the U.S. Food and Drug Administration removed the "generally recognized as safe" status for PHO essentially banning the use of these fats in food applications due to the adverse health effect connected to their consumption. It has been found that unsaturated fats can be utilized in a fat structured gel system with the addition of low-molecular-mass organic gelators, such as lecithin (Sato, 2018). Two types of interactions can occur when creating a structured gel either microtubular, which is what occurs in lecithin with the addition of water, and microplates (Sato, 2018). This gel network's formation of interactions is influenced by the supercooling and the shear that is applied to the system during gelation (Li & Liu, 2010). By altering the supercooling and agitation applied to structured gels while they are forming, physical properties such as thermal behavior, thermo-reversibility, viscoelastic properties and the capacity to entrap liquid oil can be altered (Vazquez et al., 2013; Mitre et al., 2012; Han et al., 2014). These properties are used to determine the functionalities and roles that structured gels may play in a food system (Bot & Agterof, 2006; Rueda et al., 2009; Vazquez et al., 2007). Lecithin has been shown to create a structured gel in oil systems with the addition of water to the sample since 1988 and it has been studied extensively since then (Shchipunov, 2001). The lecithins that have been studied include soy and egg (Shchipunov, 2001). In lecithin the gel formation occurs through the creation of tubular structures which occurs through the process of creating many strong hydrogen bonds (Shchipunov & Shumilina, 1997). Some of these tubular micelle-like structures have shown to create a branching network (Shchipunov & Hoffman, 1998). Overall the addition of water to a mixture of lecithin and oil will result in an increase in viscosity, but

if shear is applied to the structured gel then a thinning effect is observed (Shchipunov, 2001).

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CHAPTER III

ISOLATION OF PHOSPHOLIPIDS

Abstract

PC700 is an ingredient in the food industry that is high in PL but has other components such as ash and lactose. The purpose of this study was to isolate PL and oil soluble compounds from PC700 and to optimize the method to do so. Three emulsions were made by weight of PC700:water at 3 different levels (1:99, 5:95, and 10:90). The emulsions were characterized over the course of 30 days by measuring the stability of the emulsion by way of a Turbiscan. Emulsion stability decreased as concentration of PC700 increased ($P<0.05$). The droplet size and size distribution were also measured over the course of 30 days. As PC700 concentration increased droplet sizes increased and over the course of 30 days droplet size decreased by the day 15 mark, but an increase was observed between day 15 and day 30 with 1:99 having the largest D(3,2) followed by 10:90 then 5:95. Due to the course of time a preservative was needed and of several commercial preservatives, it was identified potassium sorbate would be the best for keeping the most similar stability to the original emulsion despite the difference in pH that was observed. An emulsion extraction method was optimized by comparing 4 different emulsion levels of PC700:water (5:95, 10:90, 30:70 and 50:50). Afterwards, ethanol was introduced in four different ratios of emulsion:ethanol by weight (1:0.5, 1:1, 1:2, and 1:3). The samples were filtered and the retentate was saved and analyzed visually for impurities and yield % was calculated. The 30:70 PC700:water with the 1:2 emulsion to ethanol was identified as reaching a visually pure sample with the highest

yield and was used for the remainder of the experiment. A hexane wash was performed by dissolving the agglomerate retentate obtained in hexane and centrifuging the sample. Once the sample separated due to centrifugation the sample was decanted and hexane was left to evaporate off both: the sediments and the hexane layer. P^{31} NMR was used to measure the PL and PL species in the sample and thin layer chromatography (TLC) was used to identify the other lipid classes in the PLI.

Introduction

The food industry uses PL frequently in product formulation. Currently an ingredient product, PC700, from Fonterra is on the market. It contains 85.0% total lipids with 59.2% of PL composition. According to the specification sheet the PL fraction of PC700 is composed of 3.0% PS, 31.0% PC, 8.7% PE, and 16.5% SM. Other components found in PC700 include 2.5% moisture, 6.6% lactose and a maximum content of 12.0% ash. Another ingredient from Fonterra is Phospholac 600, which contains more than 70% phospholipids. Arla Foods Ingredients Amba also produces polar lipid milk products known as Lacprodan-20, which contains 20% phospholipids, and Lacprodan-75, which contains 75% phospholipids. Despite the percentage of purification, these products often still contain ash, sugars or other components which limits their use as an ingredient for certain applications.

The objective of this research was to isolate milk PL from a commercially available phospholipid concentrate to allow for solubility in an oil system. The purpose of isolating milk PLs was to identify how they could be used to enhance consumer nutrition,

eliminate soy and egg allergens in current products on the market, and increase the value of current by-products in the dairy industry.

Materials & Methods

Emulsions

Sample preparation

The first step of the isolation process was to dissolve the PC700 in water to form an emulsion. Samples were prepared in replicate by using various weight ratios of PC700 to water. Three different emulsions were made 1:99 (1.5g:148.5g), 5:95 (7.5 g:142.5 g) and 10:90 (15 g:135 g.) Preservative potassium sorbate was added at a 0.2% (300.6 mg) level. All components were mixed using a magnetic stir bar in a beaker on a magnetic plate set to 1100 RPM for 30 min at ambient temperature (25 °C).

Emulsion Stability

The stability of the emulsions was measured over time using the TurbiScan Classic MA2000 (Formulaction SA, France). After making the emulsion as previously described, the samples were carefully placed in flat-bottomed TurbiScan tubes and closed with a lid. Five tubes were prepared for each emulsion. The times chosen to measure the stability of the emulsions was Day 0, 2, 5, 9, 14, 21, and 30. The data was analyzed by measuring the change in the backscattering at the top and the bottom of the tube. Samples were stored for 30 days in an incubator at 25 °C to evaluate if the emulsions will destabilize and allow for an easy isolation of the PL.

Oil Droplet Size

The oil droplet size of the emulsions was measured using Beckman Coulter LS230 Small Volume Module Plus made by Beckman Coulter in the U.S.A. The pump speed % varied, 50% is approximately 1500 ml/min, for the 1:99 emulsion a pump speed of 49% was used, for the 5:95 emulsion 50% was used, and the 10:90 emulsion had a pump speed of 47%. The various pump speeds were selected to avoid any air bubbles that may occur during the assay, as instructed by the Beckman Coulter technician team. A PIDS obscuration of 45% was used to determine the quantity of the sample needed for the assay. The $D_{3,2}$ of the droplets and droplet size distribution was recorded for all emulsions as a function of time on day 0, 15, and 30. $D_{3,2}$ can be defined as the relationship of the average surface area of a droplet divided by the volume of the emulsion.

pH

The pH of all emulsions was measured at the initial time using the HI 99161 Waterproof pH Meter for Dairy Products made by Hannah instruments in the U.S.A The pH meter was first calibrated using buffers with pH 7, Fisher Chemical SB107-500 made by Fisher Chemical in the U.S.A., and pH 4, Fisher Chemical SB101-500 made by Fisher Chemical in the U.S.A. The pH was taken 3 times for each emulsion.

Ethanol Extraction of Phospholipids (PL)

The ethanol extraction and hexane purification have a visual demonstration shown in Figure 3-1 The extraction of PL from the PC700 was tested by creating a suspension of the PC700 in water (emulsion) and then precipitating out the PL using ethanol (EtOH). Various PC700:water and EtOH ratios were tested. Four different emulsions were made

5:95 (5.5 g:104.5 g), 10:90 (11 g:99 g.), 30 70 (33 g:77 g) and 50:50 (55 g:55 g.) The components were mixed using a magnetic stir bar in a beaker on a magnetic plate set to 1100 RPM for 30 min at ambient temperature. Each emulsion ratio was then separated into four beakers of 25 g and mixed ethanol in the following ratios by weight: 1:0.5 (25 g:12.5 g), 1:1 (25 g:25 g), 1:2 (25 g:50 g) and 1:3 (25 g:75 g).

The emulsion was placed on a magnetic stir plate with a stir bar set to 700 RPM during ethanol addition and the sample was stirred for 1 min after the ethanol was completely added. The sample was then given 5 min to rest while an agglomerate or precipitate formed. The ethanol:emulsion mix was then filtered using a Buchner funnel, a 47 mm diameter microfiber glass filter paper, and a vacuum flask that was connected to a vacuum. This process allowed to separate the agglomerate from the rest of the materials in the PC700. The PL agglomerate (PLA) that remained in the filter paper was placed on watch glasses under a hood for at least 12 h to evaporate the ethanol. Samples were then moved to a desiccator where their weights were measured every 24 h for 7 days.

Hexane Phospholipid Purification

The PLA obtained from the previous step still contained some impurities, presumably, minerals. Therefore, a purification step was preformed using hexane (Figure 3-1). The hexane phospholipid purification was performed by making a 1:9 ratio of the agglomerate obtained from the previous step (AOCS Official Method Ja 3-87). The agglomerate obtained from the 30:70 emulsion using the 1:2 ethanol extraction method

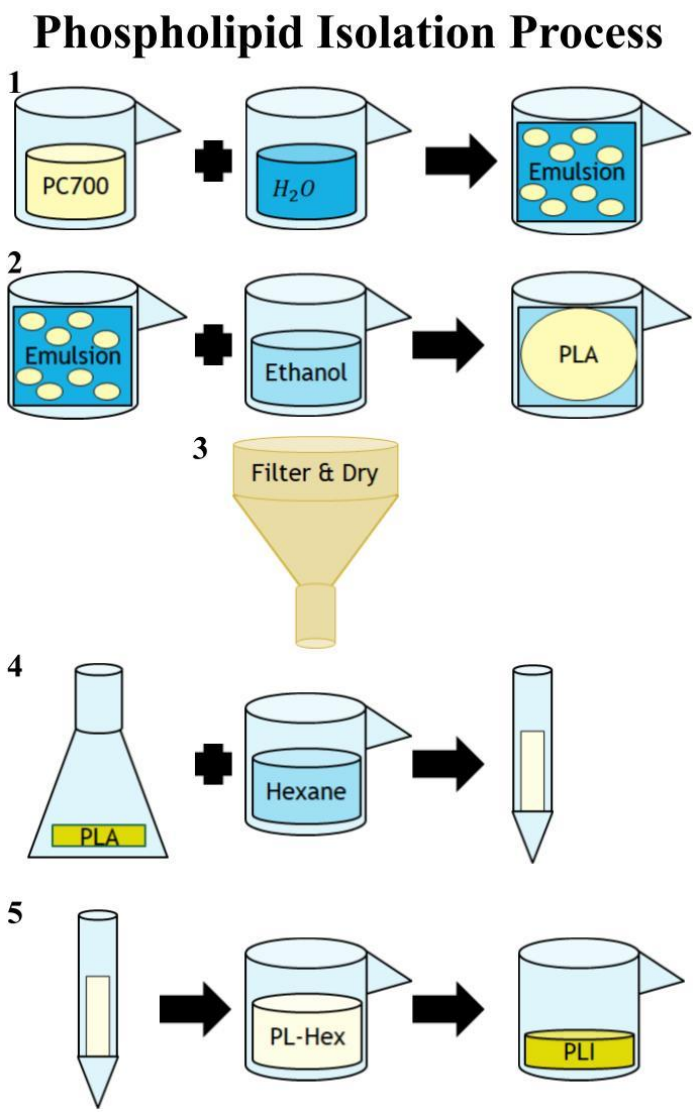

Figure 3-1: The process of phospholipid isolate (PLI) isolation from Phospholipid Concentrate 700 (PC700). (1) Create an emulsion by mixing PC700:water together at a ratio of 30:70 respectively. (2) Add ethanol to the emulsion at a ratio of 2:1 respectively to form a phospholipid agglomerate (PLA). (3) Filter and dry the PLA for 12 h using a Buchner funnel and microfiber filter. (4) Add hexane to the PLA and centrifuge the mixture in 15 ml centrifuge tubes at 1,000 x g for 5 min. (5) Decant the phospholipid hexane mixture (PL-Hex) from all sedimented material in the centrifuge tube and allow to dry for a minimum of 12 h to obtain the PLI.

was identified as giving the highest yield while still containing no visible impurities and thus was used for the remainder of the experiment. The PLA was weighed in an erlenmeyer and placed on a magnetic stir plate with a magnetic stirrer bar. The erlenmeyer was covered with aluminum foil to minimize evaporation. The sample was agitated for 10-15 min until the PLA had dissolved in the hexane leaving a solid residue suspended in the media. The suspension was then poured into two 15 ml centrifuge tubes, passing a wash of hexane over the erlenmeyer flask to remove any residuals of the hexane and sample bringing both 15 ml centrifuge tubes to a final volume of 6 ml. A Thermo Electron Corporation IEC Centra CL3R Refrigerated Centrifuge made in the U.S.A. was used with a 809 rotor at 1,000 x g, 24 °C for 5 min. A white sediment could be seen at the bottom of the centrifuge with a yellow transparent solution on top. The supernatant was separated from the sediment and was transferred into a beaker. The centrifuge tubes were then brought up to a 4.5 ml volume using hexane to wash the sediment and improve the recovery of PL and vortexed until the sediment was brought back into solution. The samples were then centrifuged again, and the supernatant was transferred to the beakers as previously described. This washing process was performed 3 times. The centrifuge tubes and the beaker were then left under a hood for at least 12 h until the hexane evaporated off. The yield for the hexane extraction was calculated using equation 3-1.

$$\frac{W_f - W_i}{W_p} * 100 = Yield\ \% \qquad [3\text{-}1]$$

Where W_i is the initial weight (g) of an empty beaker, W_f is the final weight (g) of the beaker after the polar lipids have been decanted into it and the hexane has evaporated off,

W_p is the initial weight of polar lipid dissolved in hexane (g), and 100 is to convert it into a percentage.

Nuclear Magnetic Resonance for Identification of Phospholipids

NMR was performed using a Bruker AVANCE III 500 MHz high resolution spectrometer (Bruker, Karlsruhe, Germany) with a Bruker Smart Probe for observing nuclei. The proton spectrum was recorded at 500.132 MHz and the phosphorous spectra was recorded at 202.456 MHz. The following the methods reported by Lehnhardt et al. (2001) and MacKenzie et al. (2009) were used. A detergent solution was prepared as follows: 1 g of sodium cholate (from Alfa Aesar) was dissolved in a 10 mL deuterium oxide (purchased from Aldrich)/water solution (20/80% v/v). To this was added Ethylene diamine tetraacetic acid disodium dehydrate (purchased from Mallinckrodt Pharmacueticals) (1% w/w) and Phophonomethylglycine (from Frontier Scientific) (0.3 g/L). The pH was adjusted using a Mettler Toledo Seven Compack pH/ion S220 pH meter to 7.40 using a sodium hydroxide solution (0.02 M) and the solution became clear. Four samples of PLI (20 mg) were mixed with detergent solution (750 uL). Sodium hydroxide was added to the remaining detergent solution to raise the pH to 8.51 and a further 4 samples were made. All 8 samples were sonicated using a Branson 2800 sonicator for 30 minutes. Samples were transferred into 5 mm diameter NMR tubes. Spectra were measured 25 °C using the following acquisition parameters: 196 acquisitions, 54k data points, 5000 Hz sweep width, 5.4 second acquisition time, and a 3.5 second relaxation time. All ^{31}P spectra were obtained using inverse gated proton decoupling (Bruker: igzg) to minimize the nuclear Ovenhauser effect. A line broadening of 0.2 Hz was applied to the obtained spectrum. The spectrum was processed using

MestReNova v12.0.2 from Mestrelab Research S.L. A line broadening factor of 0.2 Hz was applied in the time domain. Zerofilling was performed to increase the number of data points to 64k. Quantiative global spectra distribution using 6 improvement cycles was applied to help deconvolute overlapping peaks. Data was processed using MestreLab's MestReNova v12.01. The experiment was performed in triplicate.

Thin Layer Chromatography (TLC)

The purity of the PL obtained after isolation was evaluated by doing TLC. TLC was performed in duplicate on 4 replicates of the PLI to ensure that the total PL concentration was consistent in each extraction. TAG, cholesterol, PC, FFA, cholesterol esters (CE), MAG, 1-2 DAG, and 1-3 DAG, standards were also ran to identify all of the compounds in the sample. A TLC plate (20 × 20 cm silica gel, 60 A particle, 250 µm layer) (Whatman, Little Chalfont, Bucks, UK) was pre-washed and developed using a 100 ml mixture of chloroform and methanol at a 1:1 ratio by volume, then activated in the oven at 100 °C for 10 min. The PL (2.5 mg) samples were dissolved in 10 µl of Folch Solution (2:1 $CHCl_3$:MeOH 0.01% BHT), dried using nitrogen gas, and then dissolved in 10 µl of Folch Solution and were placed 1 cm from the bottom of the plate and the plate was air dried. Once the plate was dry it was sprayed with a 10 % w/v cupric sulfate solution in 8% w/v orthophosphoric acid until it was translucent (Baron & Coburn, 1984). The plate was heated in an oven at 145 °C for 10 min (Churchward et al., 2008). A picture was taken of the plate using a Nikon d810 Camera (2014 Ayutthaya, Thailand) and analyzed using Image Lab 6.0.1(2017 Bio-Rad Laboratories Inc.). In this software the picture color was first inverted, then the columns were identified individually. The bands and band size were identified, and the pixel count and intensity were identified and

given, allowing for the percentage of components in the sample to be identified. Peillard et al. (2019) used and developed a similar method to be able to quantify the amount volatile fatty acids in a sample using a similar software.

Statistical Analysis

A 2-way ANOVA was performed for the statistical analysis with the statistical software GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA) used for all of the statistical analyses.

Results & Discussion

Emulsions

Determining the Preservative

When emulsions were prepared without the addition of a preservative, mold growth was observed after 24 h of storage at ambient temperature. Therefore, a preservative was used to delay mold growth. Several preservatives were tested and the pH of the emulsions with and without the use of preservatives was measured and reported in Table 3-1. Microgard 730, a cultured dextrose, from Danisco was the first preservative selected since no change in the pH of the emulsions was observed. Therefore, emulsion stability was tested for the 1:99, 5:95, and 10:90 emulsions. The 5:95 and 10:90 emulsions were very stable over a period of 30 days. However, the 1:99 emulsion

Table 3-1: pH of emulsions formulated with and without preservatives at a 0.2% level for emulsions with 1 and 10% of PC700. Superscript letters indicate significant difference amongst samples (α=0.05)

Emulsion level	**Emulsion with no Preservative**	**Emulsion with Microgard 730**	**Emulsion with Microgard 200**	**Emulsion with Microgard 100**	**Emulsion with Potassium Sorbate**
1:99	4.62 ± 0.05^{e}	4.57 ± 0.01^{e}	5.60 ± 0.05^{c}	6.33 ± 0.03^{a}	6.11 ± 0.01^{b}
10:90	4.52 ± 0.01^{e}	4.57 ± 0.01^{e}	4.86 ± 0.06^{d}	4.95 ± 0.04^{d}	5.47 ± 0.01^{c}

destabilized over a time period of less than 24 h. When this emulsion was formulated without the preservative the emulsion remained stable for the first 24 h suggesting that the preservative affected emulsion stability. This process was repeated 3 times and the same results were obtained. The other preservatives used at the same 0.2% usage level, were a cultured dextrose Microgard 200, a cultured grade A skim milk powder Microgard 100, and potassium sorbate. The pH was measured for emulsions 1:99 and 10:90, with and without the preservatives, to determine the spread of the pH between emulsions when different preservatives were introduced in the emulsion system. The objective was to identify the differences in pH that the preservatives caused between the largest and lowest concentration. The pH of 5:95 was not measured because the pH would set within the span of the 1:99 and 10:90 emulsions. The samples showed flocculation with and without preservatives within 24 h, but visually the potassium sorbate was most similar to the original emulsion, was visually stable over a 24 hour period, and had the lowest distribution of pH values between the smallest and largest emulsion ratios so therefore was selected to be used despite the pH increasing 1-1.5 compared to the original

emulsion. The pH of all the emulsions with potassium sorbate had a significant change (P<0.0001) as the concentration of PC700 increased (Table 3-2). Although we began to observe about a 0.5 decrease in pH as concentration increased it was determined that this preservative along with distilled water would be the best combination in order to reflect the most representative situation for the emulsion.

Oil Droplet Size

The $D_{3,2}$ (Figure 3-2) was measured for the emulsion at days 0, 15, and 30. All emulsions showed a slight tendency in change of the $D_{3,2}$, but the 1:99 and 10:90 emulsions both showed a valley-like tendency in the change, with each time period being significantly different (P<0.0001) from each other with the exception between 10:90 on day 15 and day 30 which were not significantly different. The 5:95 emulsion showed very little variance as well as very little deviation from the average and was not significantly different (P>0.05) over the course of 30 days. Indicating that 5:95 was the least likely to change over time compared to the other emulsions. At time 0 all emulsions where significantly different showing that the concentration of PC700 increased the

Table 3-2: Change of pH in emulsions formulated with potassium sorbate as the concentration of PC700 increases. Mean values of 6 replicates and standard deviations are reported. Values with different superscripts are significantly different (α=0.05).

	Emulsions (PC700:water)		
	1:99	**5:95**	**10:90**
pH	6.11 ± 0.01^{a}	5.62 ± 0.00^{b}	5.47 ± 0.01^{c}

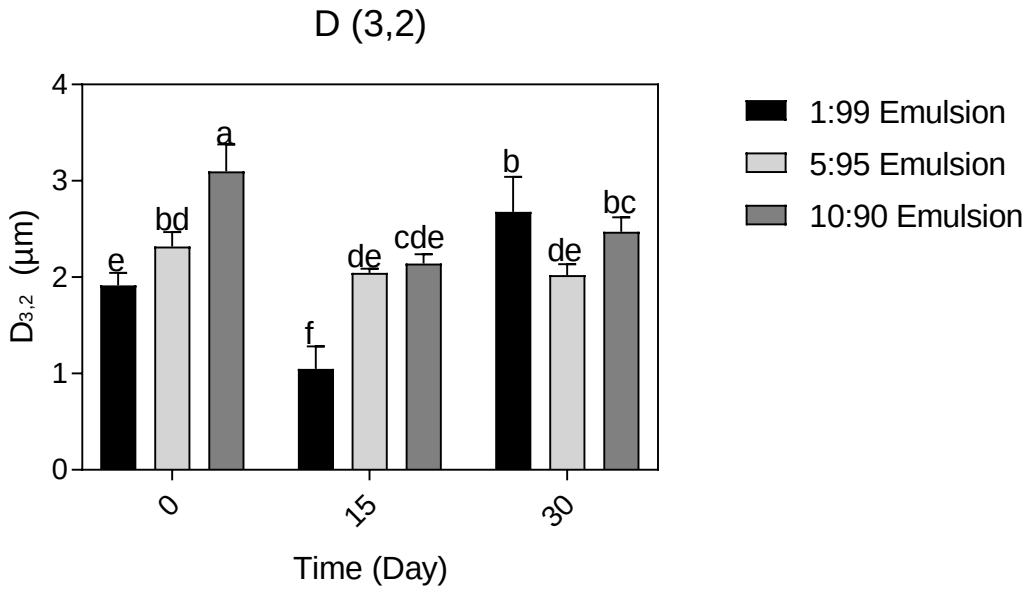

Figure 3-2: Droplet size ($D_{3,2}$ in μm) of emulsions at days 0, 15, and 30. Mean values with the different letters indicate that samples are significant different (α=0.05).

average $D_{3,2}$ size. After 15 days 5:95 and 10:90 were no longer significantly different from one another (P>0.05), although at day 30 they were once again significantly different (P<0.01). After 30 days 1:99 and 10:90 had significantly different (P<0.01) $D_{3,2}$ sizes from 5:95.

The droplet size distribution (Figure 3-3) shows the distribution of the oil droplets at each time point for each of the emulsions. In some of the original graphs showing the distribution additional peaks could be identified. These additional peaks were eliminated from the data because they were identified as being air bubbles and non-emulsified compounds. The distribution graphs show a good representation of the $D_{3,2}$ and what is happening over time to each emulsion. The common trend between the emulsions suggest

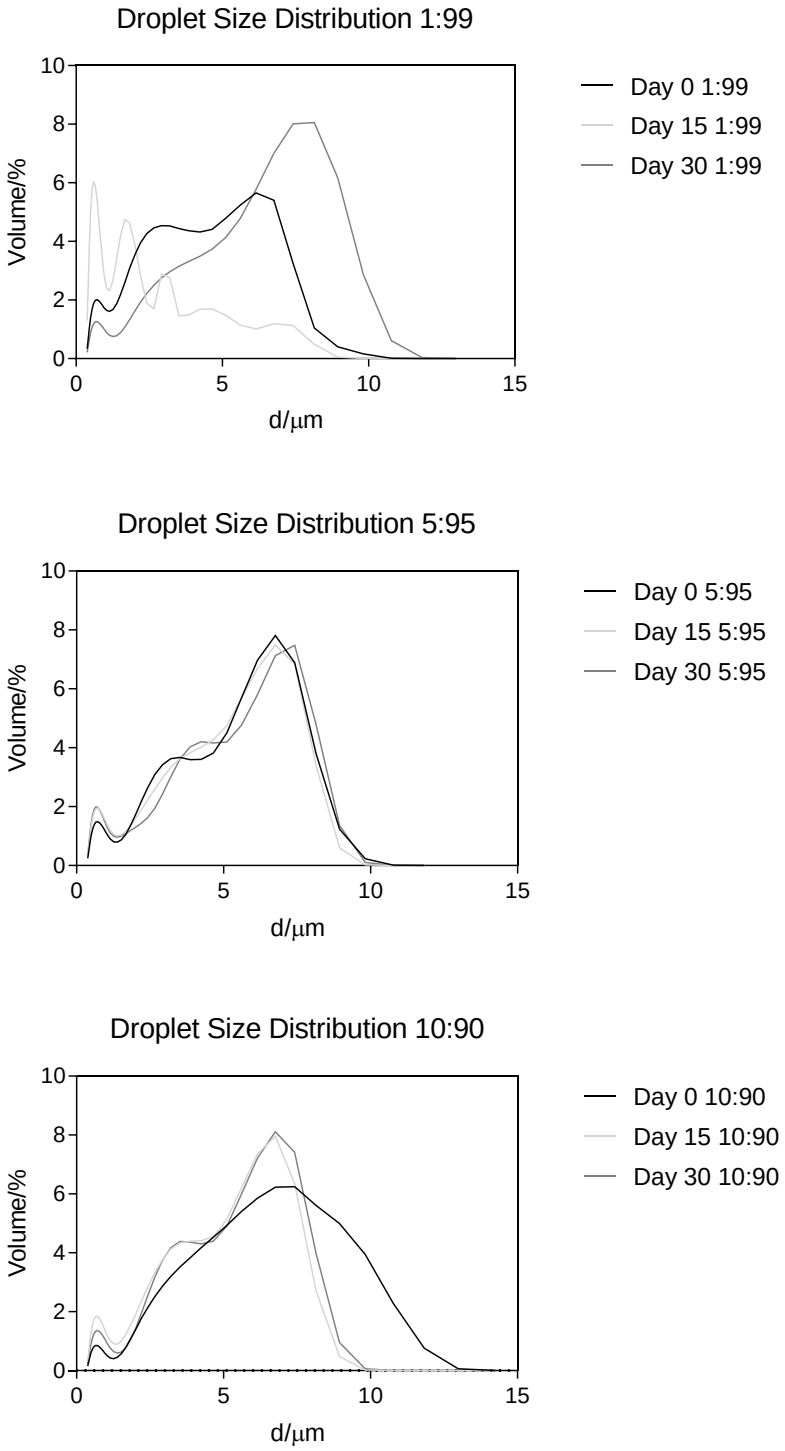

Figure 3-3: Oil droplet size distribution at day 0, 15, and 30 for emulsions 1:99, 5:95, and 10:90.

that overtime at day 15 the emulsion droplet sizes decrease from day 0 and then from day 15 to 30 emulsion droplets begin to coalesce creating larger droplet sized particles. This trend of decreasing in size for the first 15 days and then increasing for the last 15 days is most likely due to the destabilization of the large droplets resulting in destabilization and creaming thus leaving the smallest droplets left in the emulsion at day 15. Then by day 30 the smaller droplets have begun the process of coalescing resulting in a larger droplet size then what was seen at day 15. This tendency would apply to the 1:99 and 10:90 sample, while 5:95 sample showed no significant difference ($P>0.05$) between time points indicating that although the averages indicate a similar trend, this could be contributed to error in the 5:95 sample.

TurbiScan

Turbiscan data (Figure 3-4) is obtained by the backscatter %. The change in backscatter is measured over time which gives an indication of the stability of the emulsion (Figure 3-5). Figure 3-5 shows that there was not much variance in the back scatter for both zones 1 and 2 after about 40 h. Zone 1 was the emulsion at the bottom of the tube and zone 2 was the zone of the emulsions just below the meniscus of the tube. The 1:99 emulsion shows by the TurbiScan that in both zones 1 and 2 the back scatter was close to about 2.0% consistently after the first 40 h. These results are also reflected in Figure 3-5. The 1:99 emulsion was the only emulsion where zone 1 and 2 followed very closely with one another, whereas all of the other emulsions showed zone 1 having a higher % backscatter after 40 h, especially as the concentration of PC700 increases. The 1:99 emulsion shows the backscatter % being high which indicates both sedimentation and clarifying are happening simultaneously which can be seen in Figure 3-4. The 5:95

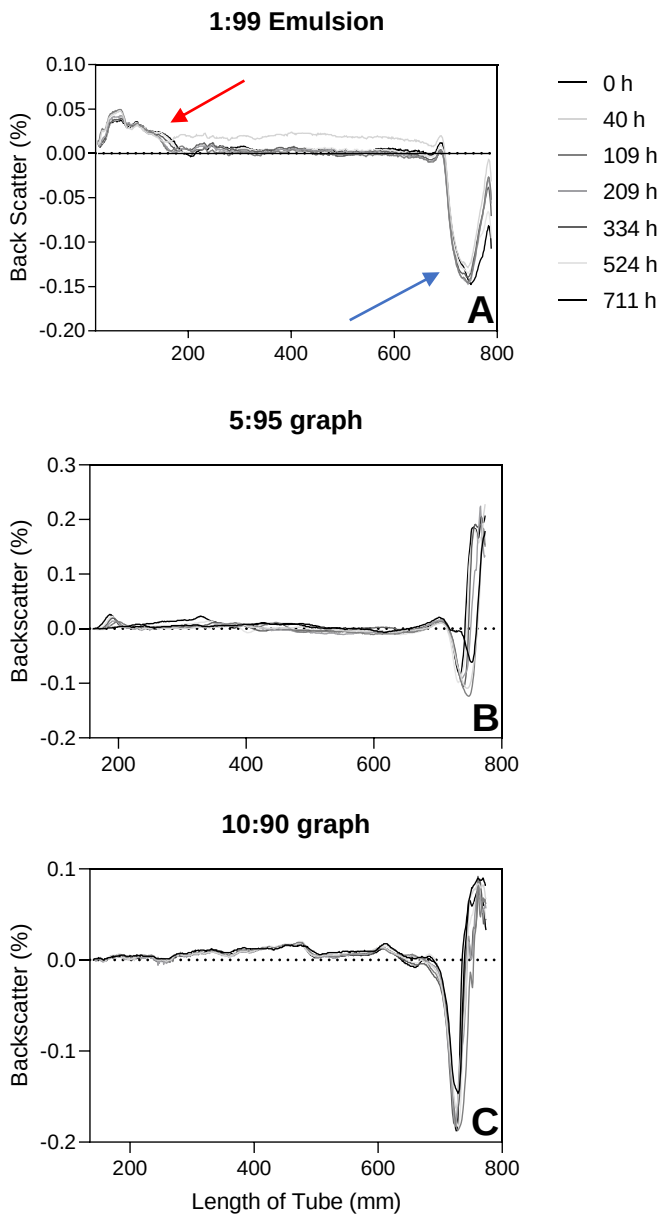

Figure 3-4: Backscattering of emulsions 1:99 (A), 5:95 (B), and 10:90 (C). Zone 1 is measuring the bottom of the tube represented by the first peak (red arrow). Zone 2 measures the top of the tube where creaming, clarifying, or flocculation may be occurring represented by the second peak (blue arrow). The similar locations were measured for the 5:95 and 10:90 emulsions as well.

emulsion sedimentation occurring very quickly after 40 h with a lower amount of creaming occurring, but after 30 days the backscatter % of both zone 1 and 2 reflected one another indicating that both clarifying and sedimentation had occurred at relatively equal amounts. In the 10:90 emulsion a very consistent back scatter in both zones was observed after 40 h although zone 1 showed a much higher value and other than at time 0 they never reached a point where the zones reflected one another. This indicates that in the 10:90 emulsion both clarifying and sedimentation occurred, but when looking at both Figures 3-4 and 3-5 it becomes clear that clarifying occurred faster than sedimentation

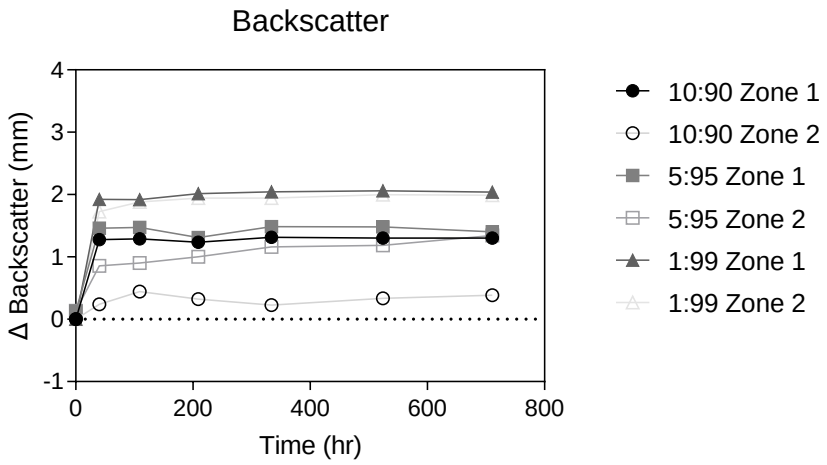

Figure 3-5: The Δ backscattering of emulsions 10:90, 5:95, and 1:99 plotted as a function of time. Zone 1 measures the bottom of the tube and the amount of sedimentation that occurs over time. Zone 2 measures the top of the tube where creaming or clarification may be occurring. In the case of the graphs shown, a trend of sedimentation and clarification are occurring simultaneously with sedimentation increasing as PC700 concentration increases.

indicating suspension of sediments. After time 0 the $D_{3,2}$ and oil droplet size distribution were fairly consistent (Figure 3-3) which was also reflected between the consistent results observed in both zones 1 and zone 2. Overall the TurbiScan tells us that as PC700 concentration increases so does the sedimentation and clarifying of the emulsions.

Ethanol Extraction of Phospholipids

Yield and Visual Appearance

The overall purpose of this experiment was to identify an optimized process that would result in the most oil soluble compounds with PL being a major component of the isolate. Due to the limited amount of PC700 only the 5:95 and 10:90 emulsions were evaluated for their stability, the higher concentrations were also very viscous reflecting a water in oil emulsion rather than an oil in water emulsion. Transferring this type of emulsion to the Turbiscan tubes and other methods would have caused potentially unreliable data to be produced. However, higher concentrations of PL:water were tested (30:70 and 50:50) in the extraction process to evaluate how these ratios would affect the isolation of PL.

A visual difference in retentate and filtrate was observed for the various extraction processes (Figure 3-6) after step 3 of Figure 3-1. Pictures reported in Figure 3-6 were taken prior to the drying process. Figure 3-7 shows the yields obtained from the EtOH extraction process. It was identified that 30:70 emulsion with a 1:2 ethanol wash was the most effective to isolate a more pure substance which was identified as having no

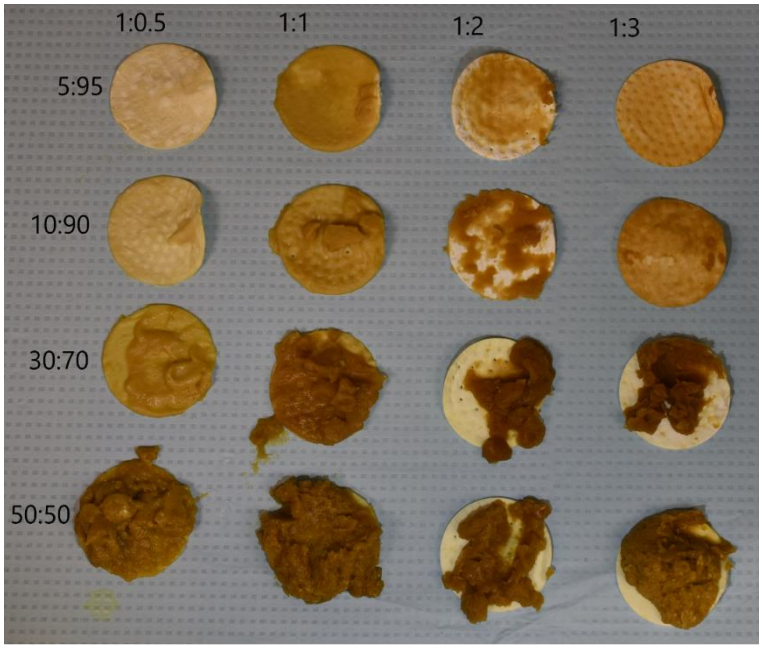

Figure 3-6: Filter papers of the retentate prior to drying. The columns are the levels of emulsion to ethanol and the rows are the levels of PC700 to water.

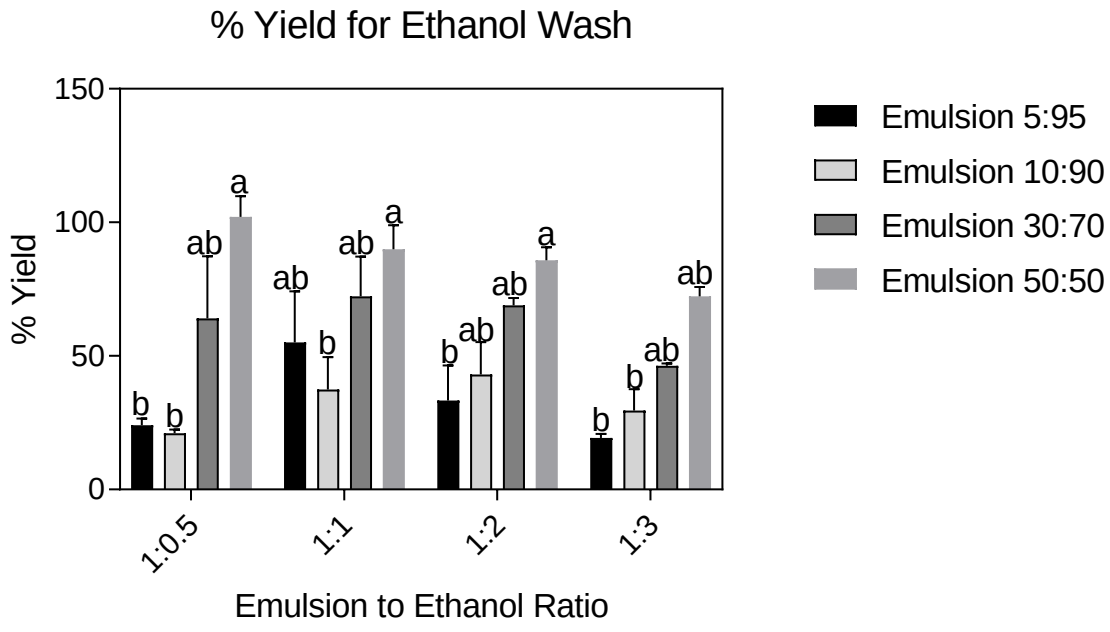

Figure 3-7: Yield (%) obtained from each emulsion (PC700:water) and ethanol extraction combination.

light brown or tan specks with the highest yield, because of this the remaining of the experiment was only performed using the 30:70 emulsion with the 1:2 EtOH wash.

Hexane phospholipid Purification

A hexane purification was performed on the 30:70 emulsion that underwent the 1:2 EtOH wash to obtain the highest concentration of PL as well as the most pure substance. The hexane purification seemed to be effective in the isolation of polar lipids. The Yield % was 40±15%. By appearance it is believed that the sample has obtained a well isolated level of PL, although NMR and TLC would be required to fully identify the qualitative and quantitative results of the sample.

NMR and TLC

The purpose of performing NMR was to quantify the polar lipids that were in the sample and to identify which PLs were present in the sample. The NMR results showed that approximately 55.0±0.5 (Table 3-3) of the agglomerate were PL, the remaining lipid classes expected could be TAG or other lipid classes. Comparing the results of the purified and extracted polar lipids with the concentration of specific polar lipids in milk, it was observed that there was an increase in the mol % significantly in PE and PC while there was a significant decrease in SM. PI and PS where in between the various ranges found in literature, so it cannot be determined if there was a significant loss through the purification process. Garcia, Murgia, Andreotti, and MacKenzie all performed the PL analysis from samples they collected from liquid milk or cream. MacKenzie also performed NMR on PC700 and other commercial products, but the PLs were not isolated from these products and so the isolated PL information was used in Table 3-3. Murgia,

Table 3-3: Composition of the phospholipid isolate (PLI) as determined by ^{31}P NMR compared to previously reported data. Data reported is the average of 4 analytical replicates. PL: phospholipid, 2LPE: lysophosophatidylethanolamine, SM: sphingomyelin, PE: phosphatidylethanolamine, Lac-PE: lactosylated phosphatidylethanolamine, 2LPC: lysophosphatidylcholine, PS: phosphatidylserine, PI: phosphatidyl inositol, PC: phosphatidyl choline, PLI: phospholipid isolate. PL species with a superscript [a] were obtained from solution with a pH of 8.5 and PL species with a superscript [b] were obtained from solution with a pH of 7.4. Reprinted with modifications from the Journal of the American Oil Chemists' Society, Cooper, Z., Simons, C., Martini, S., Retardation of Crystallization through the Addition of Dairy Phospholipids, Copyright Aug 5, 2019, with permission from John Wiley and Sons.

PL mol%	**Rep 1**	**Rep 2**	**Rep 3**	**Garcia, 2012**	**Murgia, 2003**	**Andreotti, 2006**	**MacKenzie, 2009**
2LPE[a]	3.9±1.3	5.3±0.3	3.8±0.1	-			
SM[b]	8.6±4.0	12.6±0.8	8.6±0.3	19.9	26.8	24.2	26.5±0.3
PE/ Lac-PE[b]	37.5±5.9	30.8±0.9	39.8±0.7	31.4	25.8	23.5	26.7±0.1
2LPC[b]	3.5±2.0	5.0±0.2	4.3±0.7	0			
PS[a]	3.6±0.8	3.2±0.2	3.1±0.3	11.2	1.5	3.6	11.7±0.2
PI[a]	4.2±0.5	4.6±0.3	3.6±0.1	3.6	14.0	12	7.5±0.1
PC[b]	38.3±5.0	43.9±0.5	36.9±0.4	28.7	26.8	24	26.5±0.3

Andreotti, and MacKenzie all used acetone as a principal polar solvent while the solvent used for reps 1, 2, and 3 were EtOH. Other washes were done using methanol, chloroform, or a methanol chloroform mix to continue with the extraction. Reps 1, 2, and 3 all used hexane and just used a repeated wash of hexane to obtain the final PLs. Due to the difference in solvents this could be a reason for the variance in some of the numbers. Another could be that the initial product was different, one being PC700 and the others being fluid milk. The PC700 has already undergone several processing steps which means that there would have been more opportunities for PLs to be lost throughout the process. Some variance as well would be due to the difference in percentages of each rep. If PS has a low mol% in reps 1, 2, and 3 compared with Garcia then another PL will show a higher percentage in the reps giving the perception that there are two PLs that are different in the quantities whereas the lack of one PL is what is really making the quantity to appear higher by assigning a higher % to another PL.

The results of the TLC showed that the PLI replicates contained an average of 55.1 ± 3.5 % of PL and this value was not significantly different amongst replicates or the concentration of PL found from the P^{31} PL results (P>0.05). Standards showed that the remaining components in the PLI were cholesterol (15.6 ± 0.9 %), FFA (11.6 ± 1.8%), MAG (0.9 ± 0.2%), 1-2 DAG (5.8 ± 0.4%), 1-3 DAG (3.4 ± 0.4±), TAG (4.3 ± 0.7%), and CE (3.3 ± 1.9%). (Cooper et al, 2019)

Conclusion

Milk polar lipids create stable oil in water emulsion, especially so as the concentration increases, the emulsions reflect a small oil droplet size. The polar lipids can

be extracted from a powder by creating an emulsion, breaking the emulsion using EtOH and using hexane to extract the polar lipids from a residual of minerals. The most effective emulsion to EtOH ratio found in this study was 30:70 PC700: distilled water emulsion and a 1:2 emulsion to ethanol ratio. The purification method does show a low value of SM, but there is still potential for new functionalities and health benefits that could be achieved using polar lipids from milk, knowing that SM is not a main constituent of the current plant based lecithins on the market (van Nieuwenhuyzen, 2008). The method used in this chapter results in a polar lipid extract composed mostly of PC and PE, but also includes SM, PI, PS and LPE.

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CHAPTER IV

RETARDATION OF THE CRYSTALLIZATION OF ANHYDROUS MILK FAT THROUGH THE ADDITION OF PHOSPHOLIPIDS[1]

Abstract

The objective of this work was to identify the effects that milk PL have on crystallization of anhydrous milk fat (AMF). Three mixtures were prepared by adding 0%, 0.01%, and 0.1% PL to AMF. Each mixture was crystallized for 90 min at 24, 26, and 28 °C. The SFC was measured as a function of time and fitted to the Avrami equation. Melting point, thermal behavior, viscoelastic properties, and crystal morphology were all measured at 90 min. All assays were repeated, as well as hardness, after being stored at 5 °C for 48 h. Samples containing PL showed slower crystallization as concentration increased especially at higher temperatures (26 and 28 °C). The addition of PL caused difference in crystal morphology resulting in visibly larger crystals at 90 min. The elasticity and hardness at 90 min were influenced by the addition of PL at 24 °C with lower values obtained in samples with PL compared to the AMF alone. No differences in hardness nor in elasticity was observed for samples crystallized at 26 and 28 °C. A decrease in melting enthalpy was observed in samples with PL indicating a reduction in crystallization at all temperatures which was supported by crystal morphology.

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Introduction

Polar lipids are highly used and needed in the food industry. The most common polar lipids in the industry are known as phospholipids, which consist of a lipid with a glycerol backbone, two fatty acid chains, and a phosphate group. PL are used for their emulsifying properties and to provide stability to food systems (Ahmad & Xu, 2015). For a long time, polar lipids have been known to exist in milk, but their concentration in milk is minimal (12.8-40 mg/L) (Ahamad & Xu, 2015). Currently, products obtained from the dairy industry that are high in polar lipids are used as animal feed, low profit by-products, or are reworked into different product processes at low concentrations. Some of these products that are high in polar lipids come from butter serum, which is a low profit by-product from butter and butter oil manufacturing, and the isolated fat portion from the manufacturing of whey protein isolate (Ahmad & Xu, 2015).

In the food industry there are various dairy PL products. These products are produced by various companies and generally come in a powdered form. PL products are produced to be ingredients and their PL content can range from as little as 20% PL and as much as 75% PL. Other components that may be included in these products are other lipids, lactose, and minerals.

Milk polar lipids have a different composition than common polar lipids that are currently used in the industry. The most common products that contain PL are from sources such as soy, sunflower, or egg and these polar lipids are commonly referred to as lecithins. According to the US Code of Federal Regulations (CFR) Title 21 Volume 3 a "commercial lecithin is a naturally occurring mixture of the phosphatides of choline,

ethanolamine, and inositol, with smaller amounts of other lipids" (CFR 21). There are various grades of lecithin including crude, fluidized, highly filtered, compound, chemically modified, and fractionated lecithins. Crude lecithins are often the grades of lecithin manufacturers produce. These products are standardized by usually using oil from the lecithin source to result in a consistent percentage of PL. CFR 21 does not give any limits to how much oil can be added, but soy lecithin can have as much as 40% TAG and egg lecithin can have as little as 15%. For soy lecithin the next grade would require to have less than 3% of TAG. This means that most lecithins used in the industry do not contain only phospholipids. The literature shows that the polar lipid composition in soy lecithin is 32% PC, 23% PE, 21% PI, and 9% PA. In sunflower lecithin the polar lipid composition is 34% PC, 17% PE, 30% PI, and 6% PA (van Nieuwenhuyzen & Tomás, 2008). SM and PS are polar lipids that are not commonly reported in soy or sunflower lecithins due to their low concentration but these PL are usually found in animal based lecithins (<1%) (Wendel, 2014). SM is found in both egg (1.7%) and milk PL (26.8%), but PS is not found in egg lecithin and can be only found in milk (1.5%). For example, the composition of egg polar lipids is approximately 79% PC, 17% PE, 0% PI, 0.3% PA, 1.7% SM, and 0% PS (Sotirhos et al., 1986). In milk the observed composition is 26.8% PC, 25.8% PE, 14% PI, 0.5% PA, 26.8% SM, and 1.5% PS (Murgia et al., 2003).

Lecithins are commonly used as emulsifiers, to modify the viscosity of fluids, such as melted chocolate, and as a processing aid (Nieuwenhuyzen & Tomás, 2008). Current research topics using lecithins include their use as encapsulating nutrients or pharmaceuticals in a lecithin-based mixture (Human et al., 2019), creating organogels with vegetable oils (Sato, 2018), and to control crystallization. The effects of lecithin on

lipid crystallization shows that lecithins do not influence the solid fat content of nontrans fat spreads with confectionary application, but they do influence the viscoelastic properties specifically increasing the elastic properties (Lončarević et al., 2013). In other studies, using cocoa butter an induction of crystallization, affecting the solid fat content, occurred when soybean lecithin was added at low concentrations (0.2%) but as the concentration of PL increased the rates of crystallization decreased (Miyasaki et al., 2016). A contrary effect was observed by Rigolle et al. (2015) in cocoa butter showing a delay in crystallization when sunflower and soy lecithin were added. When Smith (2000) observed the effects of PL on palm oil it was found that nucleation was delayed with the addition of PL resulting in the formation of large spherulitic crystals. When a delay is observed with the addition of PL the hypothesis is that PL are adsorbed onto the growth sites of the crystals (Vanhoutte et al., 2002) delaying the incorporation of TAG into the crystalline matrix. Overall the addition of lecithins or PL to lipid systems can cause different results depending on the source of the lecithin or PL and also on the processing condition used (Lončarević et al., 2013). Fedotova & Lencki (2008) observed the influence that PL had in milk fat crystallization during butter making. When PL were added in the form of globular milk fat to reach a concentration of 0.66% PL spherulite formation was inhibited. However, when adding soy lecithin at a level of 1.0% spherulitic growth was stimulated until 2.0% lecithin was reached which resulted in a broken up network. Overall, previous studies suggest that different combinations of fats and lecithins can induce, retard or have no effect on crystallization. The percentage of lecithin in a fat may also show varying crystallization patterns as the concentration of lecithin or PL is increased.

Studies discussed above that evaluate the use of lecithins to change the crystallization behavior of fats either use vegetable lecithins or pure phospholipids. No studies were performed with milk-based lecithins. Milk polar lipids offer a potential new functionality due to higher PS levels compared to plant based lecithins and the presence of SM. Therefore, the objective of this research was to identify if milk PL would influence the crystallization behavior of AMF and to characterize the physical properties of the crystalline network formed.

Materials & Methods

Materials

PL were isolated from a commercial source of PC700 (Fonterra, Auckland, New Zealand) using the method described below. The chemical composition of the PC700 as provided by the specification sheet was: 85.0% total lipids with 59.2% of PC700 being composed of phospholipid. The lipid fraction of PC700 was composed of 3.0% PS, 31.0% PC, 8.7% PE, and 16.5% SM. PC700 also contained 2.5% moisture, 6.6% lactose and a maximum content of 12.0% ash.

PL Extraction

The extraction of PL from the PC700 was performed by creating a suspension of the PC700 in water and then precipitating out the PL using ethanol. Preliminary experiments in our laboratory have shown that the highest yield of PL was obtained with a 30:70 ratio of PC700:water. The PC700 and the water were mixed using a magnetic stir bar in a beaker on a magnetic plate set to 1100 RPM for 30 min at ambient temperature forming an emulsion. The emulsion was then separated into four beakers of 25 g, placed

on a magnetic stir plate with a stir bar set to 700 RPM, and ethanol was added to obtain a 1:2 emulsion:ethanol ratio by weight. The sample was stirred for 1 min after the ethanol was completely added and then given 5 min to rest while an agglomerate or precipitants formed. The ethanol:emulsion mixture was then vacuum filtered using a Büchner funnel and 47 mm diameter microfiber glass filter paper, and vacuum. This process allowed to separate the agglomerate from the rest of the materials in the PC700. The PLA was scraped off the filter paper and was placed on watch glasses under a hood for at least 36 h to evaporate the ethanol. Samples were stored at 5 °C until the PLA was needed.

Hexane Phospholipid Agglomerate Purification

The PLA obtained from the previous step still contained some impurities, presumably, minerals. Therefore, a purification step was preformed using hexane. The hexane PLA purification was performed by making a 1:9 ratio of the agglomerate obtained from the previous step and hexane (AOCS Official Method Ja 3-87). The agglomerate was weighed in an Erlenmeyer flask and placed on a magnetic stir plate with a magnetic stirrer bar. The Erlenmeyer was covered with aluminum foil to minimize evaporation. The sample was agitated for 10-15 min until the agglomerate had dissolved in the hexane leaving a solid residue suspended in the media. The suspension was then poured into two 15 ml centrifuge tubes, passing a wash of hexane over the Erlenmeyer flask to remove any residuals of the hexane and sample bringing both 15 ml centrifuge tubes to a final volume of 6 ml. Samples were then centrifuged (IEC Centra CL3R, Thermo Electron Corporation, Waltham, Massachusetts, U.S.A.) using an 809 rotor at 1,000 x g and at 24 °C for 5 min. A white sediment could be seen at the bottom of the centrifuge with a yellow transparent solution on top. The supernatant was separated from

the sediment and was transferred into a beaker. The centrifuge tubes were then brought up to a 4.5 ml volume using hexane to wash the sediment and improve the recovery of PL and vortexed until the sediment was brought back into solution. The samples were then centrifuged again, and the supernatant was transferred to the beaker as previously described. This washing process was performed 3 times. The centrifuge tubes and the beaker were then left under a hood for at least 12 h until the hexane evaporated off. The obtained material after evaporation will be referred to as PLI. The process to obtain PLI (extraction and purification process) was repeated 4 times. All replicates were mixed and used for the NMR characterization described below and added to the AMF.

Thin Layer Chromatography (TLC)

The purity of the PL obtained after isolation was evaluated by doing TLC. TLC was performed in duplicate on 4 replicates of the PLI to ensure that the total PL concentration was consistent in each extraction. TAG, cholesterol, PC, FFA, CE, MAG, 1-2 DAG, 1-3 DAG, standards were also ran to be able to identify all of the compounds in the sample. A TLC plate (20 × 20 cm silica gel, 60 A particle, 250 µm layer) (Whatman, Little Chalfont, Bucks, UK) was pre-washed and developed using a 100 ml mixture of chloroform and methanol at a 1:1 ratio by volume, then activated in the oven at 100 °C for 10 min. The PL (2.5 mg) samples were dissolved in 10 µl of Folch Solution (2:1 $CHCl_3$:MeOH 0.01% BHT), dried using nitrogen gas, and then dissolved in 10 µl of Folch Solution and were placed 1 cm from the bottom of the plate and the plate was air dried. Once the plate was dry it was sprayed with a 10 % w/v cupric sulfate solution in 8% w/v orthophosphoric acid until it was translucent (Baron & Coburn, 1984). The plate was heated in an oven at 145 °C for 10 min (Churchward et al., 2008). A picture was

taken of the plate using a Nikon d810 Camera (2014 Ayutthaya, Thailand) and analyzed using Image Lab 6.0.1(2017 Bio-Rad Laboratories Inc.). In this software the picture color was first inverted, then the columns were identified individually. The bands and band size were identified, and the pixel count and intensity were identified and given, allowing for the percentage of components in the sample to be identified. Peillard et al. (2019) used and developed a similar method to be able to quantify the amount volatile fatty acids in a sample using a similar software.

Identification of PL through Nuclear Magnetic Resonance ^{13}P (^{31}P NMR)

^{31}P NMR was performed using a Bruker AVANCE III 500 MHz high resolution spectrometer (Bruker, Karlsruhe, Germany) equipped with a Bruker Smart Probe. The proton spectra were recorded at 500.132 MHz and the phosphorous spectra was recorded at 202.456 MHz. The methods reported by Lehnhardt et al. (2001) and MacKenzie et al. (2009) were used. A detergent solution was prepared as follows: 1 g of sodium cholate (from Alfa Aesar) was dissolved in a 10 mL deuterium oxide (purchased from Aldrich)/water solution (20/80% v/v). To this was added ethylenediaminetetraacetic acid disodium dehydrate (purchased from Mallinckrodt Pharmaceuticals) (1% w/w) and Phophonomethylglycine (from Frontier Scientific) (0.3 g/L). The pH was adjusted using a Mettler Toledo Seven Compack pH/ion S220 pH meter to 7.40 using a sodium hydroxide solution (0.02 M) and the solution became clear. Four samples of PL (20 mg) were mixed with detergent solution (750 μL). Sodium hydroxide was added to the remaining detergent solution to raise the pH to 8.51 and a further 4 samples were made. All 8 samples were sonicated using a Branson 2800 sonicator for 30 minutes. Samples were transferred into 5 mm diameter NMR tubes. Spectra were measured 25 °C using

the following acquisition parameters: 196 acquisitions, 54k data points, 5000 Hz sweep width, 5.4 second acquisition time, and a 3.5 second relaxation time. All ^{31}P spectra were obtained using inverse gated proton decoupling (Bruker: igzg) to minimize the nuclear Ovenhauser effect. A line broadening of 0.2 Hz was applied to the obtained spectra. The spectrawere processed using MestReNova v12.0.2 from Mestrelab Research S.L. Zerofilling was performed to increase the number of data points to 64k. Quantitative global spectra distribution using 6 improvement cycles was applied to help deconvolute overlapping peaks. The analysis was done after isolating sufficient PL required to obtain the PL mixtures and their replicates.

Mixtures

PL were added to AMF at three different concentrations: 0% PL, 0.01% PL, and 0.1% PL. To improve the solubility of the PL in the AMF, PL was added to the AMF by first dissolving it in hexane 1:9 ratio PL:hexane w/v under 700 RPM agitation. The PL in hexane solution was added to the melted AMF and mixed for 10 min at 700 RPM, left under the hood for 48 h or until all the hexane had evaporated off. The evaporation process was performed at room temperature and heated every 4-6 h to keep the sample liquid.

Crystallization kinetics – Solid Fat Content

The crystallization kinetics of the AMF with and without the addition of PL was measured by following the variation of SFC as a function of time. Each sample was melted in a microwave for 1.5 min or until liquid and placed in 5 tempered p-NMR tubes. The samples were then placed in an oven at 65 °C for 30 min. The tubes were then placed

into a water bath that were held at the crystallization temperature (T_c) (24 °C, 26 °C, and 28 °C) for 90 min. SFC was measured during 90 min every 5 min from the moment the tube was placed in the water bath (t=0) until SFC began to increase exponentially in which case SFC was measured every 2 min. When the SFC reached a plateau, SFC was measured every 5 min. The SFC was measured in triplicate runs using an NMR Minispec mq 20 analyzer (Bruker, Karlsruhe, Germany). After 90 min of crystallization, two tubes from each run were stored at 5 °C for 48 h and the SFC was measured again after this storage period to evaluate how the crystalline matrix evolved during storage at a lower temperature.

The crystallization kinetics was quantified using the Avrami equation (Marangoni, 2005) [4-1].

$$\frac{SFC(t)}{SFCmax} = 1 - e^{-kt^n} \quad [4\text{-}1]$$

Where SFC_{max} is the maximum of SFC reached when the sample reaches equilibrium, *k* is the rate of crystallization, *t* is the time, and *n* is the Avrami exponent which describes the growth and nucleation of the crystals (Marangoni, 2005). GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA) was used to fit the data to the Avrami equation.

Melting Point and Melting Behavior

The melting point and melting behavior of the crystallized samples were measured in triplicate using a DSC (DSC Q20; TA Instruments, New Castle, DE, USA). The melting points of the various AMF:PL mixtures were measured by heating 10-15 mg of the sample up to 80 °C for 30 min to erase any crystal memory; decreasing the

temperature at a rate of 5 °C/min to -20 °C and keeping the sample isothermally at -20 °C for 90 min to allow complete crystallization of the sample. Lastly, the sample was heated to 80 °C at a rate of 5 °C/min. The melting point was identified as the peak temperature (T_p) of the highest melting peak from the last heating ramp from -20 °C to 80 °C.

The melting behavior of the samples crystallized at 24, 26, and 28 °C was evaluated from the tubes used for SFC determination. After 90 min at T_c a sample (10-15 mg) was taken from the NMR tube and placed in a Tzero hermetically sealed pan and placed in TA DSC Q20 (TA Instruments, New Castle, DE, U.S.A.). The initial temperature was the same as the T_c and then increased 5 °C/min until 80 °C was reached. From all DSC graphs the onset temperature (T_{on}), the peak temperature (T_p), and change in enthalpy associated with the melting process (ΔH) were calculated.

Similar to the description for SFC the melting behavior was also measured after storing the sample at 5 °C for 48 h to evaluate how the crystalline matrix evolved during storage at a lower temperature. In this case the initial temperature in the DSC was 5 °C and the temperature increased at 5 °C/min until reaching 80 °C.

Crystal Size and Morphology

Crystal morphology was observed in each of the triplicate runs using a PLM (Olympus BX 41, Waltham, MA, USA) using a 10X magnification objective and a digital camera (model Infinity 2, Lumenera Scientific, Ottawa, ON, Canada). Pictures of the crystals were taken from the samples used to measure SFC after 90 min at T_c. Crystal microstructure was also measured after storing the sample at 5 °C for 48h.

The crystal diameter was measured from the PLM using Image-Pro Plus version 7.0 software (Media Cybernetics, USA). All crystals with a diameter of <4 μm were identified as background and were excluded from the analysis of the data. The crystals measured at 48 h were those that could be clearly identified as the crystals that formed at 90 min.

Viscoelasticity

The viscoelastic property of the samples was measured in triplicate using a rheometer (AR-G2, TA Instruments, New Castle, DE, U.S.A.). Samples were prepared by placing 30 g of sample in a 50 ml centrifuge tube and melted in a microwave for 2 min or until the sample was liquid. The sample was then placed in an oven for 30 min and then placed in a water bath held at the T_c for 90 min. The viscoelastic properties were then measured by using a 40 mm-diameter geometry that was held at T_c. The δ, G', and G" were recorded and each replicate was measured three times using a strain sweep step (0.001-10 % strain) oscillatory procedure (1 Hz) with a gap ranging from 500-1500 μm depending on how liquid the sample was. The 50 ml centrifuge tube was then stored at 5 °C for 48 h and an 8-mm core sample was taken from the centrifuge tube to measure the sample viscoelasticity. The parallel plate was set at 5 °C and an 8-mm-diameter geometry was used. Each replicate was measured three times using the same procedure as the 90 min samples and a 1,000 μm gap was used. The data used was taken from linear region at the 0.1 % strain.

Hardness

The hardness of the sample was measured in triplicate using a TA-XT Plus Texture Analyzer (Texture Technologies, Scarsdale, NY, USA). Samples were prepared by melting the mixture in the microwave for 3 min or until the sample was liquid and placing the sample in an oven at 65 °C for 30 min. The samples were then placed in 1 cm diameter disposable test tubes and placed in an incubator held at the T_c for 90 min. Samples were too soft to allow for hardness measurement after 90 min and therefore samples were stored at 5 °C for 48 h. After this storage samples were removed from the test tube, cut into 0.5 in height cylinder and tested for hardness using a 5 cm-diameter cylindrical probe using a calibrated 5 kg load. Each experimental replicate was measured 5 times.

Polymorphism

The polymorphism of all AMF samples and 0.01% and 0.1% samples crystallized at 24 and 26 °C was identified using X-ray diffraction (XRD) Philips X'Pert 3040 MPD (PANalytical, Almelo, The Netherlands) diffractometer system with a single PW3050/ 00 (θ/2-θ) goniometer. A Cu Kα radiation X-ray source was used along with a PW3123/00 Monochromator detector. The software required to utilize the equipment was X'Pert Data Collector (v2.0e) (PANalytical, Almelo, The Netherlands). The samples were crystallized using the same crystallization method used for viscoelasticity and at the 90 min mark the crystals were vacuum filtered using a Büchner funnel and a 47 mm diameter microfiber glass filter paper, and a vacuum flask that was connected to a vacuum. When the crystals where dry (>10 min of filtration) the crystals were placed on to an X-ray slide. After the analysis at 90 min the slides were stored at 5 °C for 48 h and analyzed once more.

Statistical Analysis

Statistics were performed on parameters using 2-way ANOVA and GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA) was the statistical software used for all of the statistical analyses at 90 min, excluding crystal diameter, and SFC at 48 h. Statistical Analytical Software (SAS) Studio (2019, SAS Institute Inc., Cary, NC, USA) was used to do the remaining 48 h analyses and the 90 min crystal diameter.

Results & Discussion

Crystallization Kinetics and Solid Fat Content

The SFC was plotted as a function of time during the 90 min of crystallization as can be seen in Figure 4-1. As temperature and PL concentration increased a slower crystallization was observed. Data shown in Figure 4-1 were fit to the Avrami equation where SFC_{max}, k, and n were calculated and are reported in Table 6. The SFC curves did not follow a sigmoidal shape and a maximum SFC was never reached during the 90 min at T_c. The AMF crystallized at 28 °C and the 0.1% PL sample crystallized at 26 °C could be fit to the Avrami equation but the R^2 of both samples was very low (R^2=0.74 and 0.36 respectively) and cannot be considered reliable (Table 4-1). The rate of crystallization (k) decreased as the concentration of PL increased with a decrease of two orders of magnitude for AMF crystallized with 0.1% PL at 24 °C and for obtained after 90 min (Figure 4-1) are considerably different. At 24 °C the SFC at 90 min AMF with 0.01% PL crystallized at 26 °C compared to their AMF counterparts. The SFC showed no significant difference between samples (11.0 ± 0.3%). The samples crystallized at 26 °C showed a significant difference between AMF and 0.01% PL (8.8 ± 0.4%) and 0.1% PL

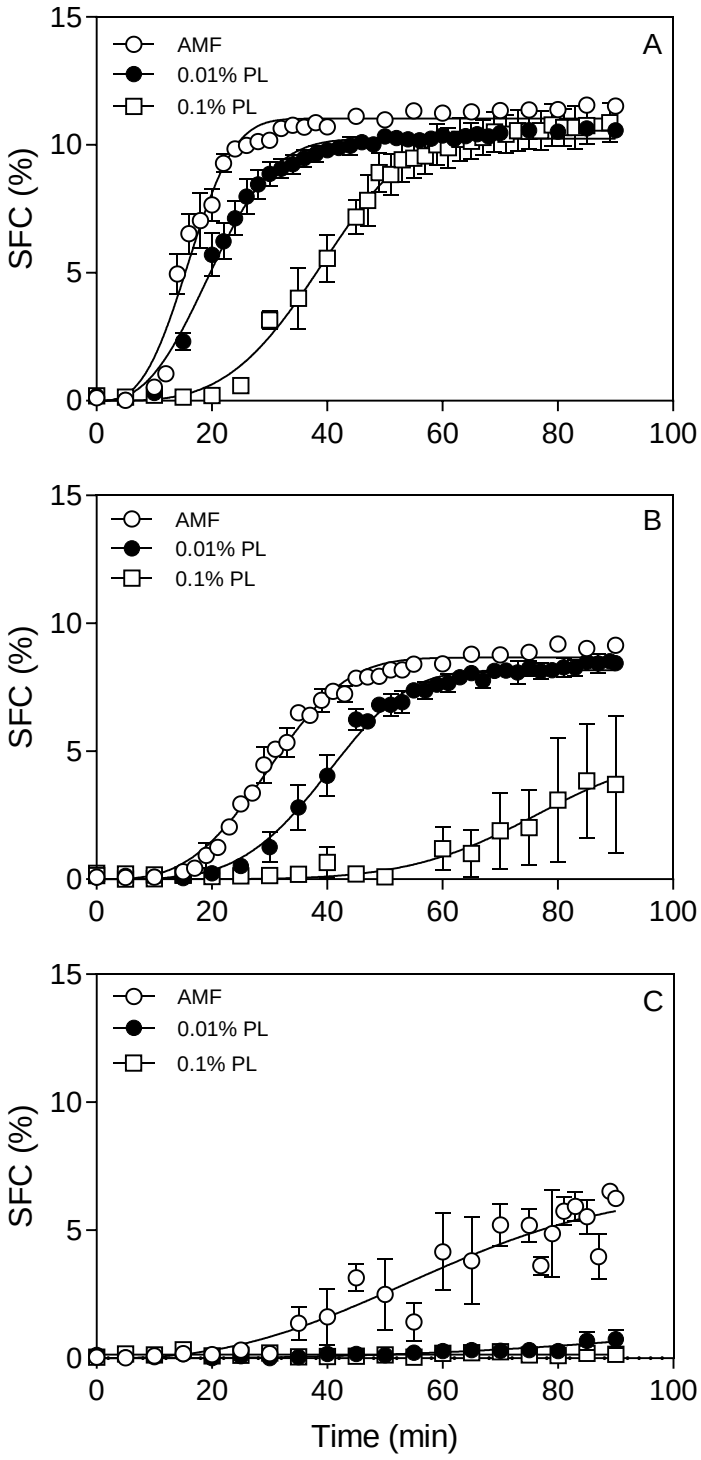

Figure 4-1: SFC of samples (AMF, AMF+0.01% PL, AMF+0.1% PL) as they crystallized over 90 min at 24 °C (A), 26 °C (B), and 28 °C (C).

Table 4-1: Parameters obtained from the Avrami fitting performed in the SFC data reported in Figure 4-1.

	Temp.	AMF	AMF + 0.01 % PL	AMF + 0.1 % PL
SFC_{max} (%)	24 °C	11.0 ± 0.1	10.2 ± 0.1	10.6 ± 0.2
	26 °C	8.7 ± 0.1	8.2 ± 0.1	4.6 ± 4.0
	28 °C	6.6 ± 1.7	N/A	N/A
k^{min-1}	24 °C	$1.7x10^{-4} \pm 1.0x10^{-4}$	$2.4x10^{-4} \pm 1.2x10^{-4}$	$1.3x10^{-6} \pm 1.6x10^{-6}$
	26 °C	$1.2x10^{-5} \pm 5.7x10^{-6}$	$2.5x10^{-7} \pm 2.5x10^{-7}$	$4.8x10^{-11} \pm 8.2x10^{-10}$
	28 °C	$2.6x10^{-5} \pm 7.0x10^{-5}$	N/A	N/A
n	24 °C	3.0 ± 0.2	2.7 ± 0.2	3.6 ± 0.3
	26 °C	3.2 ± 0.1	4.0 ± 0.3	5.4 ± 4.2
	28 °C	2.5 ± 0.8	N/A	N/A
R^2	24 °C	0.96	0.97	0.94
	26 °C	0.98	0.97	0.36
	28 °C	0.74	N/A	N/A

(3.7 ± 2.7%). Samples crystallized at 28 °C showed a significant difference between AMF (6.2 ± 0.3%) and samples that contained PL (0.1 ± 0.0%). These results show that as the concentrations of PL and temperature increased, the maximum SFC decreased. All samples for AMF and the sample at 0.01% crystallized at 24 °C had *n* values close to 3 suggesting either disc-like growth from sporadic nuclei or spherulitic growth from instantaneous nuclei. All other samples with the addition of PL that could be fit to the Avrami equation had *n* values close to 4 suggesting spherulitic growth from sporadic nuclei (Marangoni, 2012). Literature showed similar results when soy lecithin was added at a 0.2% level to cocoa butter, although the results showed less variation with all samples resulting in a mix of instantaneous disc structure or spherulitic spontaneous structures (Miyasaki et al., 2016). Vanhoutte et al. (2002) reported *n* values similar to the ones reported in our study for AMF with 0.01% PL crystallized at 24 °C, but the other samples with 0.01% PL and 0.1% PL that showed spherulitic growth from sporadic

nuclei did not compare with Vanhoutte's results. Slight differences between Vanhoutte's and our results might be due to variations in processing conditions such as temperature (25 °C), PL concentration (PL%<0.03%), and crystallization time (3 h) but also to the type of PL used. While Vanhoutte's group used soy lecithin, our work used milk-based PL. Based on the *n* values obtained in our research it is possible, that independently of the T_c used, the addition of PL induced the formation of spherulitic-shaped crystals. This type of crystal morphology is usually observed in slowly crystallized samples which is the case of the PL-based samples (Martini et al., 2002). The SFC was also measured after 48 h and no significant difference between the samples was observed (SFC=45.0 ± 0.6%; $P>0.05$). The SFC after 48 h being non-significantly different indicates that a secondary crystallization is occurring. This secondary crystallization would be due to the high super cooling that is introduced (5 °C) after 90 min, indicating that the samples with a lower SFC after 90 min (0.1% PL at 26 °C and 28 °C samples) may result in smaller crystals (Martini et al., 2002) influencing the properties of the crystalline network.

Overall, results show that the addition of milk PL delayed the crystallization of AMF. The melting point of AMF with and without PL was not statistically different with a T_p of 33.2 ± 0.1 °C. A difference in enthalpy was seen between the samples containing PL (71.1 ± 3.4 W/g) and the AMF sample (84.4 ± 1.6 W/g) which indicates that fewer crystals were formed during the crystallization with the addition of PL. Since the melting point of the samples was not affected by PL addition, it is likely that the delay in crystallization observed is due to changes that occur during crystallization at the molecular level. Previous studies have suggested that when molecules used as additives are similar to the TAGs, as would be the case of phospholipids, they can be incorporated

into the crystalline matrix and delay crystal growth. It has been shown that minor components can play an effect in the crystallization characteristics of lipids (Smith, 2000). The presence of mono- and diacylglycerols in our PL isolate can also contribute to a delay in the crystallization as previously reported by Wright et al. (2000) and Wright and Marangoni (2003).

Crystal Morphology

Figure 4-2 shows the morphology of crystals obtained for the samples. For the AMF samples crystallized at 24 °C it is evident that crystallization occurs with the formation of instantaneous nuclei forming many disc-like shaped crystals. The same behavior is observed for AMF crystallized at 26 °C but in addition to the disc-like crystals traces of spherulitic crystals can be seen. Samples crystallized at 28 °C show clear spherulites. The addition of PL promoted the formation of spherulites. As the concentration of PL increased, spherulites became bigger in size. Smith (2000) also observed similar changes in the morphology of palm oil with PL. The results showed an increase in size with the addition of PL due to the retardation of nucleation and the stunting of crystallization. Another visible difference is the very distinct Maltese-cross that appears when PL is added. No crystals were observed for samples crystallized at 28 °C with the addition of 0.1% PL and the sample at 0.01% PL had few crystals at 90 min resulting in the crystals that formed after 48 h were due to the secondary phase of crystallization during storage and not the crystallization temperature.

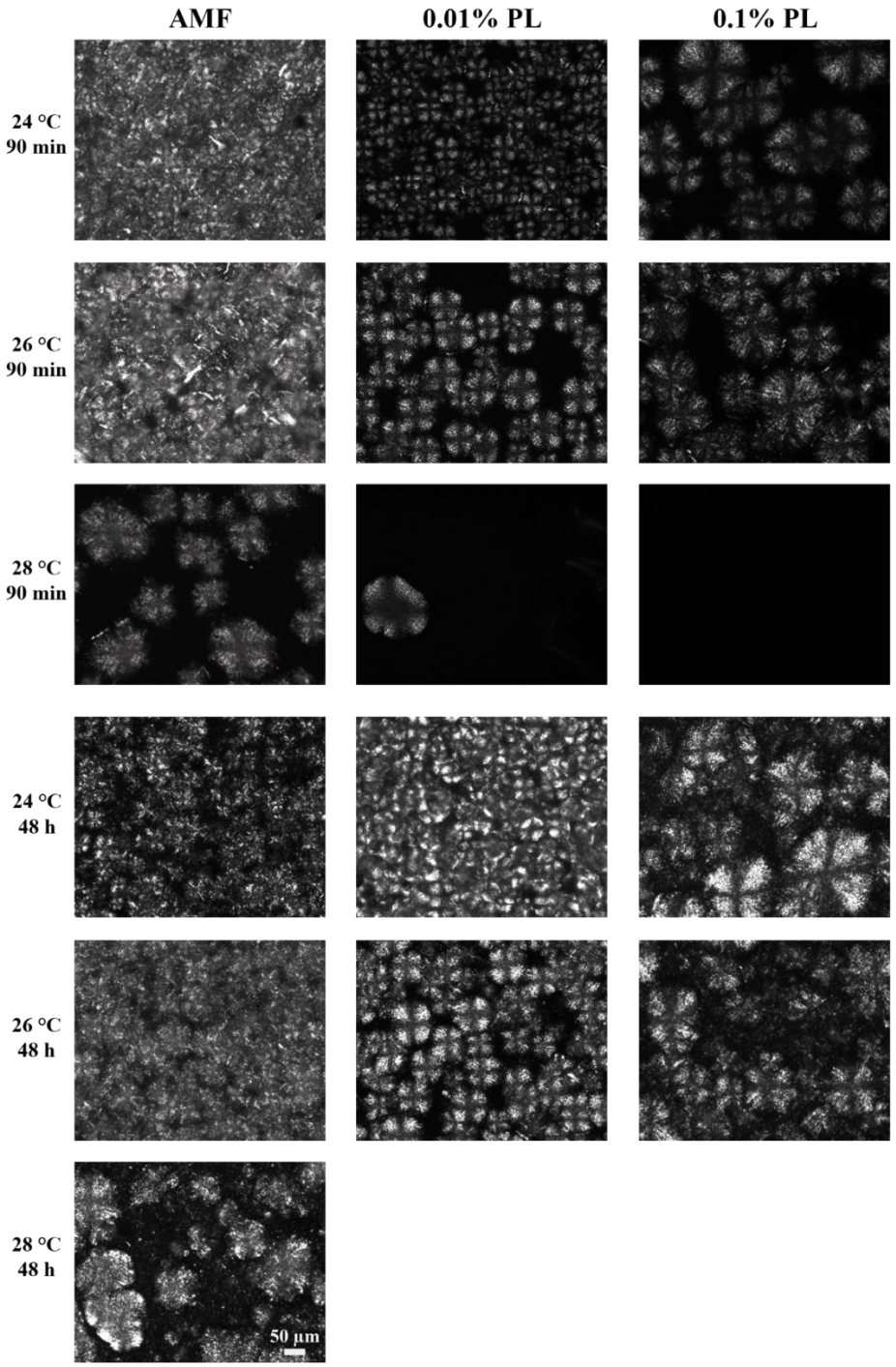

Figure 4-2: Crystal morphology of all samples (AMF, AMF+0.01% PL, AMF+0.1% PL) at 90 min at different crystallization temperatures (24 °C, 26 °C, 28 °C) and after being stored at 5 °C 48 h.

Table 4-2: Crystal diameters (μm) obtained from the PLM at 90 min after crystallization and after being stored at 5 °C for 48 h. Variables within the same time with the same letter are not significantly different (α=0.05). N/A=Data was not reported since no crystals were observed at 90 min.

Time	Temp (°C)	AMF	AMF + 0.01 % PL	AMF + 0.1 % PL
90 min	**24**	14.8 ± 2.2^{d}	54.2 ± 6.3^{ac}	96.2 ± 2.2^{b}
	26	14.6 ± 3.4^{cd}	75.6 ± 16.5^{abc}	118.4 ± 18.2^{ab}
	28	72.0 ± 8.9^{abcd}	114.3 ± 31.0^{ab}	NA
48 h	**24**	10.2 ± 0.1^{c}	52.0 ± 6.1^{b}	97.9 ± 6.5^{a}
	26	20.6 ± 2.8^{bc}	67.4 ± 7.5^{ab}	119.1 ± 20.8^{a}
	28	51.3 ± 22.5^{abc}	N/A	N/A

After measuring the crystal size diameter at both 90 min and 48 h (Table 4-2) a trend was observed, whereas the diameter increased with concentration of PL and temperature. At 90 min there was a significant difference between AMF (14.8 ± 2.2μm) and samples containing 0.01% PL (54.2 ± 6.3μm) and 0.1% PL (96.1 ± 2.2 μm) at 24 °C (P<0.0001), while at 26 °C the only a difference was seen between AMF (14.6 ± 3.4μm) and 0.1% PL (118.4 ± 18.2μm) (P=0.0020). At 48 h all samples crystallized at 24 °C were significantly different from one another (P<0.001), but the significant difference at 26 °C was only between AMF (20.6 ± 2.8μm) and 0.1% PL (119.1 ± 20.8μm) (P=0.0014), similar to the 90 min sample. It is understood that as temperatures increase crystallization rate decreases due to lower driving force for crystallization (Wright et al., 2000). The effect of retardation was due to both the addition of PL and an increase in temperature. This is also supported by Martini et al. (2002) in the theory that as the time it takes for crystals to form increases, the size of the crystals also increase. It is interesting to note that the addition of PL not only changes the crystal size but also crystal

morphology. Changes in crystal morphology could be due to difference in crystallization kinetics but also to different polymorphic forms. XRD analysis showed that AMF samples crystallized at 24, 26, and 28 °C formed a β' polymorphism with signals observed at 4.2 and 3.8 Å. When these samples were stored at 5 °C for 48 h a β polymorph was only observed for the sample crystallized at 28 °C that coexisted with the β' form (signals observed at 4.8, 4.2, and 3.8 Å). The addition of 0.01% PL induced the formation of a β and β' polymorph only for the sample crystallized at 24 °C with peaks detected at 4.6, 4.2, and 3.8 Å, but only the β' polymorphism was observed after 48 h of storage at 5 °C. The addition of 0.1% PL to AMF crystallized at 24 °C induced the formation of β' polymorphism only with signals at 4.2 and 3.8 Å. The effect of PL addition to AMF crystallized at 26 °C was somewhat contrary to the one observed at 24 °C. The addition of 0.01% of PL generated at β' polymorphism with signals at 4.2, 4.1 and 3.8 Å; while the addition of 0.1% of PL generated a mixture of β' and β polymorphs with signals at 4.8, 4.2 and 3.9 Å. These two polymorphic forms were maintained for the samples even after storage at 5 °C for 48 h.

Melting Behavior

The DSC melting profiles are shown in Figure 4-3 and the DSC parameters in Figure 4-4. As expected, T_{on} increased with T_c ($P<0.05$). The addition of PL increased T_{on} but this change was only significant for samples crystallized at 26 °C indicating that

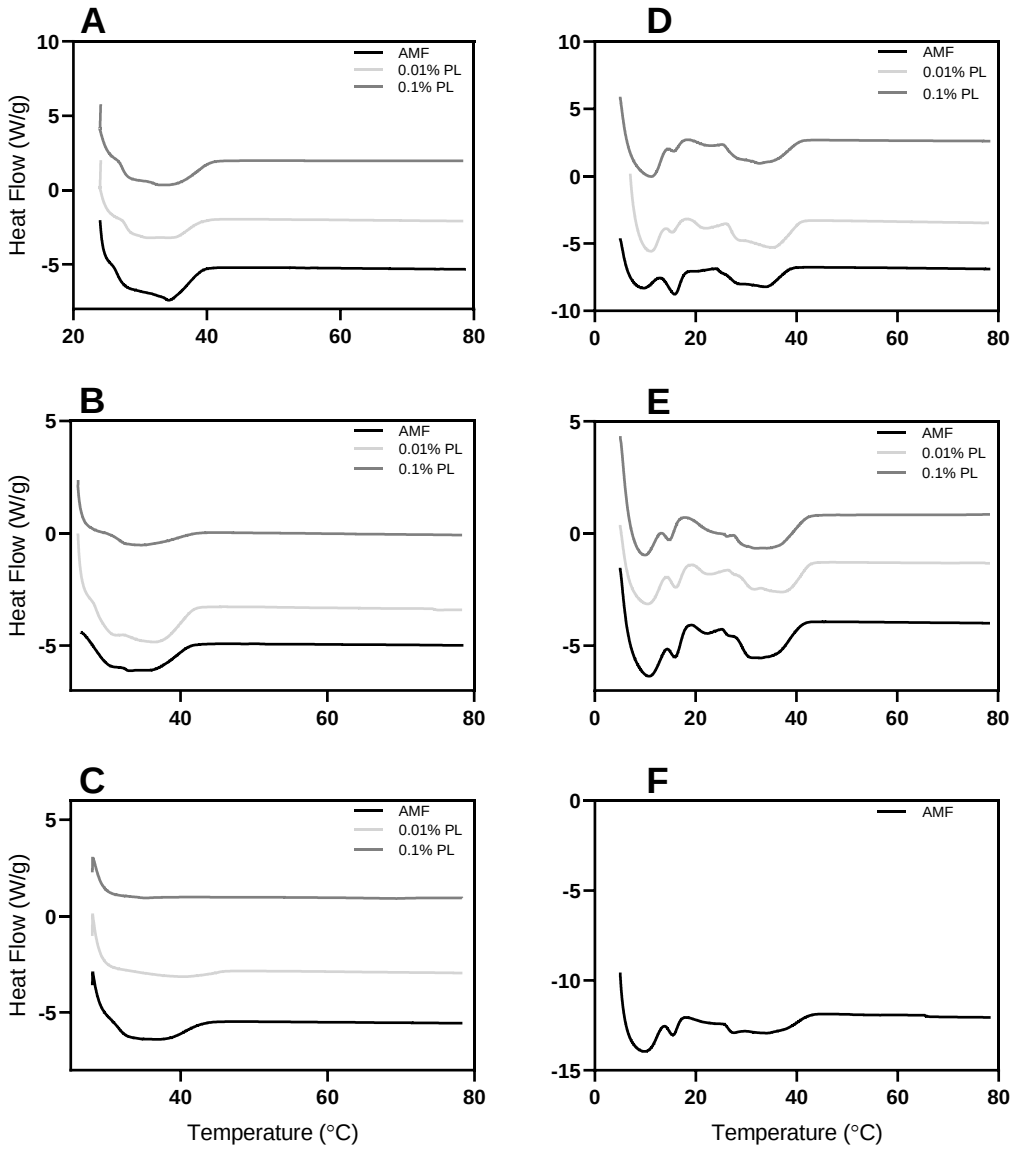

Figure 4-3: Melting profiles of all samples (AMF, AMF+0.01% PL, AMF+0.1% PL) at 90 min after crystallization temperatures 24 °C (A), 26 °C (B), and 28 °C (C). The samples were stored at 5 °C for 48 h and the melting profile was analyzed for each of the samples crystallized at 24 °C (D), 26 °C (E), and 28 °C (F).

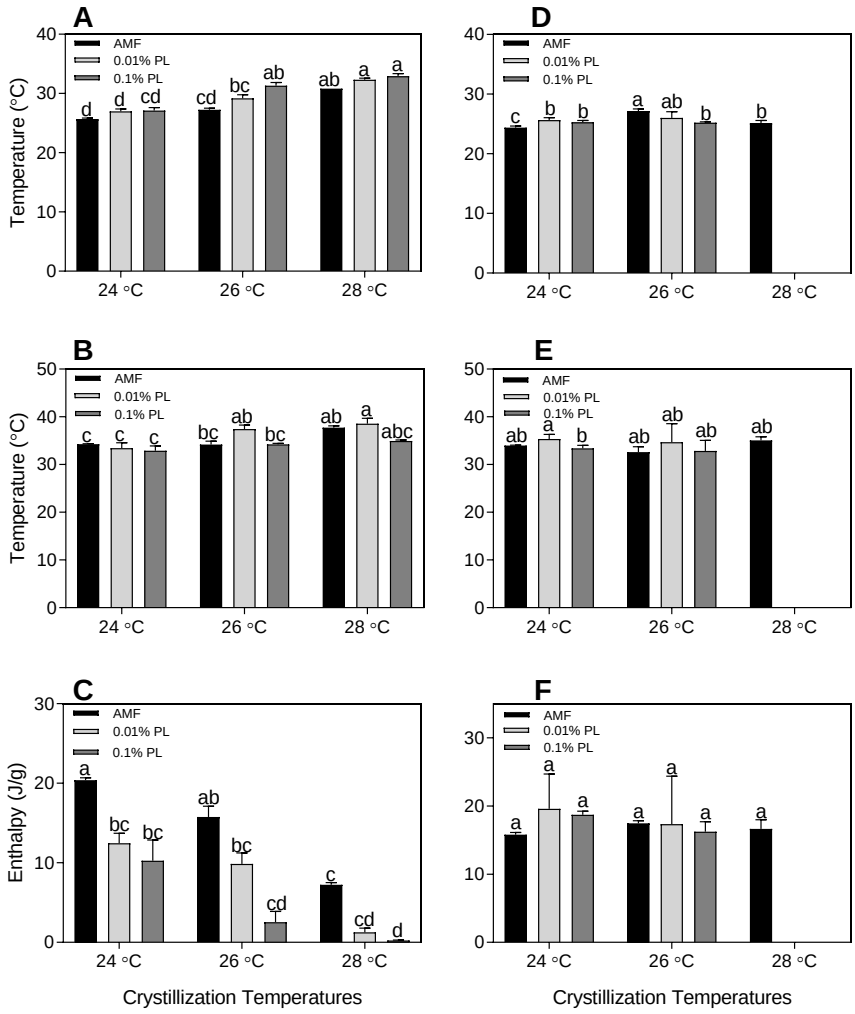

Figure 4-4: Melting parameters T_{on} (A), T_p (B), and ΔH (C) of all samples (AMF, AMF+0.01% PL, AMF+0.1% PL) at 90 min after crystallization temperatures (24 °C, 26 °C, 28 °C). The samples were stored at 5 °C for 48 h and the melting profile was analyzed for each of the samples from crystallization temperatures (24 °C, 26 °C, and 28 °C) for each of the parameters T_{on} (D), T_p (E), and ΔH (F). Data was analyzed using a Tukey multiple comparison two-way ANOVA test. Parameters within the same graph with the same letter are not significantly different (α=0.05).

milk PL had a higher influence on crystallization at 26 °C than at other crystallization temperatures (P<0.05). The reason for the significant difference at 26 °C could be that low-and high-melting point TAGs crystallize at this temperature while only high-melting point TAGs crystallize in the samples where PL was added increasing the T_{on}. Similarly, at 24 °C both low- and high-melting point TAGs have crystallized at 90 min resulting in a lower T_{on} that is not affected by the addition of PL due to the high degree of supercooling. When samples were crystallized at 28 °C only high melting TAGs are crystallizing resulting in a higher T_{on} that is not affected by the addition of PL. It is possible that at 26 °C PL are inhibiting the crystallization of low-melting point TAGs but that this effect is not seen at 28°C since low-melting point TAGs are not crystallizing at this temperature even in the samples without the addition of PL due to the low supercooling. Similar results were observed by Daels et al. (2015) and Miyasaki et al. (2016) when they added 1.5% sunflower lecithin to vegetable fats and standard lecithin (0.2%, 0.5%, & 0.8%) to cocoa butter respectively. No difference was seen in the DSC melting profiles with the addition of PL (Figure 4-3) other than peaks were less defined for 90 min samples as PL increases. Contrary to the results shown by Miyasaki there was no difference seen in T_p as concentration of PL increased. Miyasaki observed that modified and unmodified soy lecithins decreased the T_p of cocoa butter and this could easily be attributed to the cocoa butter having many polymorphic forms. It was interesting to note that there was a significant difference for the enthalpy (ΔH) of the samples at 90 min. For samples crystallized at all T_c a significant difference (P<0.05) was observed between samples that contained PL and AMF. For samples crystallized at 24 °C this difference was significant even at low concentrations of PL (0.01%). These results

suggest that as the amount of PL increased, crystallization was delayed which is in agreement with the SFC (Figure 4-1) values, and crystal morphology (Figure 4-2) previously described. Smith (2000) also observed a reduction in nucleation and crystallization when he added soy lecithin and phospholipids to palm oil. The crystallization kinetics previously discussed shows that as PL concentration increased crystallization was delayed which means fewer crystals would be present at 90 min requiring less energy to melt the crystals that had formed (Foubert et al., 2008). Literature also suggests that as enthalpy increases it indicates homogeneity throughout the crystals creating a more interactive crystal lattice (Arishima et al., 1995; Seguine, 1991; Macmillan et al., 2002; Svanberg et al., 2013). After 48 h of storage at 5 °C two melting peaks were observed (Figure 4-3): one melting peak that occurred at low temperatures (below 20 °C) and a second melting peak that occurred at higher temperatures (above 20 °C). The T_{on}, T_p, and enthalpy values reported in Figure 4-4 D, E, and F are the ones obtained for the high-temperature melting peak since this peak is the one that represents that crystals formed during crystallization at T_c for 90 min. Note that melting parameters of samples crystallized with PL at 28 °C were not reported after storage at 5 °C. Since these samples showed little to no crystallization during the 90 min at T_c results obtained after storing samples at 5 °C will not provide any additional information to our results. We expect that small crystals will be formed due to the high supercooling that do not contribute to the study of isothermal crystallization events discussed in this research. A significant difference was observed for T_{on} and T_p at 48 h. The T_{on} of AMF samples crystallized at 24 °C with PL significantly increased from 24.4 ± 0.2 °C for AMF, 25.7 ± 0.2 °C for AMF with 0.01% PL and 25.3 ± 0.2 °C for AMF with 0.1% PL. Although

there is not a significant difference at 90 min, there is a similar trend that is shown which indicates that the T_{on} at 48 h could have been highly influenced by the crystals that form at 90 min. At 26 °C a significant decrease between the T_{on} of AMF sample (27.2 ± 0.2 °C) and the 0.1% PL sample (25.2 ± 0.1 °C) was observed. This is an opposite trend compared to the one observed at 90 min at 26 °C. It is possible that the lack of crystals produced at 26 °C in the 0.1% PL sample over 90 min allows for the remaining liquid oil to create smaller crystals that may be less stable and melt faster resulting in a lower temperature T_{on}. The T_p showed a significant decrease between PL samples crystallized at 24 °C at 48 h from 35.4 ± 0.6 °C to 33.4 ± 0.4 °C for the 0.01% PL with 0.1% PL samples respectively. This would be due to an interaction effect of the PL and temperature since it can only be seen at this temperature and not the others. The enthalpy after 48 h of storage showed no significant difference between samples (P>0.05).

Hardness

The hardness was measured after storage at 5 °C for 48 h. Statistical differences were observed at 24 and 26 °C. The samples at 28 °C that contained PL were not included due to the fact that a crystallization occurred due to the storage temperature rather than the T_c. During this secondary crystallization, many small crystals were formed that contributed to the harder crystalline network for samples that had lower final SFC at 90 min (Figure 4-1). The trends show that the hardest sample was obtained at 24 °C AMF (20.1 ± 1.5 N) followed by 0.1% PL (15.4 ± 0.9 N) and by 0.01% PL sample (13.1 ± 0.6 N). Only the AMF sample and 0.01% PL sample were significantly different at 24 °C (P=0.0007). Even though a tendency of higher hardness was observed at 0.1% PL addition vs. 0.01% PL addition, these differences were not significant (P>0.05, Figure 4-

5). The decrease in hardness as a function of PL addition observed at 24 °C is due to the delay in crystallization and the increase in crystal sizes previously discussed. At 26 °C the trend changed with the sample at 0.1% PL level being the hardest (25.6 ± 2.1 N), followed by AMF (20.1 ± 0.8 N) and then 0.01% PL (16.7 ± 0.9 N). This trend supports a theory that when a sample with a low SFC is stored at 5 °C for48 h a secondary crystallization occurs causing a small crystalline network resulting in harder samples. Significant difference at 26 °C were only observed between the 0.1% PL sample and the 0.01% PL sample (P=0.0011).

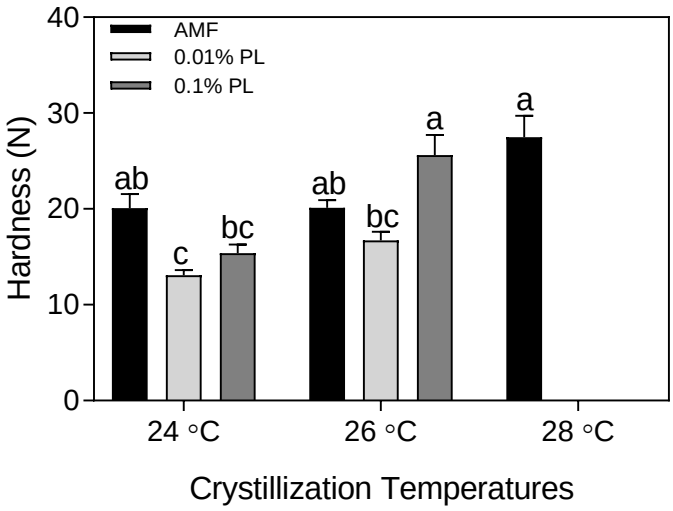

Figure 4-5: Hardness of all samples (AMF, AMF+0.01% PL, AMF+0.1% PL) after being crystallized for 90 min at crystallization temperatures (24 °C, 26 °C, 28 °C) and being stored at 5 °C for 48 h. The data was analyzed using a Tukey multiple comparison two-way ANOVA test. Samples with the same letter are not significantly different (α=0.05).

Viscoelasticity

Hardness data is difficult to interpret, especially for the samples crystallized at 28 °C and at 26 °C with the addition of PL since the secondary crystallization that occurs during storage might confound the results obtained. Therefore, measuring the viscoelasticity after the 90 min of crystallization might be a better measurement to evaluate how PL addition affects the elasticity of the sample. As the T_c increased the elastic modulus decreased due to lack of crystals in the sample (Figure 4-6). The only temperature that showed a difference between PL addition was 24 °C where the addition of PL resulted in a decreased G' (P<0.05). This could be due to the crystallization kinetics previously described. When PL were added to the sample crystallization was slowed down causing fewer crystals to be present compared to the AMF sample. When looking at the PLM of the AMF samples crystallized at 24 °C more diverse crystals were observed in AMF samples compared to the ones observed in samples with PL. G' values reported for samples crystallized at 24 °C follow the same trend as the hardness values previously discussed (Figure 4-5). G' values of samples crystallized at 26 °C followed the same trend that was observed with the hardness values at 26 °C. The viscous modulus (G") showed similar trends as the ones described for G' while no significant differences were observed for the delta values. After 48 h of storage at 5 °C G' values increased but showed no significant difference. Even after storage at 5 °C lower G' values were observed for samples crystallized at higher T_c but no effect on G' values was observed due to PL addition. Among samples stored for 48 h no significant difference in G" was observed other than at 26 °C where AMF ($5.4x10^5 \pm 1.0\ x10^5$ Pa) showed a

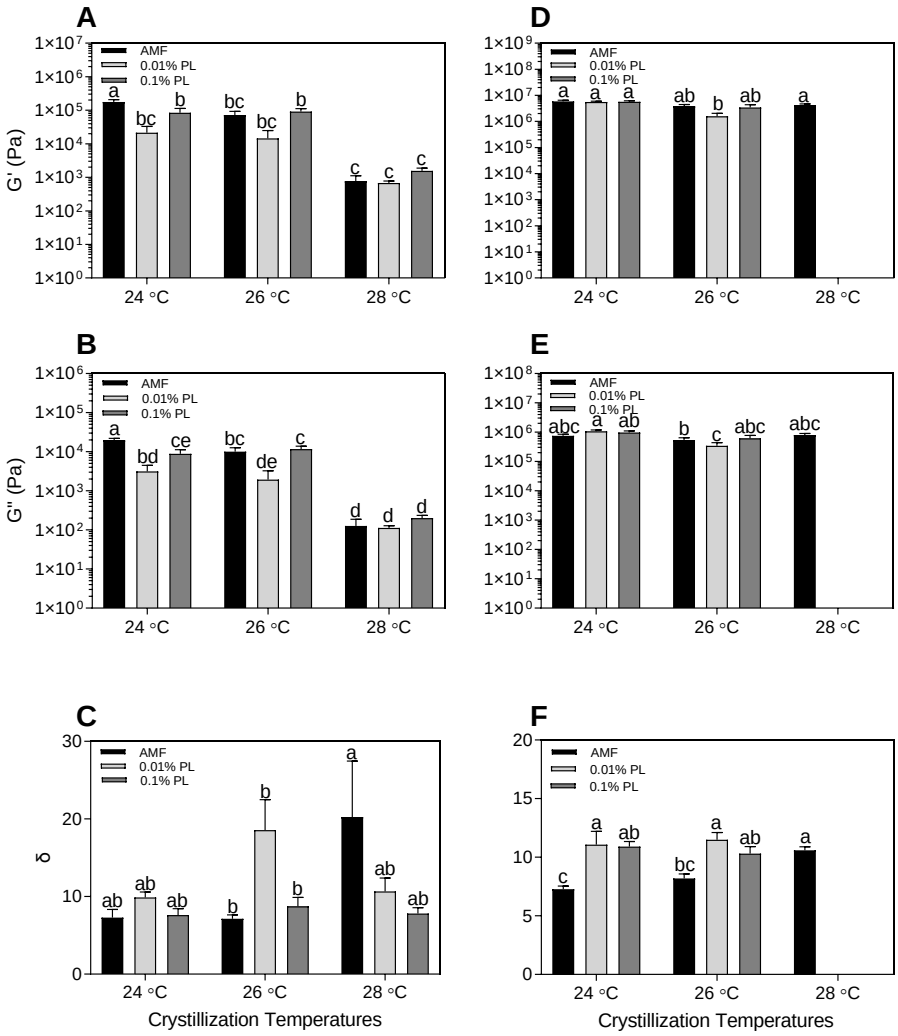

Figure 4-6: Viscoelastic parameters G' (A), G" (B), and δ (C) of all samples (AMF, AMF+0.01% PL, AMF+0.1% PL) at 90 min after crystallization temperatures (24 °C, 26 °C, 28 °C). The samples were stored at 5 °C for 48 h and the viscoelastic behavior was measured for each of the samples (D, E, and F). Data was analyzed using a Tukey multiple comparison two-way ANOVA test. Parameters within the same graph with the same letter are not significantly different (α=0.05).

higher G" than 0.01% PL ($3.4x10^5 \pm 9.9 x10^4$ Pa). This trend is the same trend reflected at 90 min, but shows a smaller difference between samples suggesting that during storage the growth of the crystals from liquid oil helped to decrease the differences between the PL samples and AMF.

Conclusion

The addition of milk PL retarded crystallization growth and reduced the amount of crystals that could be grown within 90 min. As the temperature increased the effect of PL on the delay of crystallization increased. The concentration of PL at low concentrations (0.01%) had a significant effect in terms of G', G", the 90 min enthalpy required to melt the sample, and the 48 h T_{on} at 24 °C, G" also showed to be lower at 26 °C at 90 min and 48 h, and the SFC at 90 min at high temperatures (28 °C). The 0.1% PL sample showed a significant effect on the SFC at 90 min above room temperature (26 °C and 28 °C), a decrease in the 90 min enthalpy at all temperatures, an increase in the T_{on} at 90 min, but decrease in the T_{on} at 48 h at 26 °C, an increase in the 48 h T_{on} at 24 °C, and a decrease in the G" at 90 min compared to AMF. The addition of PL also influenced nucleation and the crystal growth form. All these factors were due to the physical properties of the crystals which related to how the PL interacted with the crystals during crystallization, at 90 min, and as the samples were stored. The hardness after 48 h was influenced by the size, or lack thereof, of the crystals developed during the 90 min of crystallization. Overall it can be concluded that milk PL cause a delay in crystallization due to interaction that PL have with the crystals during crystallization.

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Chapter V

The Functional Traits of Dairy Phospholipid Gels

Abstract

In this study dairy PL gels were made using three different concentrations of PL (15%, 30%, & 45%) and soybean oil. After 24 h of storage the visual stability, morphology, SFC, melting behavior, viscosity, and oil binding capacity of the gels were evaluated. All samples showed visual stability, while the PLM showed that high concentrations of PL reduced PL mobility preventing tubular micelles to form at high concentrations of PL (45%). The SFC showed an increase with an increase in PL concentration ($P<0.0001$). The only parameter that showed significant difference ($P<0.0005$) in the DSC was the melting enthalpy between all samples. The viscosity was reported at low (0.01%), intermediate (0.1%), and high (1.0%) shear rates. Significant difference ($P<0.0001$) was observed between the 45% PL samples and the other samples at low and intermediate shear levels, but at high shear levels a significant difference was only seen between the 15% PL sample and the other samples ($P<0.0500$). The oil binding capacity showed a significant difference ($P<0.0001$) between the 45% PL sample and the other two samples.

Introduction

In the food, pharmaceutical, and cosmetic industry it is important for systems to be able to hold the proper structure of the product for consumer acceptance by way of texture and appearance, compound delivery, and functional purposes. A current method

in research to obtain structure and accomplish some of these tasks is by creating a gel in an organic solvent. According to Pehlivanoğlu et al. (2018) there are four different classes of compounds that cause gelation in organic solvents, these are known as oleogleators and are identified as forming gels by way of crystalline particles, obtaining self-assembled structures by low molecular weight compounds, self-assembled structures of polymers, and miscellaneous structure or inorganic particles. PL are a common ingredient in the food, pharmaceutical, and cosmetic industries and may serve as an oleogelator as a crystalline particle or a self-assembled structure with low molecular weight depending on the other components of the gel. PL have been shown to form a gelled system in the presence of various compounds such as soribitan tristearate (Pernetti et al., 2007), alpha tocopherol (Nikiforidis & Scholten, 2014), β-sitosterol and γ-oryzanol (Okuro et al., 2018), sucrose esters (Sintang et al., 2017), or water (Scartazzini & Luis, 1988). This functionality of PL helps to form emulsions and prevent phase separation, encapsulate compounds, and may give structure to products. Research shows that in general 95% of the PL used to form a gel must be PC. The PL source that is most commonly used to form gels comes from soy lecithin. In the dairy industry there are sources that are rich in PL, but contain high levels of impurities. Research shows that these PL can be isolated to obtain high concentrations of PL and obtain oil soluble compounds. These isolated PL sources from dairy stream show to have less than 95% PC due to the large variety of PL species that is found in milk including common ones know as PS, PC, SM, PA, PI, and PE. Currently there is little research that has been done on the functionality of dairy PL with no information on the gelling ability that they have. The

purpose of this study was to observe if dairy PL have the ability to form gels, and what influences the gelling and the physical properties of a dairy PL gel.

Materials & Methods

Isolation of PL

Phospholipids were isolated from a phospholipid concentrate (PC700, Fonterra, Auckland, New Zealand). The method used to isolate the PL was previously described in Chapter III and IV (Cooper et al., 2019). Briefly, to isolate the PL, PC700 was mixed in water at a ratio of 30:70, respectively, at ambient temperature for 30 min at 1100 RPM using a stir bar resulting in a total of a 110 g emulsion. The emulsion was separated into four beakers of 25 g and stirred at 700 RPM for 1 min at ambient temperature after the addition of EtOH at an emulsion:EtOH ratio of 1:2. Agitation was stopped and the sample was given 5 min to rest allowing for a PL agglomerate to form. The EtOH and agglomerate were filtered using a microfiber glass filter with a diameter of 47 mm, a Buchner funnel, and vacuum to separate a PLA agglomerate from the emulsion. The PLA was removed from the filter paper and allowed a minimum of 36 h to dry under a hood on a watch glass. The PLA was further purified using hexane at a ratio of 1:9, PLA:hexane. The mixture was placed in an Erlenmeyer, covered with aluminum foil to minimize evaporation, and agitated for a minimum of 10 min or until the PLA had fully dissolved. The mixture was placed into 15 ml centrifuge tubes washing the erlenmeyer with hexane to transfer any residual PL to the centrifuge tube. Samples underwent centrifugation (IEC Centra CL3R, Thermo Electron Corporation, Waltham, Massachusetts, U.S.A.) at 1,000 x g using an 809 rotor at 24 °C for a duration of 5 min. Sediments were found at the bottom of the centrifuge tube and the hexane mixture (supernatant) was transferred to a beaker.

The centrifuge tubes with the sediments were then brought to a final volume of 4.5 ml using hexane and vortexed until the sediment was suspended in the hexane. The solution was then centrifuged and decanted into the beaker. The addition of 4.5 ml hexane and vortexing the sample was repeated for a total of three times to obtain as much PL as possible. The PL/hexane solution was then left at ambient temperature for 48 h or until all the hexane was evaporated. The material obtained after these steps was called PLI.

Quantification of PL species

The quantification of PL species in the PLI obtained from the previous section was performed using P^{31} NMR, a Bruker AVANCE III 500 MHz high resolution spectrometer (Bruker, Karlsruhe, Germany) similar to what was reported in Chapter III and IV (Cooper et al., 2019) and adapted from Lehnhardt et al. (2001) and MacKenzie et al. (2009). Briefly, the phosphorous and proton spectra were recorded at 202.456 MHz and 500.132 MHz respectively. A detergent solution was prepared by mixing 1 g of sodium cholate (purchased from Alfa Aesar) and 10 ml deuterium oxide (from Aldrich) with water to create a 20:80 (v/v) sodium cholate deuterium oxide:water solution. Ethylene diamine tetra acetic acid disodium dehydrate (from Mallinckrodt paharmaceuticals) was added at a level of 1% (w/w) and with a final addition of 0.3 g/l of phophonomethylglycine (purchased from Frontier Scientific). A Mettler Toledo Seven Compack pH/ion S220 pH meter was used to adjust the pH to 7.40 by the addition of a sodium hydroxide solution of 0.02 M, becoming clear and resulting in the final detergent solution. The samples were prepared by mixing 20 mg of PL with 750 μL of the detergent in replicates of 4. The remaining detergent was mixed with sodium hydroxide to obtain a pH of 8.51 and 4 more replicates were produced. Sonication was introduced to

all samples for 30 min using a Branson 2800 sonicator. Samples were placed into 5 mm diameter NMR tubes. The acquisition parameters for the Spectra were 54k data points, 196 acquisitions, 5.4 second acquisition time, 3.5 relaxation time, and a 5000Hz sweep width at 25 °C. The ^{31}P spectra were identified by using inverse gated proton docoupling (Bruker: igzg) to minimize the nuclear Ovenhauser effect. The obtained spectra had 0.2 Hz line broadening applied and was processed using MestReNova v12.0.2 from Mestrelab Research S.L. The data points were increased to 64k by zerofilling and overlapping peaks were deconvoluted by way of quantitative global spectra distribution with 6 improvement cycles.For quantifying the individual PL species, the same method was used as described above but 12.5 mg of glyphosate was substituted with 0.3 g/l of phophonomethylglycine (purchased from Frontier Scientific).

Fatty Acid Composition

Thin Layer Chromatography

Previous research in our laboratory has shown that the PLI contained various species beyond phospholipids such as FFA, MAG, 2-DAG, 3-DAG, TAG, and CE (Chapter IV, Cooper et al., 2019). The fatty acid composition present in the phospholipids and the other compounds identified in our previous study were characterized. This characterization was performed by separating these compounds one from another using TLC and then identifying the fatty acid composition in each type of compounds. The separation of the different molecular species was performed by using a TLC plate (20 × 20 cm silica gel, 60 A particle, 250 µm layer) (Whatman, Little Chalfont, Bucks, UK). Samples were prepared using 25 mg of PLI, 50 µl of a standard composing of FFA (C21:0 7 mg/g), CE (C15:0 7 mg/g), 3-DAG (C17:0 3.5 mg/g), PC

(C19:0 7 mg/g), and TAG (C19:1 7 mg/g), and 250 µl Folch solution (2:1 $CHCl_3$:MeOH 0.01% BHT). MAG eluted in between the DAG band and PL band and therefore no standard is needed to identify the MAG. The samples were vortexed to dissolve the PLI and allow for a homogeneous solution. The TLC solvent used consisted of 80 ml hexane, 20 ml ethyl ether, and 2 ml formic acid. The fluorescent spray used consisted of 5 mg primulin and 25 ml of an acetone:water, 8:2, mixture. The TLC plate was prepared by activating it in a 55-60 °C oven for 10 min, removed and cooled. The samples were placed on the TLC plate 1.5 cm from the sides and 2 cm from the bottom, with 20 µl of sample every 2 cm each 0.5 cm apart. Samples were prepared in triplicate and each replicate was measured twice using two lanes on the TLC plate and one lane being used for the standard mixture which contained 50 µl of standard and 250 µl Folch Solution. Once all the replicates and standard mixtures were placed on the plate, it was placed in a container containing the TLC solvent and a 20x20 chromatography paper wick (Whatman, Little Chalfont, Bucks, UK). The plate was left in the container until the solvent was 1 cm from the top. The TLC plate was removed and left to dry. Once it was dry the fluorescent spray solution was applied to the TLC plate and left to dry. The dried plate was placed in a black box and UV light was used to identify the bands. Bands were identified and scraped from the TLC plate with a razor blade. The scraped material was placed in vials for further analysis.

Gas chromatography

In order to effectively quantify the FA of the lipid classes obtained from the TLC plate the FA had to be cleaved off the backbone molecules of the separate classes. To do so 630 µl of methanol and 10 N KOH was added to each vial individually and incubated

and agitated using a G24 Enviromental Incubator Shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ USA) at 55 °C for 90 min. Afterwards, 580 µl of 24 N H_2SO_4 was added and the vials were returned to the incubator and agitated at 55 °C for 90 min. Hexane was then added at a quantity of 500 µl and the samples were then centrifuged (IEC Centra CL3R, Thermo Electron Corporation, Waltham, Massachusetts, U.S.A.) at 140 x g for 5 min at 24 °C. The hexane layer was the pipetted off the top of the sample and placed in a vile where it was then dried using nitrogen, then dissolved in 100 µl of hexane. The vials were then analyzed using a GC-2010 Gas Chromatography (Shimadzu, Kyoto, Kyoto Prefecture Japan), 30mm x 0.25 mm i.d. x 0.2 µm film HP-88 column (Agilent, Santa Clara, CA, U.S.A.) and software GC Solution Postrun 2.42.00 (LabSolutions 2012) was used to analyze the peaks obtained from the gas chromatography (GC).

Phospholipid Gels

Three concentrations of PL were used to make structured lipids in soybean oil containing a final concentration of 14.93%, 29.89%, and 44.83% PL by weight resulting a final weight of 20 g. These concentrations were chosen to target approximately 15, 30, and 45% of PL in the sample. The required amount of PL was first weighed and dissolved in hexane at a 1:4 ratio, PL:hexane w/v, under agitation using a stir bar at 1100 RPM for 30 min or until the PL was fully dissolved at ambient temperature. Once PL was dissolved, soybean oil was added to the PL/hexane mix. The soybean oil (Food Club, Topco Associates LLC, Elk Grove Village, IL, USA) was obtained from a local grocery store, heated in a microwave for 1 min, and held at 75 °C for 30 min before adding to the PL/hexane mixture. After the PL, hexane, and oil were mixed the samples were left

agitating at 350 RPM using a stir bar and plate at ambient temperature for 24 h to eliminate the hexane. Agitation was applied to ensure complete elimination of hexane. Stir bars were removed and samples were left at ambient temperature for another 24 h to ensure gelling of the structured lipid was occurring and to allow any remnants of hexane to evaporate. Samples were then placed in an oven at 100 °C for 30 min to erase crystal memory and afterwards stored in an incubator at 25 °C for 24 h to induce gelation in a controlled manner. Three experimental replicates of each concentration were used for analysis.

Moisture content

The moisture content of the PLI, PL gel prior to being placed in the oven, and the PL gel after being placed in the oven at 100 °C was measured using the Karl Fishcer (KF) method adapted from (Ja 2b-87AOCS Official Method). The samples were prepared as described prior, and the KF titration equipment used was an open system Karl Fischer-Automat E 547 (Metrohm Herisau; Switzerland). The solvent was an anhydrous methanol with 99.8% purity (Sigma-Aldrich; Saint Louis, Missouri, USA) and the Aqualine Complete 5 Water Equiv.- 5 mg H2O/ml KF reagent (Fisher Scientific; Hampton, New Hampshire, USA).

The moisture content was measured by setting the parameters of the KF equipment to identify the final moisture content when an electrical current is obtained after having a voltage of 66 for 10 s under agitation to allow for the sample to be fully dissolved in the methanol. The KF was set up with a magnetic stir bar, titrator containing the KF reagent and corks to cover where samples were inserted. The assay was performed by first adding 30 ml of methanol to the cell or sufficient enough to cover the

probe. The methanol was then titrated to eliminate any residual water that may have been present. The KF factor was obtained by titrating 0.3 g of sodium tartrate dihydrate in triplicate and calculating the KF factor using the following equation [5-1]:

$$\frac{W_t * \frac{36\ g\ H_2O}{230.1\ g\ tartrate}}{V_{kf}} = KF\ factor \quad [5\text{-}1]$$

Where W_t is the weight of the tartrate that was added to the cell prior to titration and V_{kf} is the volume of KF reagent required to titrate the sodium tartrate. The W_t was multiplied by 36/230.09 to obtain the concentration of water per g of tartrate.

After calculating the KF factor 0.2 g of sample was added to the cell and titrated in triplicate. The gel samples used were 15%, 30 %, and 45%, where gel was measured in triplicate and the concentration of moisture per g PL showed no significant difference (P>0.05) indicating that moisture content between samples is not significantly different. The moisture % based off the concentration of PL was calculated by obtaining the % moisture of the sample from the following equation [5-2]:

$$\frac{F_{kf} * V_{kf}}{W} * 100 = \%\ moisture\ of\ sample \quad [5\text{-}2]$$

Where F_{kf} is the KF factor V_{kf} is the volume of KF reagent, and W is the initial weight of the sample prior to titration.

Visual stability

The structure and stability of the gels formed was observed visually after storing the samples at 25 °C for 24 h and recorded using a Nikon D810 digital camera (Minato, Tokyo, Japan). Pictures were taken to show the ability of the sample to freely flow by

flipping the sample upside down. A picture was taken for each of the three PL concentration replicates.

Solid Fat Content

The SFC was measured after storage at 25 °C for 24 h using a time domain nuclear magnetic resonance equipment (TD-NMR Minispec mq 20 analyzer (Bruker, Karlsruhe, Germany). A total of three analytical measurements were taken from each of the three experimental replicates. The sample was placed in NMR tubes and measured by placing the NMR tubes in the NMR.

Crystal Morphology

Morphology of crystals was observed using a PLM (Olympus BX 41, Waltham, MA, USA) under a 10X magnification lens and a digital camera (model Infinity 2, Lumenera Scientific, Ottawa, ON, Canada) was used to obtain pictures of the sample. Pictures were taken from the samples after being held in the incubator for 24 h at 25 °C. A total of four pictures of each of the three experimental replicates were taken.

Melting behavior

The melting behavior of samples was evaluated using a DSC (DSC Q20; TA Instruments, New Castle, DE, USA) after the sample was stored at 25 °C for 24 h. The melting behavior was measured by placing 10-15 mg of sample in a Tzero hermetically sealed pan. The pan was then placed in the DSC at 25 °C and ramped up to 110 °C at a rate of 5 °C per min. Two analytical measurements were taken from each of three experimental replicates.

Viscosity

Viscosity was measured using a rheometer (AR-G2, TA Instruments, New Castle, DE, U.S.A.) after the samples were held at 25 °C for 24 h. An 8 mm diameter parallel plate geometry was used set at 25 °C and a steady state flow procedure was used. Due to the softness of the 15% PL sample the gap was set at 800 µm to achieve good contact between the sample and the geometry, while a 1,000 µm gap was used for the 30% and 45% PL samples. Each experimental replicate was measured three times.

Oil Loss Percentage

The percentage of oil loss was calculated by weighing 1g of sample into an Eppendorf, centrifuging at 9.6 x g for 15 min, and inverting the Eppendorf with the lid open to allow any unbound oil to be drained off from the sample. The Eppendorf was weighed for a final weight and the percentage of the oil lost from the system was calculated using the following equation [5-3]:

$$OL\ (\%) = 100 - \left(\frac{W_f - W_e}{W_i} * 100\right) \quad [5\text{-}3]$$

Where W_e is the weight of the empty Eppendorf, W_i is the initial weight of the sample, and W_f is the final weight of the sample and the Eppendorf (after oil had drained off from the sample). The experiment was performed in triplicate, and each replicate was measured twice.

Statistical Analysis

A one-way ANOVA analysis was used with a Tukey comparison using GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA, USA) for all quantitative assays except for the comparison between the TLC plate PL total quantification and the P^{31} NMR PL

total quantification. For the comparison between these two methods an unpaired t-test was used in the GraphPad Prism Software.

Results & Discussion

Phospholipid Species, Concentration, and Fatty Acid Composition

Table 5-1 displays the results obtained from the P^{31} NMR and GC analyses. Previous research in our lab showed the average concentration of PL in PLI was 55.1±3.50 mol% using TLC and a pixel analytical software (Chapter IV, Cooper et al., 2019). The results obtained from P^{31} NMR (Table 5-1) validated the percentage of TLC method showing the PL mol% as being 55.0±0.5 mol% which was not significantly different from the value reported in our previous study (P=0.98). From the fatty acid

Table 5-1: Phospholipid (PL) composition of the phospholipid isolate (PLI). The PL concentration was determined using P^{31} nuclear magnetic resonance (NMR) in replicates of four. 2LPE: lysophosophatidylethanolamine, DHSM: dihydrosphingomyelin, SM: sphingomyelin, PE: phosphatidylethanolamine, Lac-PE: lactosylated phosphatidylethanolamine, 2LPC: lysophosphatidylcholine, PS:phosphatidylserine, PI: phosphatidyl inositol, PC: phosphatidyl choline, PL: phospholipid, PLI: phospholipid isolate

	2LPE (%)	**DHSM (%)**	**SM (%)**	**PE/Lac-PE (%)**	**2LPC (%)**	**PS (%)**	**PI (%)**	**PC (%)**	***Total PL in PLI***
PL (mol%)	3.1± 0.2	3.3± 0.0	21.1± 0.6	31.5±0. 8	1.6± 0.0	2.9± 0.1	2.6± 0.2	33.4± 0.1	55.0± 0.5%

composition (Table 5-2), it is important to note the high concentration of long chain saturated (C16:0 and C18:0) fatty acids. In the various fat classes it was observed that only C16:0 and C18:0 were the only fatty acids to exceed 10% in all of the lipid classes with the exception of C18:1N9 in the PL sample (32.1±1.0%) and TAG (12.0±0.4). These results are expected knowing that commonly there is an unsaturated fatty acid in the *sn*-2 position in PL (Nelson & Cox, 2013). Badings (1962) observed that 44.6 % of the fatty acids on PL were saturated with the most prevalent FA being 16:0 (15.9 %) and 18:0 (16.3 %). The high saturated FA profile indicates that crystallization is more likely to occur due to the nature of the FA. This make up is similarly seen in our sample with unsaturated fatty acids staying below 1%.

Percent Moisture of Phospholipids

The moisture % of the PLI was 6.8±0.3%. Upon the addition of PLI to hexane and oil and allowing 48 h for the hexane to evaporate, the moisture of the gels (5.3±0.2%) was no significantly different (P>0.80) between the 15%, 30%, and 45% PL samples. Similar results were observed with a decrease in moisture (2.2±0.1%) after being held at 100 °C for 30 min with no significant difference (P>0.80) between PL concentration. Godoy et al. (2015) observed that in a sample with a blend of 85% plant based lecithins/13% oleic acid/2% water reverse hexagonal phases (Hartel, 2001) were observed. This indicates that even with the low concentration of moisture in the 15%, 30%, and 45% PL samples reverse hexagonal phases are most likely present within the crystalline structure after being held at 25 °C for 24 h.

Table 5-2: Fatty acid (FA) composition of species present in the PL isolate/ FA composition was determined by separating the PL using thin layer chromatography (TLC) and analyzing the FA using gas chromatography (GC) in triplicate. The MAG, although they were detected in the TLC, had such a low concentration that it was undetectable by GC. PL: phospholipid, MAG: monoacylglycerol, 1,2-DAG: 1,2-diacylglycerol, 1,3-DAG: 1,3-diacylglycerol, FFA: free fatty acids, TAG: triacylglycerol, CE: cholesterol ester.

FA	PL (%)	1,2-DAG (%)	1,3-DAG (%)	FFA (%)	TAG (%)	CE (%)
C12:0	0.6±0.04				2.4±0.4	
C14:0	5.0±0.1		0.4±0.4	4.0±0.5	17.2±0.7	
C14:1					0.9±0.1	
C15:0	1.0±0.02	2.3±2.3	0.5±0.5	0.8±0.1	1.8±0.04	
C16:0	22.2±0.4	58.4±3.8	69.3±4.1	45.6±0.4	42.6±0.7	42.5±9.6
C16:1T	0.3±0.01				0.1±0.02	
C16:1 C9	0.8±0.0				0.5±0.02	
C17:0	0.6±0.02	1.2±0.7		1.3±0.1	0.9±0.02	
C17:1					0.2±0.1	
C18:0	16.1±0.4	33.1±3.6	27.6±2.0	42.3±1.7	17.0±0.6	52.1±9.8
C18:1T11	4.7±0.2		0.3±0.3		3.4±0.1	
C18:1N9	32.1±1.0	5.0±1.6	1.4±1.4	6.1±1.6	12.0±0.4	
C18:1N7	0.7±0.02				0.2±0.02	
C18:2N6	2.4±0.1				0.6±0.04	
C18:3N3	0.9±0.1				0.02±0.0	
C20:0	0.3±0.02				0.1±0.03	
C20:1N9	0.8±0.2					
9-11 CLA	2.8±0.8				0.3±0.1	
C22:0	3.8±1.6					
C23:2N6	0.6±0.4					
C23:0	2.6±0.1					
C24:0	1.9±0.1					

Visual Stability

No visual difference was observed between the samples after storage for 24 h at 25 °C (Figure 5-1). The samples seemed to show similar stability in preventing the mixture from flowing freely, although a more wet or oily appearance was observed as the concentration of PL decreased. Mouri et al. (2015) found similar results describing that as PL concentration increased, so did visual gel stability. Some differences observed though were that none of the gels produced by Mouri et al. (2015) had the capacity to be fully inverted. This is possibly because these authors used soybean lecithin containing almost 95%mol of PC and they also added ethanol to the gels at a level of 9% by weight. Aside from the addition of ethanol the phase diagram showed that a gel using only water and lecithin required the remaining 91% of the mixture to be made of 40-55% lecithin in order to obtain a gel, if lecithin concentration was less than this then the sample became a turbid liquid. The study performed by Mouri et al. used Pectol as another method of forming a gel with the ethanol/water/lecithin mixture which showed that gels were weakened with the increase of lecithin. In a publication by Okuro et al. (2018) a stable gel that could be inverted was obtained using a mixture of β-sitosterol/ɤ-oryzanol was weakened with the addition of lecithin rather than being strengthened. These authors showed that as soybean lecithin (over 95%mol PC) increased the gel became less stable once the concentration of PL was over 40%, which is contrary to the results observed in this study. Some of the theories for why this could be is that the PLI obtained for this study had other components such as DAG, FFA, TAG, and low concentrations of MAG which have been shown to be able induce crystallization and structure in some oil

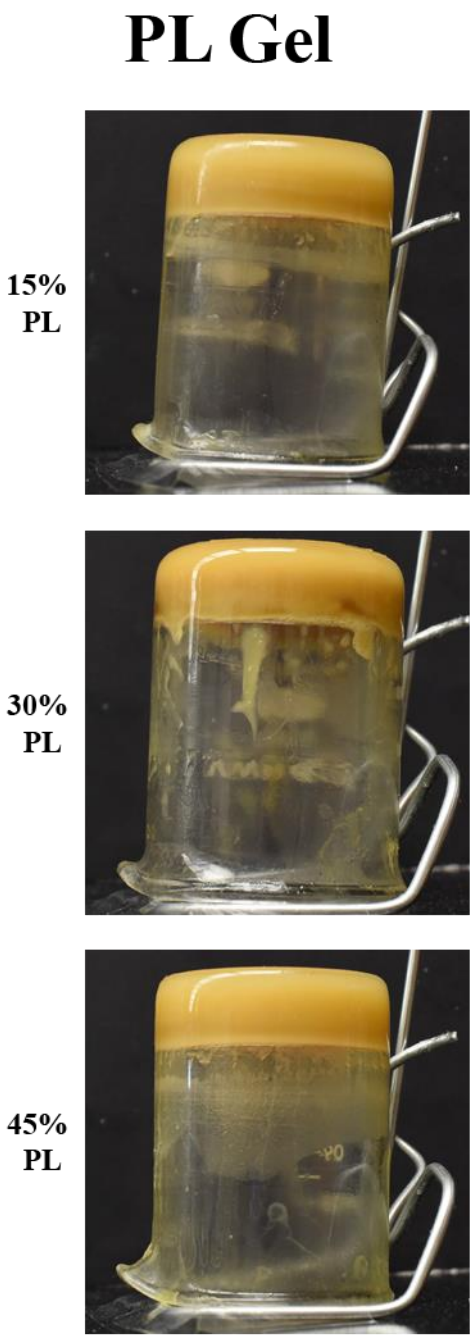

Figure 5-1: Phospholipid gels containing 15, 30, and 45 % phospholipid (PL) after being heated at 100 °C for 30 min and held at 25 °C for 24 h. The structure of all samples was strong enough to maintain its shape after being inverted.

systems (Pérez-Monterroza et al., 2014; Pehlivanoğlu et al., 2017). Another suggestion for stability as the concentration of PL increases could be due to the high concentration of saturated fatty acids of C16:0 and C18:0 in the PLI. The concentration of cholesterol also is likely to be a contributing factor to the structuring of the PL gels, cholesterol has been shown to give structure to membranes in biological systems (Nelson & Cox, 2013)

Solid Fat Content

SFC values increased with an increase in PL concentration (P<0.0001; Figure 5-2). The SFC found were 22.0 ± 0.8%, 37.5 ± 0.7%, and 48.9 ± 0.7% for the 15%, 30%, and 45% PL, respectively. This suggested that the crystallization that was occurring was highly influenced by the PL % and PLI concentration, either through the

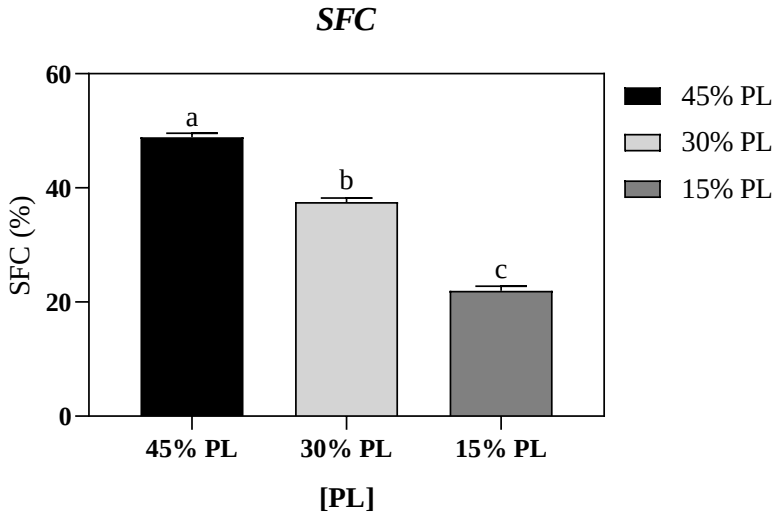

Figure 5-2: Solid fat content (SFC) of samples containing 15%, 30%, and 45% phospholipid (PL) after being melted and held at 25 °C for 24 h. Data was analyzed using a Tukey multiple comparison one-way ANOVA test. Parameters with the same letter are not significantly different (α=0.05).

crystallization of PL or PL/ other minor components inducing the crystallization. It can be suspected that in the PL the concentration of highly long chain saturated FA are aiding in the crystallization process where as in other studies it is expected that tubular formations occur due to the presence of low concentration of water interacting with PL (Pehlivanoğlu et al., 2018). Due to the low moisture content of the final gels resulting in an average of 2.2 %, it is expected that the moisture level has little to do with the structuring of the gels but rather the PL and other components of the PLI are forming crystalline particles which is why an increase in SFC is observed as the concentration of PL and PLI increase.

Crystal Morphology

When analyzing crystal morphology (Figure 5-3) the most noticeable difference between samples is the observation of the presence of well-defined structures characterized by a rectangular shape. These structures are present in the 15% and 30% samples, but they were not found in the 45% samples. In addition, these structures were shorter with higher count in samples with 15% PL (27.0 ± 3.3μm; 14.3 ± 1.6 crystals) and longer but fewer in samples with 30% PL samples (53.5 ± 6.9μm; 6.8 ± 1.8 crystals) with both crystal size and the count of the rectangular structures resulting in a significant difference ($P<0.05$). The crystal size of all the crystals was also measured with a significant difference ($P<0.001$) resulting between the 30% PL sample (3.4 ± 0.1 μm) and the other samples, 15% PL and 45% PL (2.8 ± 0.1 μm; 2.9 ± 0.1 μm) respectively, which agrees with what can be seen in Figure 5-3. According to Shchipunov (2001) PL can create rectangular tubular structures with water being trapped

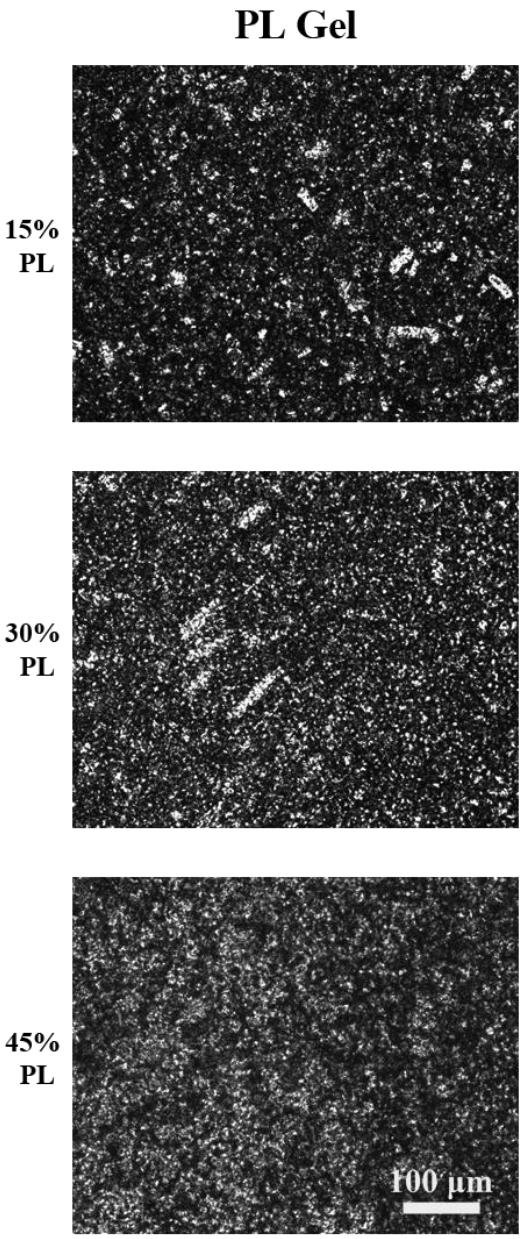

Figure 5-3: Phospholipid gels containing 15%, 30%, and 45 % phospholipid (PL) after being heated to 100 °C for 30 min and held at 25 °C for 24 h. Tubular structures were observed at 15% and 30% but not at 45%. Pictures were taken using polarized light microscopy.

on the inside of these structures, although this was suggested in soy-based PL. The observations made with PL forming the structures would be contrary to common hypothesis since at the highest concentration of PL (45%) shows that none of these rectangular structures were observed. This phenomenon can be contributed to the mobility of PL in a sample as well as the FA composition. In the samples containing higher concentration of soybean oil (15% PL and 30% PL), the sample contains enough liquid oil allowing mobility for the PL to be able to congregate and interact with one another in the observed fashion. The explanation for the 30% PL sample displaying fewer but longer tubular structures than the 15% PL sample is most likely contributed to the quantity of PL within the sample allowing a longer tubular structure to form. In the study performed by Okuro et al. (2018) a rectangular like formation was observed in the samples using hexadecane as a solvent with little visible crystallization, while crystallization can be clearly seen in samples that used sunflower oil and medium chain triacylglcyerols as a solvent. Although the description is the same the pictures of the crystals taken do not reflect one another and this is probably because the structuring of the system observed in this experiment is due to the high concentration of long chain saturated FA rather than the tubular structure entrapping water. In our study we obtained these rectangular structures in a bed of crystallized fat that could also be attributed to the crystallization of TAG, MAG, FFA, CE, and/or DAG. All of these lipid classes reflected high concentration of C16:0 and C18:0 FA suggesting that they themselves could be crystallizing at 25 °C.

Melting Behavior

The melting behavior of the samples showed broad peaks which for samples with a 45% PL concentration began at the initial temperature (25 °C) therefore not allowing for temperature onset (T_{on}) to be measured and analyzed. The peak temperature (T_p) and the change in enthalpy associated with the melting process (ΔH) are reported in Figure 5-4. Amongst all samples there was no significant difference between the T_p (53.39 ± 0.28) (P>0.05). The ΔH showed a significant difference (P<0.001) between all samples with 45% PL being the highest (14.45 ± 0.79 J/g), 30% PL the intermediate (10.11 ± 0.68 J/g), and 15% PL being the lowest (3.18 ± 0.27 J/g). This difference in ΔH, but not in T_p, indicate that the same components in samples are responsible for crystallization and the difference in visual appearance, SFC, and microstructure might be the concentration of crystallizing material. The ΔH reflects quite well the concentration of PL % and SFC indicating that PL could be responsible for the melting behavior in sample, as well as the other lipid classes discussed due to their FA composition. Okuro et al. (2018) observed similar broad peaks to those reported here but the melting peak temperatures were higher and ranged between 60-80 °C due to the different materials used in their oleogels (β-sitosterol and ɤ-oryzanol) combined with lecithin. The broad peaks may have been similar when compared to Okuro et al. (2018) data, but the T_p was much higher and the increase in T_p was identified as being higher (<80 °C) when only the other gelators (β-sitosterol and ɤ-oryzanol) were used.

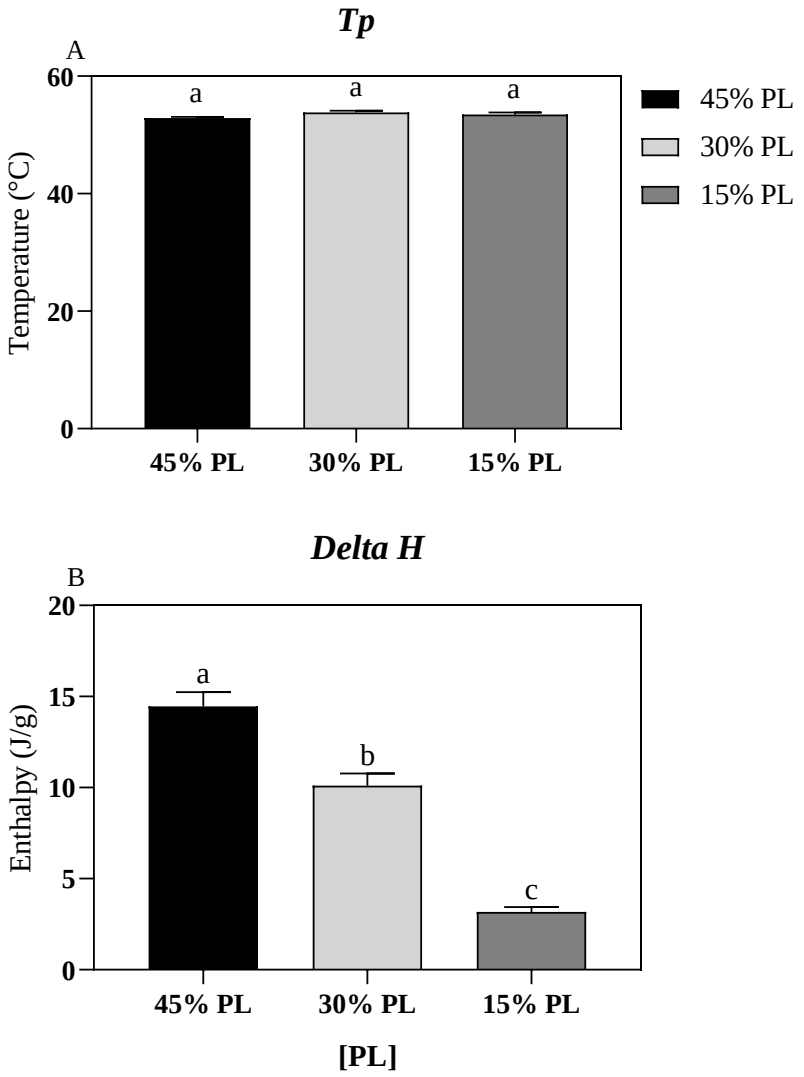

Figure 5-4: Peak temperature (T_p)(A) and change in enthalpy associated with the melting process (ΔH)(B) measured by differential scanning calorimetry (DSC) of samples containing 15%, 30%, and 45% phospholipid (PL). Measurements were taken after the sample had been melted and held at 25 °C for 24 h. Data was analyzed using a Tukey multiple comparison one-way ANOVA test. Parameters with the same letter are not significantly different (α=0.05).

Viscosity

The viscosity of the oleogels was reported at low (0.01 1/s), intermediate (0.10 1/s), and high (1.00 1/s) shear (Figure 5-5). At low and intermediate shear levels, a significant difference (P<0.0001) was observed in viscosity between the 45% PL ($2.64x10^4 \pm 2.49x10^3$ Pa*s; $2.62x10^3 \pm 3.54x10^2$ Pa*s) sample and the other two samples with 30% PL ($1.19x10^3 \pm 1.23x10^2$ Pa*s; $2.54x10^2 \pm 2.13x10$ Pa*s) and 15% PL (54.2 ± 11.6 Pa*s; 12.6 ± 27.5 Pa*s). At these shear rates no difference in viscosity was found between the 15 and 30% PL. At a high shear rate (1.00 1/s) a significant difference was observed between the higher concentrations of PL (45% PL: $3.17x10^2 \pm 3.43x10$ Pa*s; 30% PL: $1.91x10^2 \pm 7.83x10$ Pa*s; P<0.001; P<0.05) and the 15% PL sample (6.13±0.75 Pa*s) respectively. This indicated that the 30% sample can show similar viscous properties as the 15% PL or the 45% PL samples depending on the shear that it is undergoing. Mouri et al. (2015) showed comparable values in viscosity for shear rate 0.01% between the most viscous sample and the 15% PL sample, but the trend observed with an increase in PL was rather different than those observed in this study. Some contributing factors could also be other minor components found in PLI.

Oil Loss

Figure 5-6 shows that the oil loss of the 45% PL sample (11.1 ± 8.9%) was significantly lower (P<0.0001) than the one observed for the other two samples, 30% PL (72.0 ± 1.8%) and 15% PL (83.4 ± 1.8%). The explanation for this is that the PL crystallizes or induces crystallization therefore entrapping the oil within the lipid structure that forms, some to of this crystallization could also be coming from other lipid

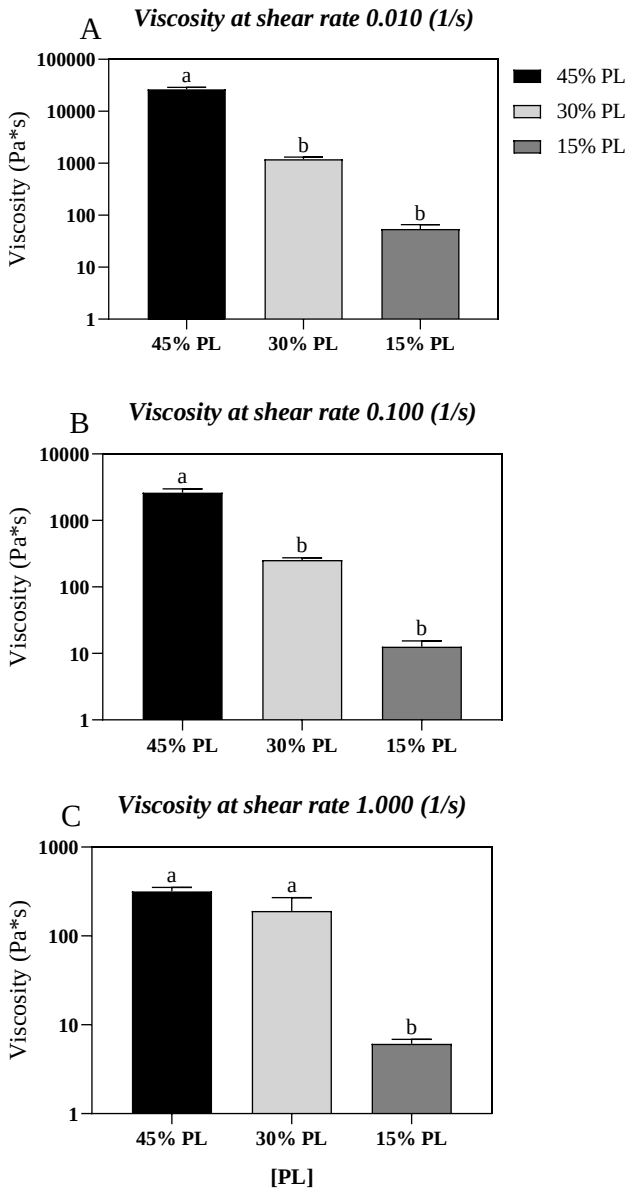

Figure 5-5: Viscosity of samples containing 15%, 30%, and 45% phospholipids (PL) at 0.01 1/s (A), 0.1 1/s (B), and 1.0 1/s (C) shear rate after being melted and held at 25 °C for 24 h. Data was analyzed using a Tukey multiple comparison one-way ANOVA test. Parameters within the same graph with the same letter are not significantly different (α=0.05).

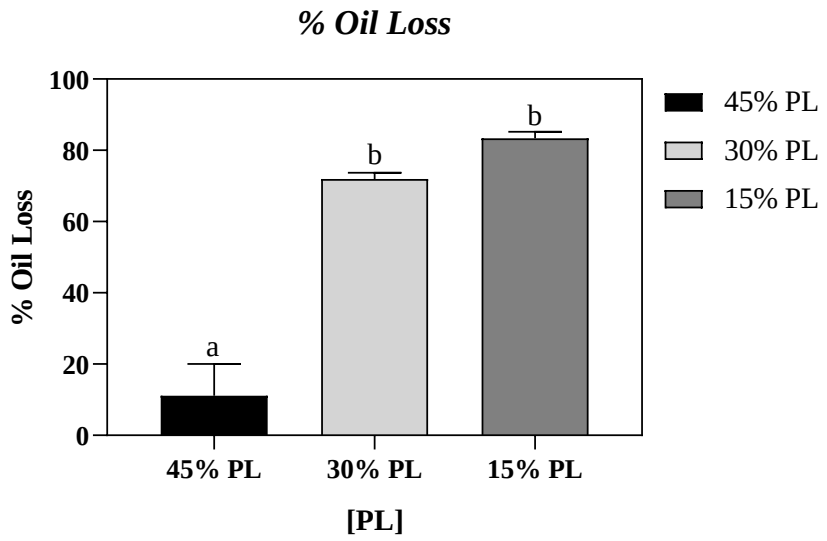

Figure 5-6: Percentage of oil lost after samples (15, 30, and 45% phospholipids (PL)) were centrifuged at 9.6 x g for 15 min and inverted for 10 min to allow free liquid oil to drain. Data was analyzed using a Tukey multiple comparison one-way ANOVA test. Parameters with the same letter are not significantly different (α=0.05).

classes that reflected high concentration of saturated fatty acids such as TAG, DAG, and FFA. MAG have also shown to be able to induce crystallization at low concentrations (Ojijo et al., 2004), our GC did not allow for any detection of fatty acids from MAG due to the low percentage of MAG that was identified in the sample. When increasing the concentration of PL it was expected for less oil to be capable of being able to escape the system due to the increased SFC and viscosity. The oil loss obtained for the 30% and 15% PL samples were not significantly different (P<0.3158).

Conclusion

Ultimately PL from a dairy source at concentrations above 15% PL allow for a gel structure to form, but for tubular micelles to form mobility of PL must be available. In concentrations of 15% or higher PL and other lipid classes of the PLI could crystallize thus increasing the solid material. As dairy PL concentration increases a higher ΔH is required to melt the crystals that form during storage, due to the PL and other lipids crystallizing. Viscosity increases with the increase of PL concentration and other lipid components, but the shear rate influences the gels depending on the concentration of PL allowing for a PL gel to be tailored for different processing. As PL and long chain saturated fatty acid content increases the ability to bind oil increases as well due to a strength in the gel allowing for entrapment to occur. Overall dairy PL influences the functional ability of gels and allows for crystal structure to be tailored by varying the concentration of PL, but when using as an ingredient for consumers the concentration should be controlled in order to ensure that saturated fat content does not exceed consumer acceptability.

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Chapter VI

Summary & Conclusions

In conclusion PL can be isolated into a PLI that contains only lipid classes and could be used as a food ingredient. PL can delay crystallization in AMF which could potentially be representative of other systems that are high in saturated fatty acids such as coconut oil or cocoa butter. PL also can create structure in soybean oil which could be reflective of other systems that contain a high concentration of unsaturated fatty acids.

The PLI could function as an ingredient since the solvents that were used are capable of being food grade therefore the process developed could easily be translated into the food industry. Future steps to prepare the process for industry would include upscaling to a pilot level and evaluating if the ratios for isolation that were determined on the lab scale translate to an industrial process in terms of yield. Aside from the need to understand the effects of upscaling on the isolation of PL, it would also be important to identify how effective the isolation process would be on other PL products or by-products. Some by-product sources could include butter serum, buttermilk, and whey protein phospholipid concentrate. Since these by-products in their raw form are liquid further research could be done identifying if it is important for the efficacy of isolation to occur when the PL by-product is in powder form or if it would be possible to isolate the PL effectively in the liquid raw form.

Now that it is understood that PL delays the crystallization of AMF it would be important to verify the influence that it has on other fats that are high in saturated fats such as cocoa butter, coconut oil, palm oil, or high melting fractions of these fats. Once

these other fats are better understood and an understanding of the functionality of PL on fats containing high concentrations of saturated fats, fat-based products containing other lecithins could be substituted with PLI or dairy lecithin to see if the influences and functionality are the same as other lecithin sources. Some examples of products that could be explored would be chocolates, mayonnaise, or margarines and shortenings.

The PL gel research could be expanded by attempting to understand how dairy PLI could create PL gels using other oils that have a high concentration of unsaturated fatty acids. Another method to extend this research would be through mixing PLI with other oleogelators to obtain an oleogel that contains less than 10% of oleogelator. Once this is better understood one could explore how dairy PLI gels could be incorporated into food products.

Other areas where this research could be extended would be in looking how PLI could be incorporated into dairy beverages, products and processes. One avenue would be improving the process of whey protein powders. Currently in industry lecithin is often incorporated in the spray drying process and the most common lecithin source currently if from soy. If one could understand if dairy PLI could be incorporated it could replace soy lecithin. Understanding PLI in dairy products, such as beverages, could eliminate allergens and add value to dairy products.

Overall this research only scratches the surface of a potential new PL ingredient source. Extending this research would secure PLI as an ingredient along with eliminating waste streams from the dairy industry, thus increasing revenue, eliminating negative environmental effects, and eliminating allergens in dairy products that already contain ingredients from dairy sources.