

MILK PHOSPHOLIPIDS EXTRACTION USING SWITCHABLE SOLVENTS

BY

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DISSERTATION ACCEPTANCE PAGE

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is dedicated to GOD for all his strength, protection, and guidance.

To my mother, father, beloved ones and mentors for their love, guidance, motivation, and
support

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ABBREVIATIONS

<i>AMF</i>	Anhydrous milk fat
<i>AOAC</i>	Association of official analytical chemists
<i>APCI</i>	Atmospheric pressure chemical ionization
<i>BM</i>	Buttermilk
<i>BMP</i>	Butter milk powder
<i>BS</i>	Beta-serum
<i>CAD</i>	Charged aerosol detector
<i>CBM</i>	Concentrate buttermilk
<i>CBM</i>	Concentrated buttermilk
<i>CLSM</i>	Confocal laser scanning microscopy
<i>CO₂</i>	Carbon dioxide
<i>C_p</i>	Heat Capacity (J kg ⁻¹ K ⁻¹)
<i>CyNMe₂</i>	N, N-dimethyl cyclohexylamine
<i>d.b</i>	Dry Basis
<i>DMCHA</i>	N,N-dimethyl cyclohexyl-amine
<i>DME</i>	Dimethyl ether
<i>DP</i>	N-dipropylamine
<i>EB</i>	N-ethyl butylamine
<i>ELSD</i>	Evaporative light scattering detector
<i>EMD</i>	Evaporative Mass Detector
<i>ESI</i>	Electrospray ionization
<i>FAMES</i>	Fatty acids methyl esters
<i>FDA</i>	Food and drug administration
<i>FE</i>	Folch extraction
<i>g</i>	Gram
<i>HPLC</i>	High-performance liquid chromatography
<i>Hz</i>	Hertz
<i>ICM</i>	Ice cream mix
<i>I_a</i>	Acoustic intensity (W cm ⁻²)

<i>kDa</i>	kilodalton
<i>kHz</i>	Kilo Hertz
<i>kPa</i>	Kilopascal
<i>LOD</i>	Limit of detection
<i>m</i>	Mass of the sample (kg)
<i>ME</i>	Mojonnier
<i>MFGM</i>	Milk fat globular membrane
<i>mg</i>	Milligram
<i>MHz</i>	Mega Hertz
<i>mL</i>	Milliliter
<i>MPa</i>	Mega Pascal
<i>MPLs</i>	milk phospholipids
<i>mV</i>	Millivolt
<i>N₂</i>	Dinitrogen
<i>ND</i>	Not Detected
<i>NFDM</i>	Non-Fat dry milk
<i>nm</i>	Nanometer
<i>pA</i>	Pascal
<i>PC</i>	Phosphatidylcholine
<i>PE</i>	Phosphatidylethanolamine
<i>PI</i>	Phosphatidylinositol
<i>Pi</i>	Power intensity
<i>PLs</i>	Phospholipids
<i>PS</i>	Phosphatidylserine
<i>RC</i>	Raw cream
<i>rpm</i>	Rotation per minute
<i>Sa</i>	Surface area (cm ²)
<i>SC-CO₂</i>	Supercritical carbon dioxide
<i>SDS-PAGE</i>	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
<i>SFE</i>	Supercritical fluid extraction
<i>SHS</i>	Switchable hydrophilicity solvents

<i>SM</i>	Sphingomyelin
<i>SPE</i>	Solid-Phase Extraction
<i>STEP</i>	Simultaneous texturization and Extraction
<i>TA</i>	Titrateable Acidity
<i>TS</i>	Total Solids
<i>TLC</i>	Thin Layer Chromatography
<i>US</i>	Ultrasound
<i>USD</i>	Ultrasound Detector
<i>UV</i>	Ultraviolet
<i>V</i>	Volt
<i>v/v</i>	Volume/Volume
<i>W</i>	Watt
<i>w/v</i>	Weight/Volume
<i>w/w</i>	Weight/Weight
<i>WPPC</i>	Whey Protein phospholipid concentrate
<i>α-La</i>	Alpha lactalbumin
<i>β-Lg</i>	Beta lactoglobulin
<i>μg</i>	Micro gram
<i>μL</i>	Micro Litter
<i>%</i>	Percentage
<i>°C</i>	Degree Celsius

ABSTRACT
EXTRACTION OF MILK PHOSPHOLIPIDS USING SWITCHABLE SOLVENT
EXTRACTION

KAAVYA RATHNAKUMAR

2021

Phospholipids (PLs) found in milk are of great interest due to their health and nutritional benefits associated with their consumption. By-products generated during the manufacture of dairy products represent a source of PLs with potential for value-added opportunities. Currently, the extraction of PLs from by-products results in overall low-efficiencies, and it involves subsequent solvent separation and lipid recovery. This dissertation studied the extraction of milk PLs from by-product streams with a tertiary amine (N, N-dimethyl cyclohexylamine, CyNMe₂) as a switchable hydrophilicity solvent. This type of solvents can be reversibly switched between a hydrophobic and hydrophilic form in the presence or absence of CO₂.

Different dairy by-products streams, including buttermilk (BM), beta-serum (BS), concentrate buttermilk (CBM), and raw cream (RC) was used to evaluate the feasibility of CyNMe₂. The extraction efficiency of CyNMe₂ ranged from 0.33-99%, depending on the type of byproduct. Remarkably, CyNMe₂ extracted up to 99% of the PLs directly from buttermilk. The extraction of PLs from BS was further studied using ultrasound prior to CyNMe₂ extraction. Overall, higher levels of acoustic intensity ($44.56 \pm 3.47 \text{ W cm}^{-2}$) prior to CyNMe₂ extraction recovered $69.07 \pm 0.11\%$ of PLs, 10-fold higher than the samples without ultrasound pretreatment. The recovered fraction of PLs mainly comprised of phosphatidylinositol (32%), phosphatidylethanolamine (30%), and sphingomyelin

(37%). The effect of the extraction temperature (25, 40, and 60°C), time (3, 10, and 18 h) and solvent ratio (3/1, 10/1, and 18/1 mL) was also studied. The highest yield obtained was $29.29 \pm 0.06\%$ of PLs at 60 °C, at minimized solvent ratio and time (3/1 mL and 3 h, respectively). At these conditions, the fraction of PLs recovered was phosphatidylinositol (59%) and phosphatidylethanolamine (35%). Insights into the extraction mechanism of the CyNMe2 was studied through various analytical measurements, such as analysis of protein-profile, particle size, zeta potential, and microstructure, Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). CyNMe2 seems to disrupt the protein-membrane through ion pair formation, releasing the PLs into the aqueous medium. Throughout this thesis, CyNMe2 shows to be an effective way to concentrate PLs from by-products.

CHAPTER 1

1.1. Introduction and objectives

The utilization of milk phospholipids (MPLs) as functional ingredients has been a topic of industrial interest over the past decades. The primary drivers for such interest have been the health benefits of MPLs beyond their basic nutrition. Improved cognitive performance, therapeutic effects, and antioxidant capacity are examples of health benefits associated with the consumption of MPLs (Ortega-Anaya & Jimenez-Flores, 2019). Dairy byproducts have been considered as a feedstock of MPLs with the most potential for producing concentrates and isolates of MPLs.

A number of byproduct streams are industrially available to recover MPLs, including buttermilk, dried buttermilk, beta-serum, and whey protein phospholipid concentrate (Huang et al., 2020). The existing methods to recover MPLs from byproduct streams include enzymatic hydrolysis of protein followed by filtration, microfiltration, ultrafiltration, supercritical fluid extraction or a combination of these technologies (Astaire et al., 2003, Costa et al., 2010, Barry et al., 2017). The recovered MPLs are further dried to produce a concentrate of MPLs, containing between 20 and 70% of MPLs with respect to the total fat (Huang et al., 2020).

On the other hand, separation methods with the ability to extract lipids from aqueous stream is relevant for process economics and commercial viability since the removal of water from streams is a prerequisite before extraction, and significantly contributes toward

the production cost. Thus, the extraction and recovery of MPLs from aqueous streams provide opportunities for the development of novel dairy ingredients.

Over the past few years, switchable solvents have been used to facilitate the extraction and subsequent separation of polar compounds (Samorì et al., 2013). Moreover, this type of solvents has the ability to extract lipids from wet materials, and they consist of either primary, secondary, or tertiary amines (Jessop et al., 2012). These solvents abruptly and reversibly switch from a hydrophobic phase to a hydrophilic form by bubbling or removing CO₂.

1.2. Thesis objectives

The overall objective of this work was to develop strategies for the extraction of MPLs from aqueous streams, using a tertiary amine (N, N-dimethyl cyclohexylamine, CyNMe₂) as a switchable hydrophilicity solvents (SHS). The specific objectives of this thesis were:

- To evaluate the feasibility of extraction and separation of MPLs from different dairy matrices using CyNMe₂ as a switchable hydrophilicity solvent (chapter 3),
- To improve the extraction of beta-serum using ultrasound prior to CyNMe₂ extraction (chapter 4),
- To evaluate the effect of time, temperature, and solvent ratio on the CyNMe₂ extraction of MPLs from beta-serum (chapter 5),
- To understanding the mechanism of CyNMe₂ extraction (chapter 6),
- To evaluate the applicability of beta-serum in the manufacture of ice-cream (chapter 7).

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CHAPTER 2

LITERATURE REVIEW¹

2.1. Relevance of phospholipids

Phospholipids are surface-active, amphiphilic molecules, consisting of both the hydrophilic tail and hydrophobic head (Contarini and Povo, 2013a). Because of this amphiphilic character, they are used as emulsifier, wetting agent, solubilizer, and liposome former (Hoogevest et al., 2014). PLs are complex polar lipids that contain a phosphate group and two fatty acids esterified to a glycerol backbone.

In milk, PLs are naturally present within the fat globule embedded with the epithelial cell plasma membrane, forming a complex structure known as the milk fat globular membrane (MFGM). This type of arrangement causes the milk fat to be emulsified and dispersed within the milk (Costa et al., 2010). MFGM contains proteins, minerals, neutral lipids, and enzymes (Danthine et al., 2000). Phospholipids are perhaps the most significant compound within the MFGM, accounting for about 4.49 g PLs 100 g⁻¹ of fat (Avalli & Contarini, 2005). Glycerophospholipids and sphingolipids are quantitatively the most important PLs in milk. They account for about 0.5-1% of the total MPLs, and they are mostly present in the MFGM. Phosphatidylcholine (PC) and sphingomyelin (SM) are outside the membrane, while phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) are found in the inner surface of the membrane (Contarini and Povo, 2013a).

¹ A version of this chapter has been submitted as book chapter in *Non-thermal Processing Technologies for the Dairy Industry*

During the processing of dairy products, PLs are separated from the membrane and the resulting stream is known as byproduct. For instance, the churning of cream during the manufacture of butter produces a liquid rich in PLs known as buttermilk. Similarly, the manufacture of anhydrous milk fat produces B-serum as the main by-product. MPLs are gaining more importance because of the nutritional and health benefits and their unique composition in PLs. The composition of PLs in different dairy by-products is represented in **Table 2.1**.

Table 2.1. Approximate composition of phospholipids in different dairy products (Burling and Graverholt, 2008).

Product	Fat (%)	PLs (mg/g fat)
Cream	22.1	8.6
Milk	3.0	3.5
Butter	82.0	1.9
Buttermilk	0.2	45

MPLs have exhibited nutraceutical properties due to the unique profile of PLs. **Table 2.2** exemplifies the profile of MPLs. PS is associated with cognitive function and releasing stress and is replaced by inactive cholesterol as the brain ages (Castro-Gomez et al., 2017), while SM effectively inhibits colon tumors (Kuchta-Noctor et al., 2016). It also reported that consuming PLs helped in reducing cardiovascular diseases, restoring immunological defences (Hernell et al., 2016), reduced cholesterol absorption (Timby et al., 2015), and total liver lipids (Küllenberg et al., 2012). Apart from the nutritional benefits, milk phospholipids as natural emulsifiers in food products. It is also used in the infant formula for infants growth and brain development (Huang et al., 2020b).

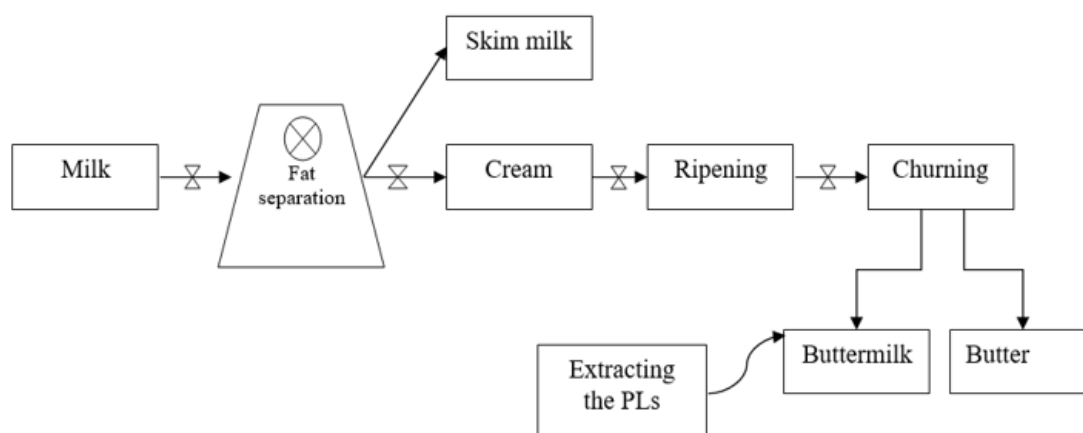
Table 2.2. Relative Composition of PLs (Huang et al., 2020)

Phospholipids (PLs)	Soy	Egg	Milk
Phosphatidylcholine (PC)	34	75	27
Phosphatidylethanolamine (PE)	21	15	25
Sphingomyelin (SM)	0	2	24
Phosphatidylserine (PS)	0	1	18

2.2. Methods for concentrating the phospholipids

2.2.1. Buttermilk derivatives

Buttermilk is a by-product obtained from the churning of butter which has a rich amount of milk fat globular membrane (MFGM). **Figure 2.1** illustrates the process for manufacturing of butter and by-products obtained.

**Figure 2.1.** Schematic of the manufacture of butter and processing.

The major challenge in the isolation and concentration of the MFGM is skim milk solids, mostly the casein micelles, which inhibit the concentration process. Another study

used a two-step process to polar lipids were concentrated using microfiltration and supercritical fluid extraction (SFE) for the buttermilk derivative (Astaire et al., 2003). The SFE with the optimal condition (375 bar, 77°C; 20 g min⁻¹ CO₂ flow rate; and 75 min extraction time) was used to extract the nonpolar lipids from the filtered and spray dried samples, where the temperature did not significantly impact the yield. With SFE, the total fat was reduced by 38%. It was observed that particle sizes decreased after the filtration and extraction processes (Astaire et al., 2003). Other researchers reported using ultrafiltration and SFE to obtain a whey buttermilk powder enriched in milk fat globule membrane PLs (Costa et al., 2010). **Figure 2.2** illustrates the extraction of MPLs using enzymatic and membrane filtration.

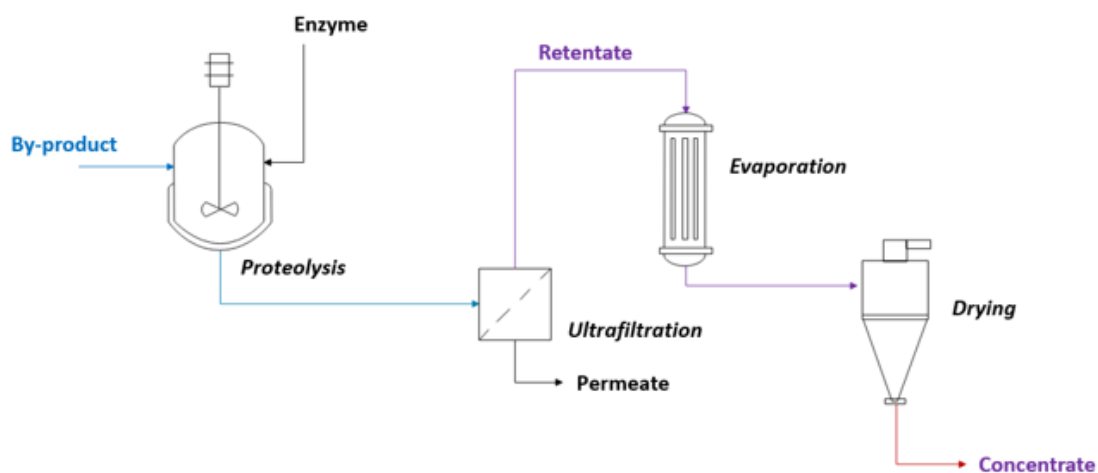


Figure 2.2. Illustration of the extraction of MPLs using enzymatic and membrane filtration.

The whey buttermilk originally contained 25% of protein, 16% of lipids include 2% of PLs. It was concentrated by ultrafiltration and diafiltration. The retentate was then spray dried and submitted to SFE (350 bar and 50 °C). Most of the lactose and ash were removed

by SFE and ultrafiltration, and there were 73% of protein, 21% of lipid, including 61% of PLs in dried whey buttermilk. In a recent study, PLs have been extracted from ultrafiltrate buttermilk powder (BMP) (Barry et al., 2017). SFE and ethanol extracted the ultra filtrated BMP with $11.05 \pm 0.02\%$ PLs as co-solvent. The optimal conditions used were $40\text{ }^{\circ}\text{C}$, 300 bar, and 20% of ethanol and increased PLs concentration to $56.24 \pm 0.07\%$ on the dry base. Others have concentrated 56% of PLs from reconstituted buttermilk using enzymatic hydrolysis of protein before microfiltration, followed by supercritical carbon dioxide with ethanol as co-solvent (Barry et al., 2017). Another investigation on the concentration of PLs from buttermilk powder showed a five-fold increment in the PLs content by microfiltration coupled with supercritical carbon dioxide (Spence et al., 2009). High purity of phospholipid-rich extract of 76% (wt/wt) was achieved at SFE condition of $60\text{ }^{\circ}\text{C}$, 30 MPa, and 15% (wt/wt) ethanol from the buttermilk powder using a pure sequential SC-CO₂ and ethanol-modified SC-CO₂ was carried out, the first one was to separate the non-polar lipids, followed by concentrating the PLs in the second fraction (Ubeyitogullari & Rizvi, 2020).

2.2.2. Beta-serum

Beta-serum (BS) is a by-product obtained from the phase inversion of anhydrous milk fat. Researchers have been extracted nonpolar and polar lipids from BS (Catchpole et al., 2008). BS contains approximately 60% fat, and it has more components from MFGM than buttermilk. Price et al. (2019) applied zinc and calcium acetate with mild heat treatment, and adjusting the pH precipitated the PLs and protein into a pellet fraction and further used the ethanol to extract the PLs. With ethanol extraction, PLs recovery of $97.7 \pm 1.7\%$ from

the zinc acetate precipitate and $94.9 \pm 3.7\%$ from calcium acetate precipitate was achieved. Catchpole et al. (2008) used Dimethyl ether (DME), and CO_2 were both used as solvents. DME can extract both neutral and polar lipids. Spray-dried beta-serum first extracted most neutral lipids by supercritical fluid extraction (SFE) with neat CO_2 under 300 bar and 40°C . The PLs enriched residual was then re-extracted by SFE with DME under 40 bar and 60°C . The polar lipid extracts were analyzed and contained 70% of PLs. The study also stated that the spray-dried sample could be subjected to CO_2 and then DME or DME and then CO_2 .

2.2.3. Whey protein phospholipid concentrate (WPPC)

Whey Protein phospholipid concentrate (WPPC) is also known as pro-cream. It is a by-product produced from the processing of cheese obtained during the micro-filtration of whey protein isolate (Levin et al., 2016). Whey protein PL concentrate contains 60 to 70% whey protein, and 10 to 30% of the total lipid content is PLs. The study by Sprick et al. (2019) found that a 2-step SFE process with neat S-CO_2 and ethanol as a co-solvent is an efficient extraction method for concentrating a PL-rich fraction from Whey Protein Phospholipid Concentrate (WPPC), obtaining 26.26 g of total PL/100g of fat at 35.0 MPa, 40°C , and 15% ethanol. Simultaneous texturization and use of ethanol to extract the PLs was developed by Price et al. (2018). The optimum processing conditions for a combined 5-stage sequential extraction for producing a PL-enriched lipid fraction were 70% ethanol at 70°C , total lipid recovery, total PL recovery, and PL content achieved was 40.7, 58.1, and 45.8%, respectively. An enriched fraction of α -lactalbumin and β -lactoglobulin was obtained from whey protein concentrate using heat and CO_2 using a pressure of 4140 kPa

and 5520 kPa, temperature 64°C for a residence time of 10 min was obtained a recovery of 55 % and 78% that of α -La and β -Lg (Tomasula et al., 1998). Many studies reported the methods to enhance the functional properties of buttermilk, beta serum and WPPC. Physicochemical treatments, including microfiltration, ultrafiltration, and supercritical fluid extraction, have been applied to increase the concentration of PLs.

2.3. Switchable hydrophilicity solvents (SHS)

SHS is a new class of solvents with unique behavior. These solvents, made from either primary, secondary, and tertiary amines, can extract lipids from wet materials. The Philip G. Jessop group developed these green solvents at Queens University, Canada (Jessop, 2017). These are often referred to as Smart solvents as they can abruptly switch from water-immiscible (hydrophobic) form to water-miscible (hydrophilic) form by simply bubbling with the CO₂ (Jessop et al., 2010a). **Figure 2.3** represents the switchable hydrophilicity solvents (SHS) extraction process.

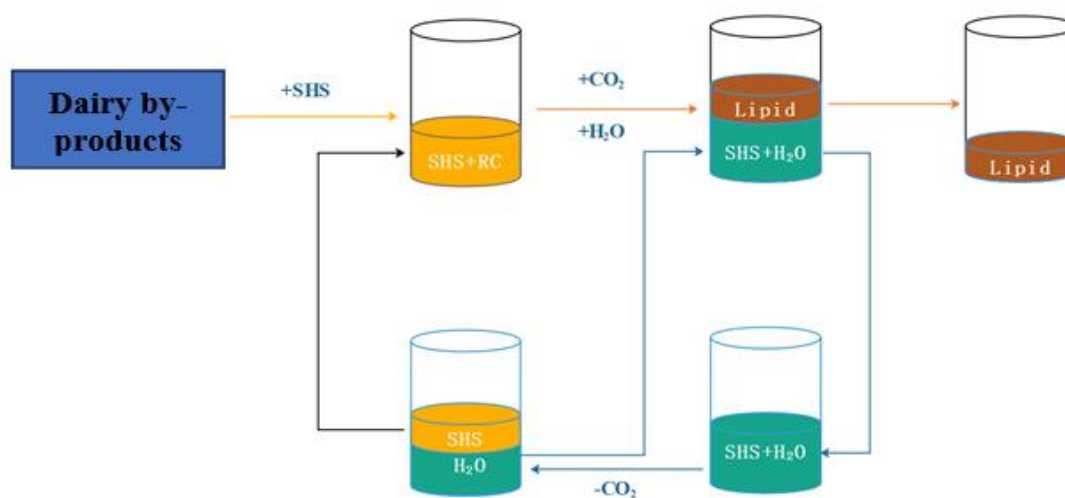


Figure 2.3. Schematic diagram of Switchable solvent extraction.

The switchable hydrophilicity solvents can be tested for their behavior based on the phase formed. If an organic liquid forms one phase when mixed with water before CO₂ is added, it is called monophasic and therefore not considered SHS. If a mixture of an organic compound and water forms two phases before CO₂ is added and forms one phase after CO₂ is added, it is an SHS. The change in miscibility is because of an acid-base reaction between either hydrated CO₂ or carbonic acid in the carbonated water, and the SHS gives a hydrophilic ammonium bicarbonate salt (Jessop et al., 2010b). Removing the CO₂ and inserting the N₂ goes back to its original amine and can be reused for the further extraction process.

2.3.1. Switchable solvents in the extraction of lipids

Hexanes and other hydrocarbons are becoming a health concern in recent times and environmental damage. Distillation is a common practice for removing solvents; therefore, this process has various disadvantages. This process requires a lot of volatile solvents leading to vapour emission contributing to smog formation (Jessop, 2017) and the other concern is it requires a large input of energy. Therefore, it would be desirable to obtain a non-distillation process for separating these solvents from the process. Replacement of these solvents has led to the discovery of these green solvents.

Switchable solvents such as primary, secondary, or tertiary amines are applied to extract the lipids from the microalgae. Investigations by Samori et al. (2013) showed that the utilization of N,N dimethyl cyclohexylamine (CyNMe₂), a tertiary amine for extracting lipids from wet algal cultures or samples. The low volatility, intermediate polarity and low water solubility behavior of the solvent contribute to higher extraction efficiency compared

to the organic solvents such as chloroform and methanol. The fatty acids methyl esters (FAMES) from the amine yields higher than the algal pellets subjected to CHCl_3 -MeOH extraction. This was because the amine had access to all the structural lipids, which are resistant to extraction with CHCl_3 -MeOH. Boyd et al. (2012) studies on freeze-dried samples of *Botryococcus braunii* microalgae used N, N-dimethyl cyclohexylamine extracted up to 22 wt.% crude lipids relative to the freeze-dried cell weight, compared to conventional chloroform and methanol. Secondary amines such as dipropyl amine and ethyl butylamine were found to extract lipid material from aqueous slurries of fresh, unbroken microalgae wet *Desmodesmus* algae slurries (Du et al., 2013). Oil was directly extracted from wet microalgae slurries without the need for drying and milling and subsequently recovered the extracted oil and solvent by simple phase splitting, using CO_2 as a trigger. An energy-efficient lipid oil extraction from algae was obtained using this method without drying or cell disruption. Efficient lipid extraction from the oleaginous yeast *Yarrowia lipolytica* was done using N-ethyl butylamine (EB), N-dipropylamine (DP), and N,N-dimethyl cyclohexyl-amine (DMCHA) elsewhere (Yook et al., 2019). It was observed that DMCHA and EB effectively extracted lipids from *Y. lipolytica*, resulting in up to 13% higher lipid yields than conventional organic solvent extraction using a chloroform/methanol mixture. This study improved the energy efficiency and economic feasibility of an oleaginous yeast-based biodiesel production process.

New polyethoxylated tertiary amines (methoxy ethyl) dibutylamine, N (methoxy ethoxy ethyl) dibutylamine and N (methoxy diethoxy ethyl) dibutylamine were synthesized as a replacer of DMHCA tested on the marine microalga (Samori et al., 2014)

Nannochloropsis gaditana, well known for its ability to produce high level of triacylglycerols. N-(methoxyethoxyethyl) dibutylamine resulted in being a valid candidate since it is hydrolytically stable, less volatile, and slightly less toxic than DMCHA towards aquatic organisms. SHS opens the possibility of recycling the algal culture medium, thus reducing the overall cost and water consumption and nutrients in third-generation biofuel plants. N, N,N tributylpentanamidine, as an SHS, was used to extract soybean oil from crushed soybeans (Phan et al., 2009).

In conclusion, unlike organic solvents, it does not require a distillation process, and the reusability of the solvent makes it a more sustainable process. These switchable solvent systems provide an outlook to a cost and energy-efficient lipid recovery of lipids for fuels from algae. Switchable solvents can be advantageous as media for reactions, extractions or separations (Jessop and Subramaniam, 2007), especially when in a multi-step chemical process, solvent are used for a specific reaction step and must be removed entirely before the next step is carried out Phan et al.(2008). CyNMe₂ appeared to be the best option, considering the following features expected for a “good” SHS amine, which should: (i) be immiscible with water (in the neutral form) and become miscible with water upon switching with CO₂; (ii) be liquid at room temperature (in the neutral form); (iii) have high boiling and (iv) have low toxicity.

2.4.Ultrasound assisted extraction

Ultrasound (USD) generates a sound wave that ranges from 20 kHz to 100 MHz using a transducer that results in the particles compression and rarefaction in the medium and

collapsing of bubbles causing cavitation (Guimaraes et al., 2019). The increasing temperature and pressure implode the bubbles creating high waves of shear energy and turbulence in the cavitation (Abeyasinghe et al.2020). USD can selectively separate and fractionate components, induce a range of sound effects, and enable complete usage of the byproducts and exploit the individual components specific functional properties (Leong et al., 2018). The USD has various potential applications in the dairy industry, such as homogenization, crystallization, enhancing the milk fat separation, and bioactive components. The enhancement of milk fat separation by the USD is obtained at an equal or higher frequency range 400 kHz (Torkamani et al., 2014; Guimaraes et al., 2019). When the USD is applied on fat globules, the pressure builds up in the container or chamber walls that act as pressure antinodes because of the radiation force, thereby increasing flocculation or coalescence occurring at these sites (Juliano et al., 2011). The increase in the floccule size of the fat globules causes a faster velocity rise and a separation speed (Leong et al., 2014).

Figure 2.4 illustrates the experimental setup and mechanism of ultrasound-assisted extraction. The phospholipids from the whey powder separated using the acoustic intensities 400 and 1000 kHz at different specific energy inputs 23–390 kJ kg⁻¹ were studied by Torkamani et al. (2016). The process enhances the gravity separation of fat at a maximum power draw after 30 min , which was 4 to 5 times higher than the non-sonicated treatment resulting PL concentration of the whey powder was $59.8 \pm 2.8 \mu\text{g } 100 \text{ g}^{-1}$ of powder. It was also interesting to note from this study that even at highest specific energy inputs (390 kJ kg⁻¹) USD did not promote oxidative reactions in the whey powders.

Ultrasound pretreatment as a cell disruption method to enhance the lipid extraction from the algae cells before solvent extraction was studied by Ellison et al. (2019). Reduced extraction time with enhanced lipid recovery of 0.21% was obtained from the *Nannochloropsis oculata* (microalgae) using USD compared to the conventional Bligh and Dyer method (Adam et al., 2012). Ultrasound exposure for 5 min was considered one of the most effective pretreatment methods (Lee et al., 2010).

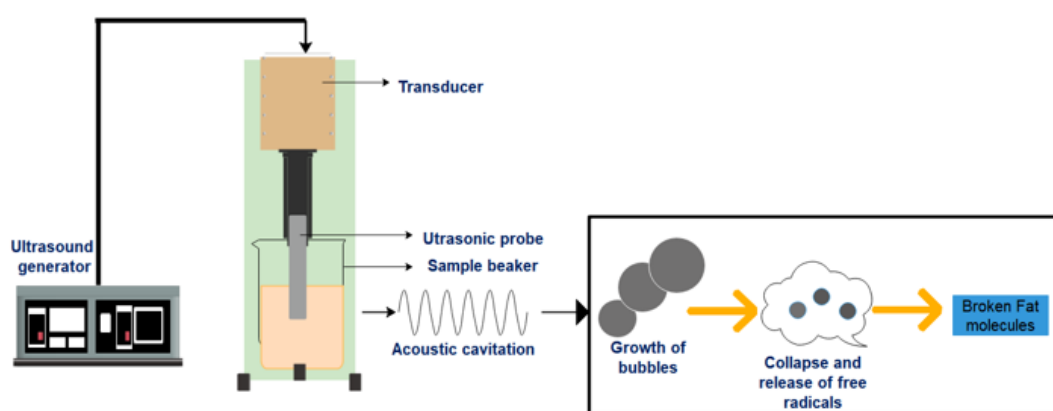


Figure 2.4. Illustration of Ultrasound-assisted extraction.

The ultrasound extraction process includes parameters like amplitude, frequency, ultrasonic power to obtain the desired cavitation effect. The optimal choice of ultrasound frequency and intensity also should be considered (Wen et al., 2018). The actual power dissipated in a glass vessel will be calculated using the formula (Jambrak et al., 2014; Yanjun et al., 2014):

$$P_{diss} = mC_p \left(\frac{dT}{dt} \right) \quad (2.1)$$

Where P_{diss} (W) is the actual ultrasound power dissipated, m - a mass of the product (kg), C_p - specific heat capacity of the dairy by-product ($J\ kg^{-1}\ k^{-1}$); $(\frac{dT}{dt})$ - is the change of temperature ($^{\circ}C$) for the time (s). The power intensity ($W\ m^{-2}$) is the one that the medium experiences at the surface of the probe per unit area of the horn tip, can be calculated using:

$$P_i = \frac{P_{diss}}{\pi r^2} \quad (2.2)$$

Where P_i - power intensity ($W\ m^{-2}$); r - radius of the horn tip (m); ε - energy density ($W\ cm^{-3}$) is the amount of ultrasound energy per unit volume of the sample and can be used as a reference to scale up the system (Tiwari, 2015):

$$\varepsilon = \frac{P_{diss}}{Volume} \quad (2.3)$$

2.5. Analytical methods for phospholipids

2.5.1. Thin Layer Chromatography(TLC)

TLC is a simple chromatography method for identifying synthesized mixtures by separating the compounds in the samples. The difference in analyte affinities includes the TLC polarities, development solvent, and compounds in the spot made the balance of intermolecular forces lead solvent to move the solute on the plate. **Figure 2.5** represents the polar and non-polar lipids separated on the TLC plate. This technique has been widely used for determining the identity of compounds, individual components in a sample, and purity of compounds. In the food industry, researchers used TLC to identify different neutral lipids

and polar lipids. The solvent mixture for detecting PLs comprises chloroform, methanol, and deionized water; hexane, ethyl acetate, acetic acid is the solvent used to detect neutral lipids (Hutchins et al., 2008). Usually, compounds separated on the plate are colorless; visualization methods are needed to observe development. UV light and iodine vapor are the two most common methods. The spots interfere with fluorescence, show dark color on glowing silica gel plate under the UV light, and mark the spots by pencil to know the locations. For iodine vapors, since most organic compounds will turn to dark color with iodine, spots are easily observed after placing the plate into iodine vapors. Visualization of reagents such as ferric chloride, potassium permanganate, and ninhydrin could also make separated spot visible (Jork et al., 1990).

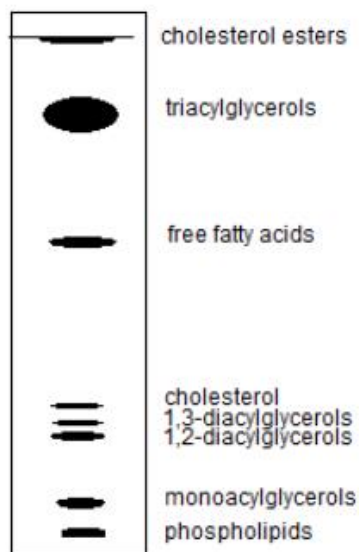


Figure 2.5. Thin-layer chromatography separation of simple lipids (Touchstone, 1995).

2.5.2. High Performance Liquid chromatography (HPLC)

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

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

HPLC is utilized to isolate the PLs into various fractions using different detectors such as UV or evaporative light scattering detector (ELSD) (Fagan and Wijesundera, 2004, Avalli and Contarini, 2005). Recently, a new hybrid detector called the charged aerosol detector (CAD) has been into use. In lipidomic, it is necessary to use very sensitive methods to detect and quantify all the lipids present even at low concentrations (Markey et al., 2017). The operation principle is based on two steps: the first involves nebulizing the HPLC column effluent and evaporating the solvents. These steps are the same as that of the ELSD. The second part is the ionization of the aerosol particles by impacting the positively charged nitrogen (N_2^{+}) obtained by corona discharge. The amount of ion charged is then detected by an electrometer (Brunelli et al., 2007). CAD shares the same mobile phase nebulization principle as the ELSD but uses a charge transfer for solute detection that provides improved performances makes it more sensitive and precise (Contarini and Povolito, 2013a). A detailed mechanism behind the operation is reported elsewhere (Dixon and Peterson, 2002).

Hazotte et al. (2007) compared different universal detectors such as ELSD, atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and charged aerosol detectors (CAD), in which the CAD shows a linear response from 5 to 500 $\mu\text{g/mL}$. When compared to the ELSD, the improvement factor was 2.7 to 12 for all low concentration components. For the complex lipid sample, this study shows that at low concentration, CAD provides better performances than ELSD and has a sensitivity near to mass spectroscopy according to the solute. CAD is the only detector tested that detects all lipids and, at the same time, to have a linear response on two orders of magnitude. This universal detector is very easy to use in micro liquid chromatography, has good sensitivity, and the high temperatures can improve its limit of detection (LOD). Consequently, CAD has the potential to become the new detector of choice in the developed analytical methods for lipids in addition to the mass spectrometer. Ramos et al. (2008) analysis of *Leishmania* membrane phospholipids using CAD and ELSD showed that improved detection of phospholipids at low levels was obtained using CAD and was more informative than ELSD. CAD is compatible with linear calibration over a 10-fold range, and it is more precise than ELSD.

HPLC, coupled with CAD, was applied to detect milk phospholipid fractions from the cow milk (Kielbowicz et al., 2013). The separation of the compounds of interest was achieved on a diol stationary phase with a mobile phase consisting of 13% HCOOH, hexane and 2-propanol in a 19 min elution program, including 10 min equilibration of the column. The total amount of PLs in the milk samples ranged from 22.7 to 31.3 mg 100 mL⁻¹ of milk. Five classes of PLs were detected of which phosphatidyl-choline (PC) was the

predominant 45%, followed phosphatidylethanolamine (PE) 34%, phosphatidylserine (PS) 8.6%, phosphatidylinositol (PI) 12% and sphingomyelin (SM) 5%. The optimization on the separation of PLs for different dairy products such as skim milk, cream, butter, buttermilk, butter oil and butter serum was demonstrated (Barry et al., 2016). Separation and identification were obtained using N₂ as the nebulizing gas and the silica column size 150 x 3 mm. The detector temperature was set at 40°C, while the sample chamber was maintained at 15°C. The mobile phases used were dichloromethane, MeOH: triethylamine/acetic acid buffer, pH 3.5. The operating pressure was maintained at 241.32 kPa, and the scale output range was 500 pA. These were some parameters that were set for CAD. Well defined peaks were observed for each PLs, and the concentration of PLs was in line with literature previously published by (Avalli and Contarini, 2005, Rombaut et al., 2005). The increase in the PLs values for PI and PS is $9.63 \pm 0.08\%$ and $10.10 \pm 0.02\%$ as from the total PL, respectively, which increases between 2% and 7% compared with published data (Avalli and Contarini, 2005). This was attributed to the detection sensitivity and precision offered by the CAD. Therefore, CAD offers a fine-tuning of pH and run time produced chromatograms that demonstrated stable retention times and were reproducible in duplicate runs on different, thus creating a robust methodology for PL analysis.

2.6. Microscopic methods

2.6.1. Confocal laser scanning microscopy (CLSM)

Phospholipids compose the backbone of the milk fat globular membrane (MFGM), in which the proteins are embedded. Thus the organization of the phospholipids likely plays a crucial role in protein-lipid interactions. Different microscopy techniques have been used

to elucidate the MFGM structure, such as electron microscopy, immunomicroscopic and biochemical techniques, freeze etching, and freeze-fracture immunocytochemistry. However, some of these techniques are time-consuming and might alter the MFGM structure or introduce artifacts. The introduction of Confocal laser scanning microscopy (CLSM), a noninvasive technique is used in recent times to determine the MFGM. Phospholipids are saturated with longer and symmetric fatty acid chains more readily adopt the liquid-ordered structures. The ability to see this phase separation with CLSM results from the fact that the exogenous fluorescent dye-labelled phospholipid partitions exclusively into the liquid disordered phase, thus leaving the liquid-ordered domains “dark”. The large dye molecule is attached to either the headgroup or fatty acid chain of the phospholipid, thus preventing it from packing tightly enough to enter the liquid-ordered phase.

The CLSM technique involving the use of exogenous probes and dyes at low concentration has been shown to not introduce significant perturbations of the native biological structure of the membrane (Gallier et al., 2010a). The milk fat globules (Rd-dope), an exogenous dye that has high photostability, is considered a suitable choice for CLSM. This substance is a headgroup labelled phospholipid probe, which can be incorporated with minimal perturbation into the phospholipids layer of the milk fat globule membrane Gallier et al.(2015). The spatial resolution limit of the CLSM is on the order of one-half of the wavelength of the light used excited the fluorophore at 559nm Gallier et al.(2010). Since the MFGM is 10 to 20nmthick (Walstra and Jenness, 1984), the thickness of the membrane is overestimated, and it is not possible to observe the trilayer structure of

the MFGM. Since the MFGM is complexed with proteins at the surface, the use of FCF green is utilized. It is a dye that electrostatically attracts the charged groups present in the proteins. Nile Red is an oxazone dye used to stain lipid droplets (Greenspan et al., 1985; Lopez and Ménard, 2011). It is specific to triglycerides and lipid vesicles inside the MFGM.

Research by Lopez et al. (2010) have used CLSM, and fluorescent dye (Rd-dope) labelled phospholipids to elucidate lipid rafts' presence, areas rich in cholesterol and sphingomyelin observed in cell. The human milk fat globules were also stained using the fluorescent probes Rd-dope, FCF and Nile red, which shows that spatial heterogeneity in the human milk fat globule membrane (MFGM), with the lateral segregation of SM in liquid-ordered phase domains of various shapes and sizes surrounded by a liquid-disordered phase composed of the glycerophospholipids in which the proteins are dispersed (Lopez and Ménard, 2011). The Sphingomyelin accounts for about 35-45% of mature breast milk. This provides a way for MFGM-based ingredient concentrated from by-products such as bovine buttermilks could be added to infant formulas as a functional ingredient to benefit the specific composition, structure and functions of the MFGM (Rasmussen, 2009, Ortega-Anaya and Jimenez-Flores, 2019).

2.7. References

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CHAPTER 3

EXTRACTION OF DAIRY PHOSPHOLIPIDS USING SWITCHABLE SOLVENTS: A
FEASIBILITY STUDY²**3.1. Abstract**

A tertiary amine (N, N-dimethyl cyclohexylamine, CyNMe₂) was used as a switchable hydrophilicity solvent (SHS) for extracting phospholipids (PLs) from raw cream (RC), buttermilk (BM), concentrated buttermilk (CBM), and beta-serum (BS). The SHS extractions were performed with varying solvent–sample weight ratio at room temperature. The extracted PLs using CyNMe₂ were recovered by bubbling CO₂ at atmospheric pressure, switching the CyNMe₂ into its respective salt. For comparison, the PLs were also extracted using Folch (FE) and Mojonnier (ME) extraction. The extraction efficiency of SHS varied from 0.33% to 99%, depending on the type of byproduct. The SHS extracted up to 99% of the PLs directly from BM, while only 11.37% ± 0.57% and 2.66% ± 0.56% of the PLs were extracted with FE and ME, respectively. These results demonstrate the applicability of SHS for the extraction of PLs from dairy byproducts.

3.2. Introduction

Phospholipids (PLs) are found as lipid bilayers in all plant and animals cells membranes (Küllenberget al., 2012). The term PLs refers to a class of complex polar lipids containing a phosphate group and two fatty acids esterified to a glycerol backbone. The phosphate group is linked to a polar group such as choline, ethanolamine, or serine (Contarini et al., 2013). PLs derived from milk are primarily rich in sphingomyelin (SM) and

² A version of this chapter has been published in *Foods*, 8(7), 265.

phosphatidylserine (PS), two of the most highly bioactive PLs (Pimentel et al., 2016). Health benefits associated with the consumption of PLs include reduced incidence of cardiovascular diseases, cholesterol adsorption, reduced gastrointestinal infections, and improved immunological function (Küllenberg et al., 2012; Avalli and Contarini., 2005).

Dairy PLs are embedded with epithelial cell plasma membranes forming a complex structure known as milk fat globule membrane (MFGM). Such arrangements allow the milk fat to be emulsified and dispersed within the milk (Costa et al., 2010). In raw milk, PLs account for about 0.5-1% of the total milk fat, and about 60-70% of the PLs are located in the MFGM, depending on the variety, season and lactation stage (Contarini et al., 2013). On the other hand, the content of PLs in dairy foods is strongly influenced by the manufacturing steps (Ali et al., 2018). An investigation on measuring the concentration of PLs in 31 dairy foods showed values of PLs from 0.1 to 25% of the total milk fat (Rombaut et al., 2005).

Dairy byproducts represent a natural source of PLs with great potential for isolation and further commercialization. Isolation of PLs from byproduct streams involves various steps (concentration, extraction, solvent separation, lipid recovery, and fractionation) within the entire process, which results in low-overall efficiency and therefore economically unviable. Instead, concentrates of PLs obtained from dairy byproducts such as buttermilk has been a research priority in the past few years (Rombaut et al., 2005; Astaire et al., 2003; Barry et al., 2017; Spence et al., 2009). Earlier, it has been highlighted the limited applicability of the PLs concentrates when comparing with the industrial

application of PLs derived from lecithin (Price et al., 2018). Thus, it is desirable to develop technology for producing isolated PLs fractions derived from dairy byproducts.

Recently, a new class of solvents, namely switchable hydrophilicity solvents (SHS), have been developed to facilitate the extraction and subsequent separation of lipidic materials (Jessop et al., 2012). These type of solvents are made of either primary, secondary, or tertiary amines, and they are capable of extracting lipids from wet materials. SHS abruptly and reversibly switch between a hydrophobic form (poorly miscible with water) and a hydrophilic form (miscible with water). The polarity switch of these solvents is triggered by simply bubbling or removing CO₂ (Jessop et al., 2010). The underlying principle behind the change in polarity is due to the formation of an ammonium carbonate salt in the presence of CO₂, while upon removing the CO₂ with nitrogen, the carbonate salt returns to its original form of amine. Earlier, an investigation employed N,N,N'-tributylpentanamidine as an SHS for the extraction of soybean oil (Jessop et al., 2010).

Similarly, other authors extracted lipids from freeze-dried microalgae using a tertiary amine (N, N-dimethyl cyclohexylamine, CyNMe₂) as an SHS (Boyd et al., 2012). More importantly, the SHS was removed from the extract without distillation. In summary, the existing literature on the extraction of lipids via SHS reveals that CyNMe₂, a commercially available amine, has tunable hydrophilicity meaning that it can be switched from the hydrophobic form into hydrophilic form by adding CO₂. The present research aimed to evaluate the feasibility of extraction and separation of PLs from different dairy matrices using CyNMe₂ as a switchable hydrophilicity solvent.

3.3. Materials and methods

3.3.1. Dairy Byproducts

The tested dairy matrices were raw cream (RC), buttermilk (BM), concentrated buttermilk (CBM), and beta-serum (BS). The RC was obtained from the Davis Dairy Plant at South Dakota State University (Brookings, SD). A portion of the cream was churned at 4°C to obtain BM using a laboratory-scale churner (TM 31 Thermomix, Vorwerk LLC, Thousand Oaks, CA). The CBM was obtained by freeze-drying BM using a benchtop freeze drier (Model 117, Labconco Corporation, Kansas City, MO). The BS was obtained from a local plant (Valley Queen, Milbank, SD. N, N-dimethyl cyclohexylamine (99%, Sigma Aldrich), phospholipid mixture for HPLC (Soybean, P3817-1VL, Sigma Aldrich), methanol (99.9%, Sigma Aldrich), chloroform (99.9%, Sigma Aldrich), hexane (99.9%, Sigma Aldrich), HPLC grade water (Sigma Aldrich), histological grade ethanol (Sigma Aldrich), petroleum ether (95%, Fisher Scientific), ethyl ester (99%, Fisher Scientific), phosphomolybdic acid hydrate (99%, Alfa Aesar), TLC silica gel plate (TLC silica gel 60 F254, EMD Milipore, Burlington, MA) and activated silica gel (Silica gel 60 G, EMD Milipore) were purchased from commercial suppliers.

3.3.2. Compositional Analysis

A sample of each matrix was tested for total solids, protein, fat, lactose, and pH. The total solids was determined gravimetrically according to the methodology described elsewhere (AOAC, 1998). The protein content on a total nitrogen basis was determined by Kjeldahl method. The fat content was measured gravimetrically according to the method of Mojonnier fat extraction. The concentration of lactose was determined using HPLC

following the methodology reported by elsewhere (Amamcharla et al., 2011). The pH was measured in 10 mL of the sample using an Orion Versa Star Pro (Thermo Fisher Scientific, Waltham, MA).

3.3.3. Conventional Extraction

The extraction of total lipids was performed using Folch (FE) and Mojonnier (ME) extraction. The FE was conducted following the guidelines reported elsewhere (Martínez-Monteaquedo 2015). Briefly, each FE consisted of 1 g of sample mixed with 20 mL of chloroform: methanol solution (2:1, v/v). Then, the mixture was vortexed for 5 min, followed by centrifugation at 4200 X g for 5 min. The lower phase (lipids dissolved in chloroform) was transferred to a test tube, where the chloroform was evaporated at 45°C under nitrogen flow. The extraction via Mojonnier was conducted according to the methodology reported elsewhere (Gallier et al., 2010) with some modifications. One gram of sample was transferred into a Mojonnier test tube and diluted with 6 mL of deionized water. The diluted sample was mixed 1.5 mL of NH₄OH, 10 mL of ethyl alcohol, 25 mL of ethyl ester, 25 mL of petroleum ether, and few drops of phenolphthalein indicator. Then, the mixture was vigorously shaken and centrifuged for 5 min. After centrifugation, the lipid phase was poured into an aluminum pan, where the organic solvents were evaporated by heating the pan at 65°C. A second extraction was performed using with 5 mL of ethyl alcohol, 15 mL of ethyl ether, and 15 mL of petroleum ether. The dried lipids were weighted, and the extraction efficiency was expressed as the percentage of total lipids recovered according to Equation (3.1).

$$\text{Total lipids (\%)} = \frac{\text{Weight of recovered lipids}}{\text{Weight of sample}} \cdot 100 \quad (3.1)$$

3.3.4. Switchable Solvent Extraction

Figure 3.1 is a schematic depiction of the lipid extraction via SHS from different dairy matrices. Each extraction consisted of 1 g of sample added into a 20 mL vial containing either 3, 6, or 12 mL of CyNMe₂.

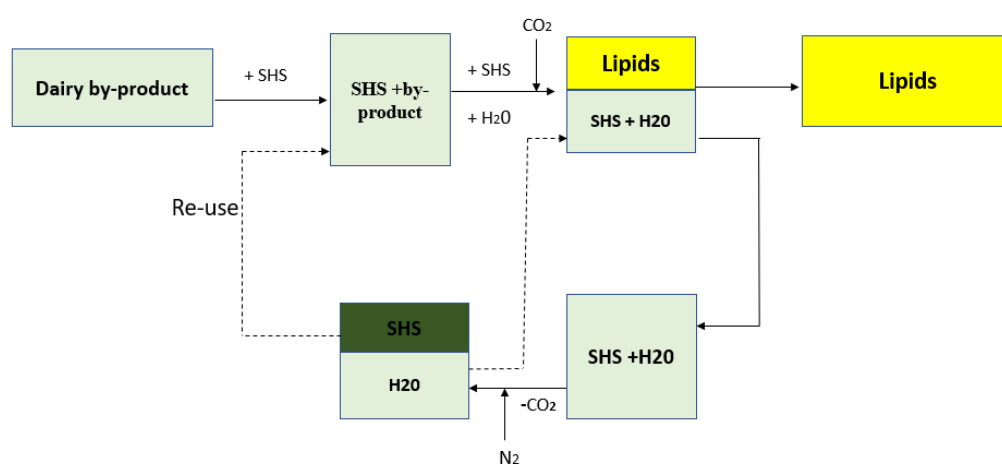


Figure 3.1. Schematic diagram for the extraction of lipids from dairy byproducts using switchable hydrophilicity solvent. The dotted lines represent water and SHS that can be reused at the end of the process.

Figure 3.2 illustrates the SHS extraction of PLs, where the mixture (solvent/matrix) was stirred at room temperature for 18 h (step (1) in **Figure 3.2**). Afterward, an equimolar amount of water was added to maintain the stoichiometry of the reaction (step (2)) followed by bubbling CO₂ at room temperature (step (3)) until the layer of CyNMe₂ and water combined (usually 3-4 h), leaving the lipid layer at the top of the vial. The presence of CO₂ converted the CyNMe₂ into its respective salt that is water soluble, switching the hydrophobicity form of the amine into the hydrophilicity form of the bicarbonate salt where

the layer of lipids is at the top of the mixture (step (4)). Three mL of hexane was added to dissolve the lipid layer, and subsequently transferred to a test tube. The hydrophilic mixture made of CyNMe₂ and water was separated into their respective component by removing the CO₂ until the layers were visible formed. The separated hydrophobic CyNMe₂ and water were recovered for further lipid extraction.

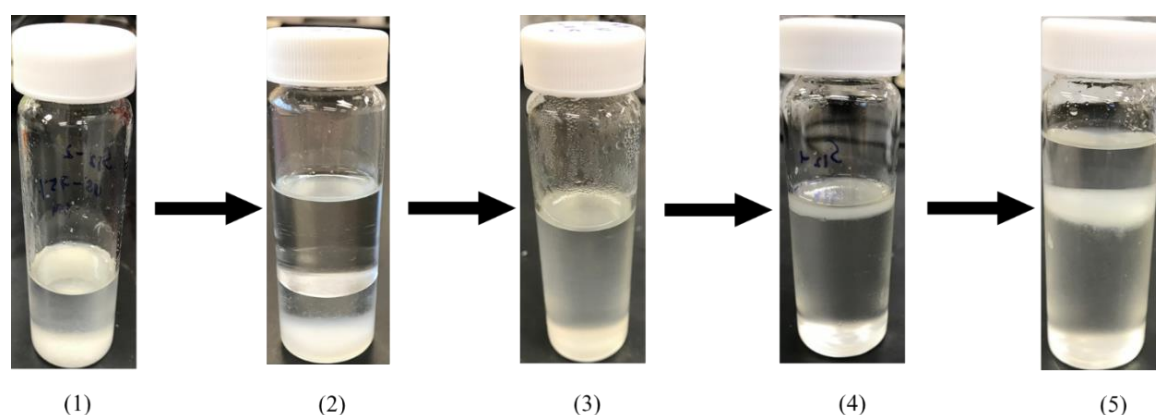


Figure 3.2. Extraction of lipids from dairy byproducts with N,N-dimethylcyclohexylamine (CyNMe₂) as a switchable hydrophilicity solvent (SHS): (1) SHS with dairy sample after 18 h of extraction; (2) addition of water; (3) after bubbling CO₂ to separate the lipid phase; (4) lipids on the top and CyNMe₂ on the bottom; and (5) addition of hexane to facilitate the separation of lipids.

3.3.5. Fractionation of Extracted Lipids

The extracted lipids were fractionated via solid-phase extraction (SPE) following the methodology reported elsewhere (Donato et al., 2011). A SPE column (1×10 cm) made of activated silica gel was first conditioned with 10 mL of chloroform: methanol mixture (95:5, v/v). A portion of the extracted lipids (100 mg) was dissolved in 1 mL of the chloroform: methanol solution and run through the conditioned column. The neutral lipids were eluted with 20 mL of chloroform: methanol (95:5, v/v). The PLs were recovered using 10 mL of methanol, followed by 10 mL of chloroform: methanol:water mixture

(5:3:2,v/v/v). Afterward, the solvents from the PLs fraction were evaporated under vacuum at 40°C. The recovered PLs were expressed as a percentage of the extracted lipids, according to Equation (3.2).

$$\text{Recovered phospholipids (\%)} = \frac{\text{Weight of dried fraction}}{\text{Weight of lipids}} \cdot 100 \quad (3.2)$$

3.3.6. Thin Layer Chromatography (TLC)

The recovered PLs were quantified on TLC silica gel plates. A mixture of chloroform: methanol: water (65:25:4, v/v) was used as a mobile phase for the detection of PLs. A solution of molybdophosphoric acid (5%, w/v) in ethanol was used as spray reagent, followed by heating at 180°C for 10 min (Sajilata et al., 2008). Clear visualization of the detection of spots was obtained under UV-light.

3.3.7. Statistical Analysis

Each extraction was carried out in triplicate, and the mean values for total lipids and recovered PLs were compared using Tukey's test ($p < 0.05$). The statistical analysis was carried out using Sigma plot software V11 (SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1. Compositional Analysis

shows the compositional characteristics of the different dairy matrix used in this study. The evaluated parameters were within the expected range for each matrix. On the other hand, substantial variations in the total solids (8-91%), lactose (3-8%), and fat (4-27%)

were observed between the different matrices. Such variations allow us to evaluate the feasibility of SHS in extracting PLs under different environmental factors.

Table 3.1. Composition of dairy by-products used for extraction of phospholipids.

Parameter	Dairy Matrix			
	Raw Cream	Butter Milk	Concentrated Butter Milk	B-Serum
Total Solids (%)	51.77 ± 0.18	9.63 ± 0.03	91.81 ± 0.06	8.17 ± 0.34
Protein (%)	0.24 ± 0.01	0.33 ± 0.01	1.44 ± 0.07	0.18 ± 0.01
Fat (%)	27.47 ± 1.60	4.09 ± 0.41	5.09 ± 0.63	4.05 ± 0.04
Ash (%)	0.61 ± 0.02	0.94 ± 0.08	6.26 ± 0.17	1.04 ± 0.39
Lactose (%)	3.06 ± 2.73	3.98 ± 0.39	8.69 ± 0.28	6.91 ± 0.15
pH	6.58 ± 0.01	6.38 ± 0.01	5.05 ± 0.02	6.74 ± 0.01

3.4.2. Total Lipid Extraction

The extracted total lipids from different dairy matrices (RC, BM, CBM, and BS) using three different methods (FE, ME, and SHS) are shown in **Figure 3.3**. Overall, the type of dairy matrix strongly influenced the amount of total lipids extracted. For RC (**Figure 3.3a**), the FE and ME showed no significant difference in the amount of total lipids recovered (28.98 ± 1.36 and $27.47 \pm 1.60\%$, respectively). Contrary, lower values of total lipids were obtained with CyNMe₂ at SHS/RC ratio 3/1 and 6/1 (21.84 ± 1.15 and $22.32 \pm 1.75\%$, respectively). On the other hand, the total lipids recovered from RC increased to $29.24 \pm 1.38\%$ with increasing the SHS/RC ratio to 12/1. Such values of extracted total lipids were not significantly different from those obtained with FE and ME (**Figure 3.3a**). Moreover,

FE and ME required a substantially higher ratio of the solvent/matrix (20/1 and 50/1, respectively) than that of SHS/RC of 12/1. Further increment of the SHS/sample ratio did not result in higher values of extracted total lipids (data not shown).

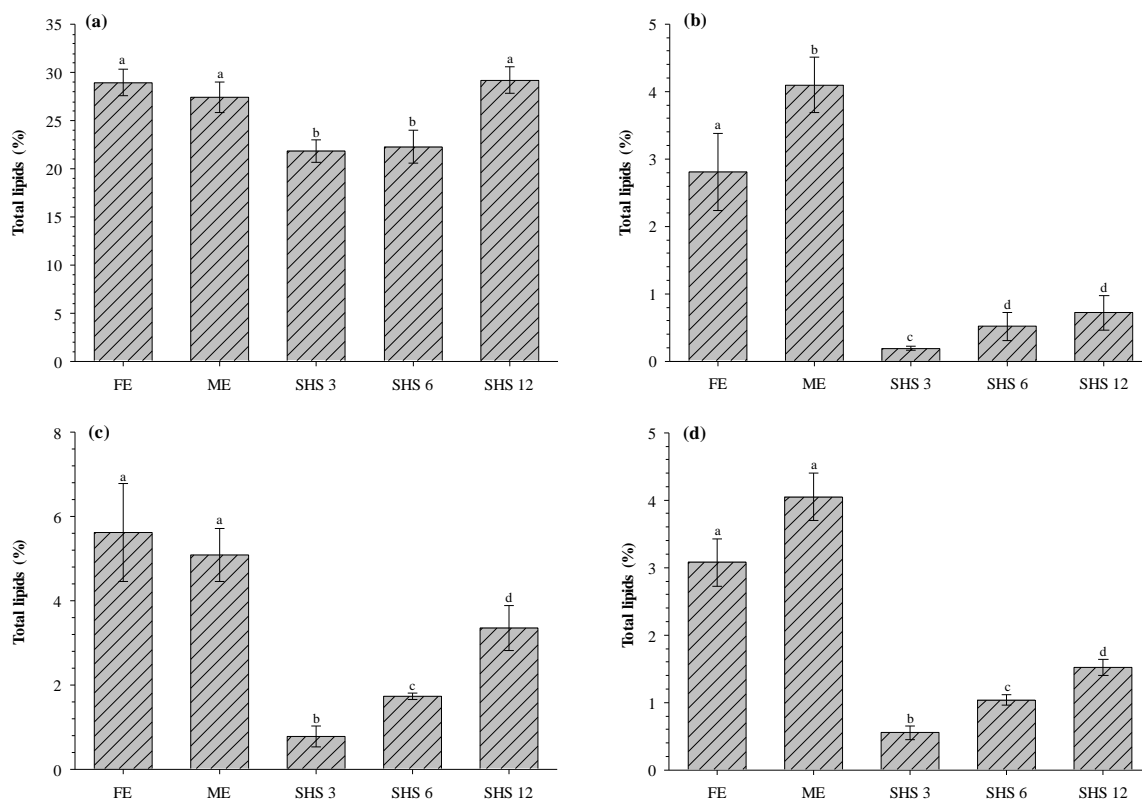


Figure 3.3. Comparison of different extraction methods (Folch, Mojonnier, and switchable hydrophilicity solvent) on the lipids recovered from (a) raw cream, (b) buttermilk, (c) concentrated buttermilk, and (d) beta-serum. FE – Folch extraction; ME – Mojonnier extraction; SHS 3, 6, 12 – switchable hydrophilicity solvent ratio 1/3, 1/6, 1/12, respectively. Mean \pm standard deviation within each column with different letters (a-d) are significantly different ($p < 0.05$) according to Tukey test.

Figure 3.3b shows the extracted total lipids from BM using FE, ME, and SHS (at 3/1, 6/1, and 12/1 ratio). The highest values of extracted total lipids were obtained using ME ($4.09 \pm 0.41\%$), followed by FE ($2.81 \pm 0.57\%$). The extraction values via SHS were rather low (0.18-0.71%), showing an increasing tendency with the solvent/matrix ratio. Similar

trends were observed for the extraction of total lipids from CBM (**Figure 3.3 c**), where the extraction with SHS increased (0.77 ± 0.25 to $3.34 \pm 0.54\%$) with the solvent/matrix ratio. However, the highest extraction was obtained using FE ($5.62 \pm 1.16\%$), followed by ME ($5.09 \pm 0.63\%$). CBM through either freeze-drying or spray drying has been used as a feedstock for the concentration of lipids using supercritical CO₂. **Figure 3.3d** shows the extraction of total lipids from BS using FE, ME, and SHS. The highest extraction of total lipids was obtained with ME ($4.05 \pm 0.35\%$) followed by FE ($3.07 \pm 0.35\%$), while lower values of extraction were obtained with SHS (0.55-1.52%). BS is a byproduct obtained through phase inversion from an oil-in-water to a water-in-oil emulsion (Catchpole et al., 2008).

3.4.3. Phospholipids Recovered

The extracted total lipids were fractionated with SPE to calculate the amount of recovered PLs. **Figure 3.4** shows the recovered PLs from different dairy matrices (RC, BM, CBM, and BS) using three different methods (FE, ME, and SHS). In the case of RC, the recovered PLs with FE, ME, and SHS 3/1 ranged from 0.28-0.30% and no significant difference was detected between extraction methods. On the other hand, the recovered PLs with SHS 12/1 was slightly higher ($0.33 \pm 0.01\%$) than any other extraction method. Other authors reported similar values of recovered PLs (0.40%) from cream using FE (Rombaut et al., 2007).

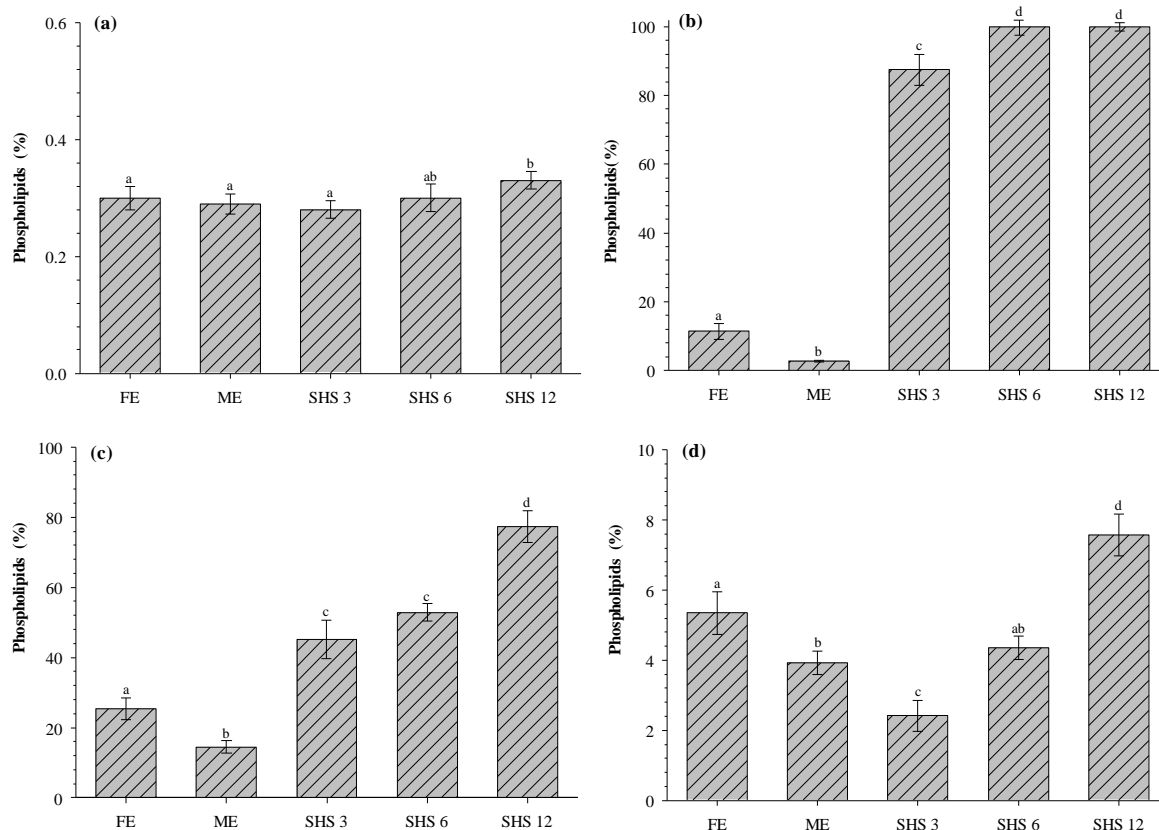


Figure 3.4. Comparison of different extraction methods (Folch, Mojonnier, and switchable hydrophilicity solvent) on the phospholipids recovered from (a) raw cream, (b) buttermilk, (c) concentrated buttermilk, and (d) beta-serum. FE – Folch extraction; ME – Mojonnier extraction; SHS 3, 6, 12 – switchable hydrophilicity solvent ratio 1/3, 1/6, 1/12, respectively. Mean \pm standard deviation within each column with different letters (a-d) are significantly different ($p < 0.05$) according to Tukey test.

The recovered PLs from BM (**Figure 3.4b**) using SHS were remarkably higher compared with that obtained from FE and ME. The use of CyNMe₂ as a SHS remarkably extracted 87.50 ± 4.50 , 99.93 ± 2.50 , and $99.96 \pm 1.21\%$ at a ratio 3/1, 6/1, and 12/1, respectively (**Figure 3.4b**). Contrary, only 11.37 ± 2.31 and $2.66 \pm 0.26\%$ of the PLs were recovered using FE and ME, respectively. In the case of CBM, 25.33 ± 3.10 and $14.41 \pm 1.78\%$ of the PLs were recovered using FE and ME, respectively. Interestingly, the use of SHS substantially increased the amount of recovered PLs (45.21 ± 5.51 , 52.84 ± 2.45 , and

$77.27 \pm 4.51\%$ at a ratio of 3/1, 6/1, and 12/1, respectively. **Figure 3.4d** shows the recovered PLs from BS, where the highest recovered amount was obtained using SHS 12/1 ($7.57 \pm 0.59\%$) followed by FE ($5.34 \pm 0.61\%$). Contrary, the lowest amount of recovered PLs was found in those samples extracted with SHS 3/1 ($2.41 \pm 0.44\%$) and ME ($3.92 \pm 0.33\%$).

3.4.4. PLs TLC Characterization

A representative TLC image of the buttermilk PLs is shown in **Figure 3.5**. There are four visible color bands in the TLC plate, which represents the individual PLs (phosphatidyl ethanolamine, PE, phosphatidylserine, PS, phosphatidylinositol, PI, phosphatidylcholine, PC, and sphingomyelin, SM). Similar results were also confirmed the presence of individual PLs for raw cream, buttermilk powder, and processed milk (Gallier et al.,2010). The recovered PLs using SHS clearly outperformed the conventional extraction methods, judging by the bands corresponding to individual PLs.

3.5. Discussion

The extraction with CyNMe₂ as an SHS results in a higher amount of recovered PLs, regardless of the dairy matrix with recovery values ranging from 0.33 to 99%. Traditional extraction methods such as FE combine organic solvents with polar alcohol that disrupts the hydrogen bonding and electrostatic forces between the polar lipids and proteins, creating holes in the membrane. Such a combination of organic solvent and polar alcohol somehow enabled the non-polar solvent to enter the cells and interact with the hydrophobic neutral lipids (Du et al.,2015).

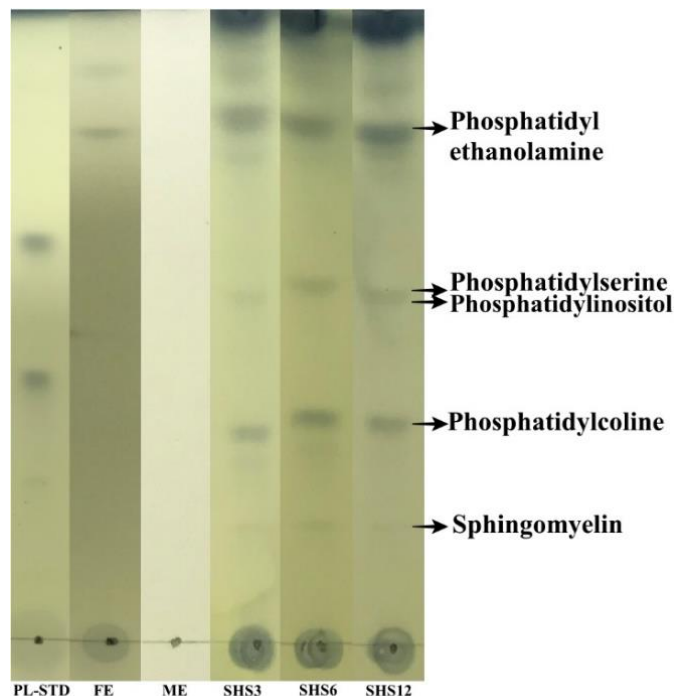


Figure 3.5. Representative thin-layer chromatography (TLC) plate of buttermilk showing the migration of the recovered phospholipids. PL-STD is the standard mixture of phospholipids; FE – Folch extraction; ME – Mojonier extraction; SHS 3, 6, 12 – switchable hydrophilicity solvent ratio 1/3, 1/6, 1/12, respectively.

In the ME, a strong base (NH_4OH) is used to digest the protein and release the fat, which is subsequently dissolved with a mixture of organic solvents (Gallier et al., 2010). However, the majority of PLs remained within the ammonia phase rather than the organic solvent phase. Remarkably, the extraction using CyNMe₂ as an SHS resulted in higher values of recovered PLs despite not having polar alcohol or a denaturing agent. CyNMe₂ is a tertiary amine that is commercially available, having very low miscibility with water under nitrogen atmosphere (atmospheric conditions), and it becomes hydrophilic in the presence of CO_2 . CyNMe₂, in the presence of CO_2 , forms a bicarbonate salt that is soluble in water, while in the absence of CO_2 , the carbonated salt is converted back into CyNMe₂

(Rombaut et al.,2007). The chemical reaction responsible for the immiscibility change is represented in **Figure 3.6**.

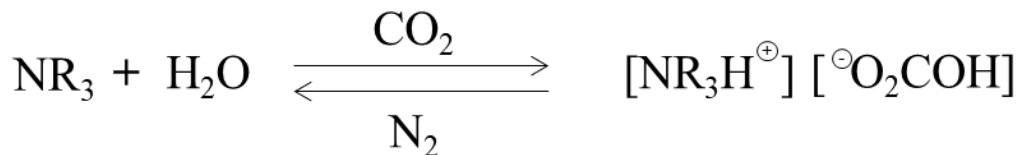


Figure 3.6. Reaction mechanism from switching hydrophilicity of N,N-dimethylcyclohexylamine. Adapted from (Duet al.,2015).

The efficiency of CyNMe2 in extracting PLs directly from dairy matrices was tested by varying the solvent/matrix ratio. The majority of research using a tertiary amine as an SHS involves the use of CyNMe2. This has been exemplified elsewhere (Boyd et al.,2012), who employed CyNMe2 to extract and isolate lipids, including triacylglycerol, diacylglycerol, monoacylglycerol, and free fatty acids from algae (*Botryococcus braunii*). These authors reported values of recovered lipids up to 22% of the dry cell weight at room temperature. The use of CyNMe2 for extracting PLs from raw cream is rather low (0.28-0.31%). This is because the PLs, along with the membrane proteins, emulsify the fat creating thick cell walls that makes the extraction of PLs very difficult. The extraction of PLs from RC may be improved by the application of mechanical treatment prior to extraction, which is beyond the scope of this work.

The performance of the SHS indirectly extracting PLs from BM was quite remarkable, judging by the unprecedented amount of recovered PLs (99%). Direct extraction of PLs from a dilute stream offers logistical and financial advantages over conventional organic solvent extraction methods. Extraction of lipids directly from dilute media without

pretreatment has been documented elsewhere (Samorì et al., 2013), who used CyNMe₂ to extract lipids directly from wet algae (up 80% water content) and reported extraction yields in the range of 31-57%.

The current literature on the utilization of dairy PLs deals with the development of concentrates as end-product, and no information is available regarding isolated fractions of PLs. In the absence of such information, reports dealing with the concentrates of dairy PLs obtained through a combination of technologies (enzymatic hydrolysis, microfiltration, ultrafiltration, and supercritical carbon dioxide) were used for comparison with our experimental findings. It has been developed a two-step process for obtaining a concentrate of PLs (up to 19%) from reconstituted buttermilk using microfiltration and supercritical carbon dioxide extraction (Astaire et al., 2003). Similarly, other authors concentrated 56% of PLs from reconstituted buttermilk using enzymatic hydrolysis of protein prior microfiltration followed by supercritical carbon dioxide with ethanol as co-solvent (Barry et al., 2017). A concentrate containing 60% of PLs was obtained by ultrafiltration of whey buttermilk prior to spray drying. The final concentration of PLs was then obtained after extraction using supercritical carbon dioxide (Costa et al., 2010). An investigation on the concentration of PLs from buttermilk powder showed a 5-fold increment in the PLs content by microfiltration coupled with supercritical carbon dioxide (Spence et al., 2009). More recently, (Price et al., 2018) extracted 58% of the total PLs from whey protein phospholipid concentrate using simultaneous texturization and extraction (STEP) under optimum conditions (5-stage sequential extraction using ethanol at 70°C). All these approaches have led to the enrichment of PLs in the byproduct stream rather than extraction from their

respective matrix. We have investigated an alternative way to concentrate and isolate dairy PLs, where the values of recovered PLs were 0.33 ± 0.01 , 7.57 ± 0.59 , 77.27 ± 4.51 , and $99.96 \pm 1.20\%$ for RC, BS, CBM, and BM, respectively. Our results are in agreement with the earlier reports elsewhere (Samorì et al., 2013), suggesting that SHS can outperform conventional extraction and concentration methods by directly extracting lipids from dilute streams. Further quantification of the individual PLs would be of relevant interest in future investigations. Research in this area is scarce, and it offers opportunities for further studies in the field of process development, micromixing, optimization, and development of new types of SHS. It is worth to mention that CyNMe2 (N,N-dimethylcyclohexylamine) is an amine authorized by the FDA as an Indirect Food Additive (CFR:177.2600). Nevertheless, other relevant aspects need further investigation, such as amine recovery and purity of the PLs fraction as well as structural modification to minimize potential impact in biological systems. The application of other amines as SHS is an active research area, including dimethylethanolamine, spermine, and spermidine.

3.6. Conclusions

For the first time, the feasibility of extracting phospholipids directly from dairy byproducts was evaluated using CyNMe2 as an SHS. The SHS extracted up to 99.96% of the PLs directly from BM, while only between 2 and 11% of the PLs were extracted with conventional methods. Further optimization of other extraction parameters (temperature, time, type of system, and matrix) is needed for the development of solvent systems that maximizes the quality of the separations.

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CHAPTER 4
IMPROVEMENTS IN THE EXTRACTION OF MILK PHOSPHOLIPIDS FROM
BETA-SERUM USING ULTRASOUND PRIOR TO TERTIARY AMINE
EXTRACTION³

4.1. Abstract

We report improvements in the extraction of milk phospholipids (MPLs) from beta-serum, a dairy byproduct, by applying ultrasound prior to a tertiary amine extraction. Three acoustic intensities (15.53 ± 1.20 , 31.76 ± 2.46 , or 44.56 ± 3.47 W cm⁻²) were applied for 4 min before the amine extraction (*N,N*-dimethylcyclohexylamine, CyNMe2). The extracted lipids were fractionated by solid phase-microextraction, and the recovered MPLs were quantified by HPLC-CAD. An acoustic intensity of 44.56 ± 3.47 W cm⁻² followed by CyNMe2 extraction (12/1, solvent to ratio) yielded $69.67 \pm 3.45\%$ of MPLs, while only $7.57 \pm 0.59\%$ were recovered without ultrasound. The fraction of MPLs was made of phosphatidylinositol (32%), phosphatidylethanolamine (30%), and sphingomyelin (37%). Scanning electron images and particle size revealed significant disruption of the complex arrangement between membrane proteins and MPLs, which may help to release the MPLs into the aqueous medium.

4.2. Introduction

Milk phospholipids (MPLs) are a diverse group of over 30 structurally related compounds located within the milk fat globule membrane (MFGM) (Contarini & Povolo, 2013; Ortega-Anaya & Jiméñez-Flores, 2019). MPLs are relevant to human nutrition since

³ A version of this chapter has been published in *LWT-Food Science and Technology*, 2021, 141, 110864

their intake has shown beneficial cognitive performance effects (Hellhammer, Waladkhani, Hero, & Buss, 2010). Moreover, the therapeutic ability of MPLs have been documented in experimental arthritis in rats (Hartmann et al., 2009), the anticancer activity of colon cancer cells (Kuchta-Noctor, Murray, Stanton, Devery, & Kelly, 2016), antioxidant activity in vitro assays (Huang, Brennan, et al., 2020), and protection against gastrointestinal infections (Küllenberg, Taylor, Schneider, & Massing, 2012). Bioactivity of MPLs has been recently reviewed elsewhere (Küllenberg et al., 2012; Ortega-Anaya & Jimenez-Flores, 2019). Phospholipids are classified according to their chemical structure, namely glycerolphospholipids and sphingolipids (Contarini & Povolo, 2013). Phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) are examples of glycerolphospholipids, while sphingomyelin (SM) is the dominant species of sphingolipids (Ali et al., 2019). Bovine milk fat contains a high proportion of SM and PS (about 24 and 12% of the total PLs, respectively), two of the most bioactive phospholipids (Gassi et al., 2016). The concentration of MPLs, as well as their architecture within the milk fat globule, has already been elucidated in previous research (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Zheng, Jimenez-Flores, & Everett, 2014; Zheng, Jimenez-Flores, Gragson, & Everett, 2014).

Beta-serum (BS) is the main byproduct obtained during the manufacture of anhydrous milk fat. It is the remaining aqueous stream separated from the phase inversion of concentrated cream, and it is characterized for its relatively high concentration of MPLs (Cathpole, Tallon, Grey, Fletcher, & Flethcehr, 2008). Thus, beta-serum represents a rich source of MPLs with promising potential for extraction and subsequent commercialization

(Cheng, Rathnakumar, & Martínez-Monteagudo, 2019; Huang, Zhao, et al., 2020). Direct extraction of MPLs from BS has not been economically feasible in the past due to the numerous steps involved in the extraction and subsequent separation (Cheng et al., 2019). Instead, concentrates of MPLs are commercially available containing from 20 to 70% of MPLs with respect to the total fat (Huang, Zhao, et al., 2020). For instance, a powder containing 61% of MPLs was obtained by two-step concentration involving microfiltration of whey buttermilk followed by extraction via supercritical carbon dioxide (Costa, Elias-Argote, Jiménez-Flores, & Gigante, 2010). An investigation reported a concentrated fraction of MPLs (up to 56%) using enzymatic hydrolysis of protein prior to microfiltration of buttermilk and subsequent extraction via supercritical carbon dioxide (Barry, Dinan, & Kelly, 2017). A final concentration of 58% of MPLs was obtained from whey protein phospholipid concentrate using five-stage sequential extraction (Price, Fei, Clark, & Wang, 2018). A lipidic fraction containing about 70% of MPLs was obtained from BS using solvent fractionation (Price, Wan, Fei, Clark, & Wang, 2020).

Recently, the use of CO₂ responsive species as tunable solvents has been an attractive alternative for the extraction of lipids (Alshamrani, Vanderveen, & Jessop, 2016). A tertiary amine (N, N-dimethyl cyclohexylamine, CyNMe₂) has shown to be an efficient tunable or switchable solvent (Jessop et al., 2011). The speciality of CyNMe₂ is the ability to switch on and off from water-immiscible (hydrophobic) form to water-miscible (hydrophilic) form in the presence or absence of CO₂. The change in miscibility is because of a reversible acid-base reaction between hydrated CO₂ or carbonic acid in the carbonated water and the amine (Alshamrani et al., 2016). Cheng et al. (2019) demonstrated the

feasibility of extracting MPLs from different dairy matrices using CyNMe₂ as a switchable hydrophilicity solvent. The efficiency of CyNMe₂ strongly depended on the type of dairy matrices, where about 7% of MPLs were extracted directly from BS (Cheng et al., 2019).

Ultrasound prior to CyNMe₂ represents a potential way to improve the yields of MPLs from dairy byproducts. This is because frequencies between 20 and 30 kHz generates microjets and shear forces within the liquid, and such mechanical effects can break cell walls, which might improve the mass transfer and overall extraction yields (Vilkhu, Mawson, Simons, & Bates, 2008). Advantages and applications of ultrasound-assisted extraction have been reviewed elsewhere (Ashokkumar, 2015). The use of ultrasound prior to the extraction of MPLs has not been reported in the literature. The objective of this work is to evaluate the influence of ultrasound prior to the tertiary amine extraction of MPLs from beta-serum.

4.3. Materials and methods

4.3. 1. Materials

N,N-dimethylcyclohexylamine (99%, Sigma Aldrich, St. Louis, MO, USA), methanol (99.9%, Sigma Aldrich), chloroform (99.9%, Sigma Aldrich), hexane (99.9%, Sigma Aldrich), HPLC-grade water (Sigma Aldrich), activated silica gel (Silica gel 60 G, EMD Millipore), phospholipid mixture (Soybean, P3817-1VL, Sigma Aldrich), Rd-dope (Avanti Polar Lipids Inc., Alabaster, AL, USA), Laemmli buffer (BioRad, Hercules, CA, USA), Tris-acrylamide gels (4–15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad), Tris/Glycine/SDS Buffer (Bio-Rad), Protein TM Kaleidoscope standards (Bio-Rad), and

2-mercaptoethanol (Fisher Scientific) were purchased from commercial suppliers. Beta-serum was obtained from a cheese plant (Valley Queen, Millbank, SD). The BS was divided into 500 mL portions stored at -20°C until further use. The BS was thawed by immersing the bottles in a water bath at room temperature.

2.2. Compositional analysis

The BS was tested for total solids, total protein, fat, and pH. The total solids were determined gravimetrically at 103°C for 15 h using a laboratory oven (Isotemp oven, Iowa, USA), following the methodology reported elsewhere (Cheng et al., 2019). The total protein was obtained through Kjeldahl method, while the fat content was determined using Mojonnier method (Gallier, Gragson, Cabral, Jimenez-Flores, & Everett, 2010). The pH was measured using Orion Versa Star Pro (Thermo Fisher Scientific, Waltham, MA), and the lactose was measured using HPLC following the methodology reported elsewhere (Amamcharla & Metzger, 2011). Briefly, an HPLC instrument (Beckman Coulter Inc.) with a multichannel wavelength scanning detector (190–600 nm, System Gold 168 detector) was used to determine the concentration of lactose. A refractive index detector (RI-2031, Jasco Corporation, Hachioji, Japan), an ion exclusion column (ROA-Organic Acid H+ 8%, Phenomenex Inc., Torrance, CA) heated at 65°C were used for the separation of lactose. A sulfuric acid solution at a concentration of 0.013 N was used as a mobile phase.

4.3.2. Ultrasound pretreatment

One hundred g of BS were ultrasonicated for 4 min using a 20 kHz sonicator (U1P1000hd, Hielscher Ultrasonics, Teltow, Germany). The ultrasound horn (21 mm length and 3.0 cm² of surface area) was immersed in the test sample, covering about two-thirds of the length. The tested peak to peak amplitude was 50, 75, and 100%. For all

treatments, the initial temperature was kept constant (21°C), and the temperature rise during sonication was recorded using a K-type thermocouple connected to a data logger (Omega Engineering Inc., Stamford, CT). The tested conditions were selected based on preliminary experiments (Rathnakumar & Martinez-Monteagudo, 2020). The rise in the sample temperature due to acoustic cavitation was 17.4 ± 0.4 , 32.6 ± 0.3 , and 48.5 ± 0.2 °C for 50, 75, and 100% peak to peak amplitude, respectively. Each tested amplitude was converted into acoustic intensity (I_a , W cm^{-2}), according to Equation (4.1) (Margulis & Margulis M, 2003).

$$I_a = \frac{m \cdot C_p \cdot \frac{dT}{dt}}{S_a} \quad (4.1)$$

where m is the mass of the sample (kg), C_p is the heat capacity of the sample ($\text{J kg}^{-1} \text{K}^{-1}$), $\frac{dT}{dt}$ is the rate of temperature rise determined experimentally, and S_a is the surface area of the ultrasound emitting surface (cm^2). The resulting acoustic intensities were 15.53 ± 1.20 , 31.76 ± 2.46 , and $44.56 \pm 3.47 \text{ W cm}^{-2}$.

4.3.3. Extraction methods

The extraction of MPLs from BS was carried out in two steps: i) solvent extraction and ii) lipid fractionation. The solvent extraction was performed via Folch and CyNMe₂ extraction, following the methodology reported elsewhere (Cheng et al., 2019). For the Folch extraction, 1 g of BS was mixed with 20 mL of chloroform: methanol solution (2:1, v/v). The mixture was vortexed for 3 min, followed by centrifugation at room temperature (4200×g) for 5 min. The upper phase was then discarded, while the lower phase was

transferred to a test tube, where the chloroform was removed at 45 °C under nitrogen flow. In the case of CyNMe₂, 1 g of the sonicated samples were transferred to a 20 mL vial containing three different solvent to sample 3, 6, or 12 mL of CyNMe₂. The treatments were named as CyNMe₂-x, with x refers to the solvent to sample ratio. The mixture (solvent/sample) was stirred at room temperature for 18 h. Afterward, an equimolar amount of water was added to maintain the stoichiometry of the reaction, followed by bubbling CO₂ at room temperature until the layer of CyNMe₂ and water combined, leaving the lipid layer at the top of the vial. Then, 3 mL of hexane was added to dissolve the lipid layer and subsequently transferred to a test tube, where the hexane was evaporated under nitrogen flow at 30 °C. The dried lipids were used to calculate the total lipids according to Equation (4.2).

$$\text{Total lipids (\%)} = \frac{\text{weight of dried lipids}}{\text{weight of sample}} \cdot 100 \quad (4.2)$$

In the second step, the extracted lipids were fractionated to recover the MPLs using solid-phase extraction (SPE). An activated silica gel column (1 cm × 10 cm) was used for the separation, following the methodology reported elsewhere (Cheng et al., 2019). Dried lipids (100 mg) were dissolved in 1 mL of chloroform: methanol solution (95:5, v/v) and run through the SPE column previously conditioned with 10 mL of the chloroform:methanol solution. The MPLs were recovered with 10 mL of methanol followed by 10 mL of chloroform: methanol: water (5:3:2 v/v/v). Finally, solvents were evaporated at 40°C under vacuum, and the MPLs fractions were calculated using Equation (4.3). The extracted MPLs were stored at -20 °C until further analysis.

$$\text{Total Phospholipids (\%)} = \frac{\text{weight of dried fraction}}{\text{weight of lipids}} \cdot 100 \quad (4.3)$$

4.3.4. Physical determinations

4.3.4.1. Particle size distribution

A ZetaSizer Nano ZS (Malvern Instruments Ltd, Cambridge, UK) was used to determine the particle size distribution of treated samples, according to the methodology reported elsewhere (Ma, Yang, Zhao, & Guo, 2018). Firstly, 10 μL of the test sample was transferred to a disposable cuvette (DTS 0012, Sigma-Aldrich, St Louis, MO, USA), where it was diluted 100x with deionized water. Diluted samples were transferred to the measuring chamber and equilibrated at room temperature for 120 s. A scattering angle of 170° with a refractive index of 1.46 was used to determine the average size and distribution of particles within a range of 0.6–6000 nm.

4.3.4.2. Zeta potential

Untreated and treated samples were analyzed for the zeta-potential using a Zetasizer Nano (Malvern Instruments Ltd., Cambridge, UK). Diluted samples (100x) were transferred to a disposable polycarbonate cuvette (ATA scientific, DTS1061). Measurements have repeated a minimum of 10 times per run with a minimum of 7 runs.

4.3.5. Microstructure

4.3.5.1. Confocal laser scanning microscopy

An Olympus FV1000 inverted confocal laser scanning electron microscope (CLSM) (Olympus America Inc., Center Valley, PA) was used to analyze the microstructure of sonicated samples. According to the methodology described elsewhere (Gallier et al., 2010). A drop of the test sample was placed in the concavity of the slide containing 50 μ L of agarose (0.5% w/v in deionized water). CLSM images were captured at 40x magnification, and the excitation of the Rd-dope was achieved at 559 nm using emission from a diode laser. All images were acquired at room temperature.

4.3.5.2. Scanning electron microscopy

An aliquot of 2000 μ L of the sonicated sample was placed in a microscope slide and dried under vacuum at 40 °C for 15 h (Isotemp Vacuum, model 280A, USA). Samples were mounted on circular aluminium stubs with double-sided sticky tape, coated with gold to a thickness of 12 nm (Sengar, Rawson, Muthiah, & Kalakandan, 2020). The structure was observed and subsequently captured using a scanning electron microscope (model S-3400N Hitachi, Ltd. Tokyo, Japan) at an accelerating voltage of 7 kV.

4.3.6. Analytical determinations

4.3.6.1. Gel electrophoresis

The protein profile was determined using Sodium Dodecyl Sulfate Polyacrylamide gel (SDS-PAGE) under reducing conditions as reported elsewhere (Primacella, Wang, & Acevedo, 2018). Briefly, 10 mL of sample was mixed with 40 mL of acetone. After the

precipitation of the proteins, the mixture was placed in a freezer at $-4\text{ }^{\circ}\text{C}$ for 1 h and centrifuged (3600 rpm) (Jouan CR412, Jouan Inc., Winchester, VA, USA) for 15 min at $0\text{ }^{\circ}\text{C}$. The supernatant was discarded, and the pellets were dissolved in 0.1 M phosphate buffer, pH 5.0 (Rathnakumar, Anal, & Lakshmi, 2017). Then, 5 μL of dissolved pellets were transferred into a vial containing 4.75 μL of 2x Laemmli sample buffer (Bio-Rad, Hercules, CA), and 0.25 μL of 4% 2-mercaptoethanol (Fisher Scientific, Hampton, NH). Dissolved pellets were heated at $90\text{ }^{\circ}\text{C}$ for 5 min. Upon cooling, 10 μL of the preparation was loaded into Tris-acrylamide gels (4–15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad). Gels were run for 1 h at 200 V using Tris/Glycine/SDS buffer (Bio-Rad). Then, the gels were removed and stained using Bio-safe Coomassie G-250 stain (Bio-Rad) and destined to get the protein pattern. The individual proteins were estimated based on the molecular weight using the standard from BIO-RAD (Precision plus protein standards, 161–0375), and the protein bands were identified following the methodology reported elsewhere (Ye, Singh, Taylor, & Anema, 2004). The gels were scanned using Bio-5000 Microtek (Microtek, Taiwan) and processed using the software Scan Wizard Bio properties.

4.3.7. Quantification of phospholipids profile

The phospholipid profile was determined by a UHPLC system (Dionex Ultimate 3000, Thermo Scientific) coupled to a charged aerosol detector (CAD, Dionex Corona Veo RS, Thermo Scientific). The methodology reported by (Braun, Flück, Cotting, Monard, & Giuffrida, 2010) was followed with modifications. Briefly, frozen samples were removed from cold storage and allowed to reach room temperature. Then, MPLs were dissolved in

1 mL of chloroform: methanol (9:1, v/v). Dissolved MPLs were separated using a binary phase and two identical silica columns (4.6 mm × 250 mm, 5 μm particle size, Synchronis, Thermo Scientific). Mobile phase A consisted of ammonium acetate at a concentration of 3 g L⁻¹, while acetonitrile-methanol (100 + 3, v/v) was used as mobile phase B. A gradient run (t) of 52 min was used to separate the major classes of MPLs using the following conditions: 5% A and 95% B at t = 0 min; 5% A and 95% B at t = 2 min; 25% A and 75% B at t = 35 min; 25% A and 75% B at t = 40 min; 5% A and 95% B at t = 41 min; 5% A and 95% B at t = 52 min. An overall flow rate of 1 mL min⁻¹, and the column oven was kept at 55°C. Fifty μL of resuspended samples were injected. The CAD was set to a power of 1.0, and the data acquisition rate was set at 2 Hz with a filter constant of 3.6. The evaporator temperature was held at 35°C. A corona nitrogen gas generator and air compressor (Peak Scientific Instruments, Billerica, MA, USA) supplied N₂ gas to the detector. A standard curve was generated with the major classes of phospholipids at different concentrations, including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). The peaks corresponding to each standard were integrated using Chromeleon software Version 7 (Thermo Scientific). Each sample was analyzed in duplicates under the same conditions. The five major phospholipids were identified by their retention time and were quantified by comparing their peak area with a standard curve.

4.3.8. Statistical analysis

All extraction conditions were carried out in triplicates, and the mean values for the total lipids and recovered MPLs were compared using Tukey's test ($p < 0.05$). The

statistical analysis was carried out using Sigma plot software V11 (SPSS Inc., Chicago, IL, USA).

4.4. Results and discussion

4.4.1. Composition of beta-serum

Table 4.1 shows the gross composition of BS, where the major compound on a dry basis within the BS was lactose ($43.88 \pm 0.88\%$), followed by total protein ($20.98 \pm 1.01\%$) and fat ($14.39 \pm 0.83\%$). The literature of the chemical characterization of BS showed a quite variable composition, from 40 to 45, 30–33, and 8–22% for lactose, protein, and fat, respectively (Cheng et al., 2019; Huang, Zhao, et al., 2020). The concentration of MPLs in BS is relatively high ($14.67 \pm 0.43\%$ with respect to the total fat), making it a suitable source of MPLs for extraction and subsequent isolation. However, the concentration of MPLs from BS requires the removal of lactose, which is the predominant compound, and proteins (caseins and whey protein).

Table 4.1. Compositional analysis of beta-serum.

Parameter	Value
pH	6.65 ± 0.01
Total solids (%)	9.96 ± 0.75
Total protein (% , dry basis)	20.98 ± 1.01
Total fat (% , dry basis)	14.39 ± 0.88
Lactose (% , dry basis)	43.88 ± 1.65
Ash (% , dry basis)	4.82 ± 0.28
Total phospholipids (g per g of fat)	14.67 ± 0.43

4.4.2. Total phospholipids

The extraction of total MPLs without ultrasound pretreatment produced extraction yields of 5.34 ± 0.35 , 2.81 ± 0.15 , 5.36 ± 0.55 , and $7.75 \pm 0.67\%$ for Folch, CyNMe-3, CyNMe-6, and CyNMe-12, respectively (**Figure 4.1**). Cheng et al. (2019) reported similar yields of MPLs extracted from BS using Folch and CyNMe2 (5% and 2–7%, respectively). Folch extraction involves the disruption of hydrogen bonds and electrostatic forces within the membrane proteins by the action of ethanol, which allows the organic solvent to dissolve the MPLs. The CyNMe2 extraction consists of an organic base of medium polarity and strong basicity (Vanderveen, Durelle, & Jessop, 2014). CyNMe2 has low miscibility with water, and it converts into a hydrophilic in the presence of CO₂. Essentially, the organic base (CyNMe2) is converted into bicarbonate salt by CO₂, where the bicarbonate salt is soluble in water, and it can be recovered for further used. Alshamrani et al. (2016) reviewed the fundamentals of CO₂-responsive species, including CyNMe2. On the other hand, the use of ultrasound before extraction resulted in higher yields (8–69%), increasing the acoustic intensity. At an acoustic intensity of $15.58 \pm 1.20 \text{ W cm}^{-2}$, the yield of MPLs was 8.36 ± 0.41 , 17.29 ± 0.39 , 20.31 ± 1.01 , and $24.87 \pm 1.24\%$ for Folch, CyNMe3, CyNMe-6, and CyNMe-12, respectively. Increasing the acoustic intensity to $31.76 \pm 2.46 \text{ W cm}^{-2}$ yielded higher values of extracted MPLs (19–52%), being higher for the CyNMe2-12 extraction. A similar trend but more pronounced was observed at an acoustic intensity of $44.56 \pm 3.47 \text{ W cm}^{-2}$, where the highest extraction values of MPLs was obtained for CyNMe2-12 ($69.07 \pm 3.45\%$).

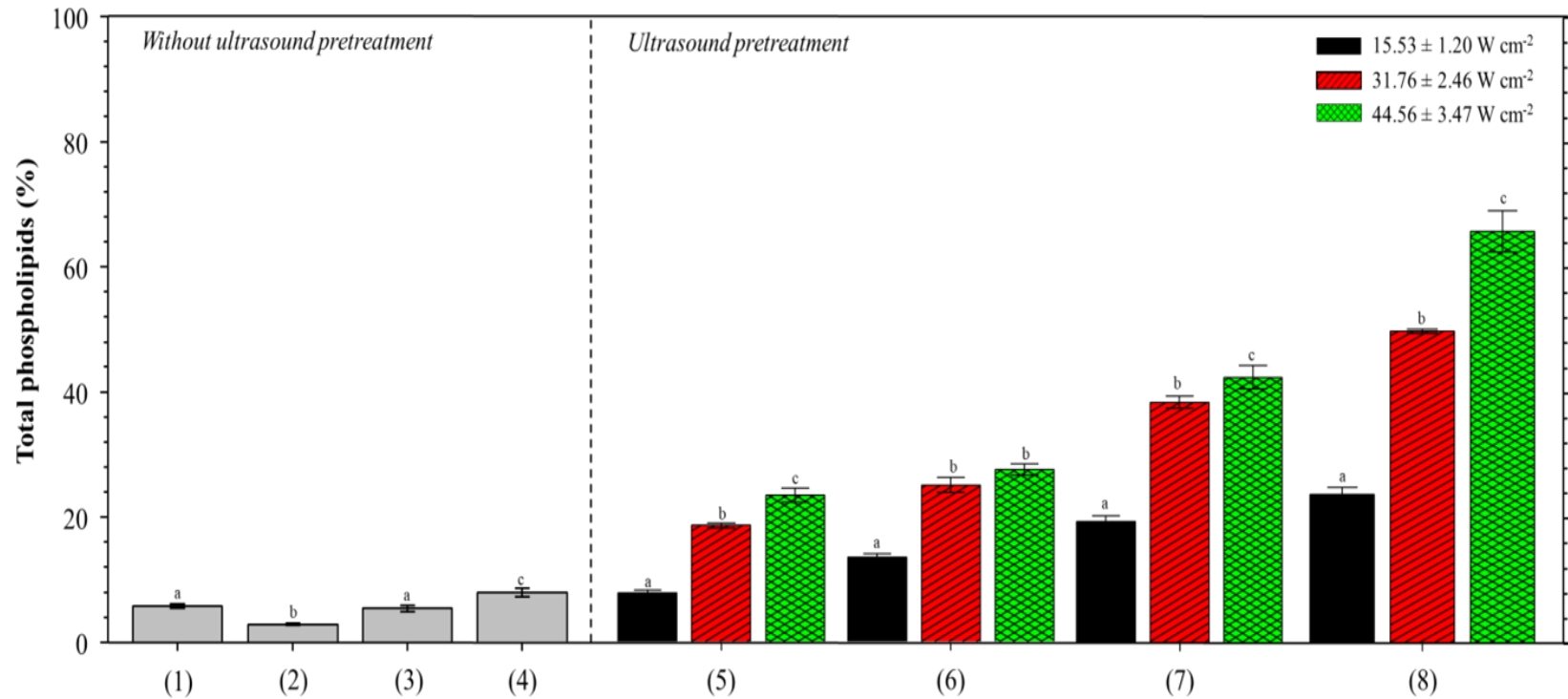


Figure 4.1. Comparison of the different extraction treatments on the yield of total lipids from beta-serum. (1) Folch extraction, (2) CyNMe₂ extraction at solvent to sample ratio of 3/1, (3) CyNMe₂ extraction at solvent to sample ratio of 6/1, (4) CyNMe₂ extraction at solvent to sample ratio of 12/1, (5) ultrasound prior Folch extraction, (6) ultrasound prior CyNMe₂ extraction at solvent to sample ratio of 3/1, (7) ultrasound prior CyNMe₂ extraction at solvent to sample ratio of 6/1, and (8) ultrasound prior CyNMe₂ extraction at solvent to sample ratio of 12/1.

During sonication, acoustic cavitation (formation, growth, and collapse of bubbles) occurs within the liquid, and it creates shear forces of large magnitude (Ashokkumar, 2015). Such forces can break the complex arrangement between membrane proteins and MPLs, releasing the MPLs into the aqueous medium.

The highest concentration obtained in this study was $69.67 \pm 3.45\%$ with respect of the fat content. Costa et al. (2010) concentrated MPLs up to 61% via ultrafiltration followed by supercritical carbon dioxide. A concentrate containing up to 19% of MPLs was obtained using a two-step process that consisted of nanofiltration and supercritical carbon dioxide (Astaire, Ward, German, & Jimenez-Flores, 2003). A fraction of MPLs (58%) was produced after five-stage sequential extraction with ethanol (Price et al., 2018). A product containing 36% of MPLs was obtained by the addition of zinc and calcium acetate with thermal treatment prior to ethanol extraction (Price, Fei, Clark, & Wang, 2020). Price, Fei, et al. (2020) reported a fraction containing 70% of MPLs acetone fractionation. Ubeyitogullari and Rizvi (2020) reported a MPLs fraction containing 76% using sequential supercritical carbon dioxide and ethanol supercritical carbon dioxide.

4.4.3. Particle size and zeta potential

Prior to the ultrasound, BS displayed three distinctive peaks, where the highest intensity corresponded to a size of 324, 63, and 93 nm (**Figure 4.2**). Each peak accounted for 80, 12, and 8% of a total distribution of particles, respectively. Contrary, the ultrasound pretreatment shifted the distribution of particles to a broader and smaller distribution that

spanned from 600 to 90 nm, with an average size between 162 and 172 nm (**Table 4.2**).

Such behavior was observed regardless of the applied acoustic intensity.

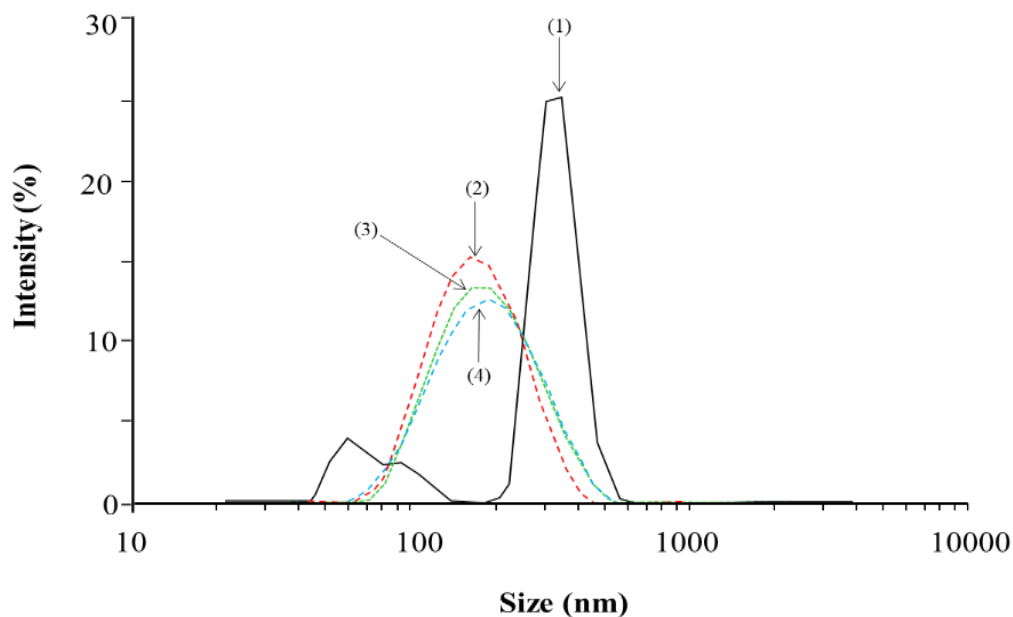


Figure 4.2. Particle size distribution before and after ultrasound pretreatment. (1) untreated beta-serum; (2) ultrasound pretreatment at an acoustic intensity of $15.5 \pm 1.2 \text{ W cm}^{-2}$, (3) ultrasound pretreatment at an acoustic intensity of $31.7 \pm 2.4 \text{ W cm}^{-2}$, and (4) ultrasound pretreatment at an acoustic intensity of $44.5 \pm 3.4 \text{ W cm}^{-2}$.

Table 4.2. Zeta potential and average particle size of beta-serum before and after ultrasound pretreatments.

Sample	Zeta potential (mV)	Average particle size (nm)
Untreated beta-serum	$-37.8 \pm 5.98a$	$579.7 \pm 16.46a$
Ultrasound at $15.5 \pm 1.2 \text{ W cm}^{-2}$	$-38.8 \pm 6.03a$	$171.9 \pm 12.17b$
Ultrasound at $31.7 \pm 2.4 \text{ W cm}^{-2}$	$-30.2 \pm 5.69ab$	$163.1 \pm 13.02b$
Ultrasound at $44.5 \pm 3.4 \text{ W cm}^{-2}$	$-27.1 \pm 5.42b$	$172.1 \pm 15.62b$

Mean \pm standard deviation within each column with different letters are significantly different ($p < 0.05$) according to Tukey test

Average particle size as well as their distribution (**Table 4.2** and **Figure 4.2**, respectively) seem to support the role of ultrasound pretreatment. **Table 4.2** shows the values of the zeta potential of BS before and after sonication. Overall, the values ranged from -27 to -38 mV, showing a slightly increasing tendency with the acoustic intensity.

4.4.4. Microstructure

The microstructure as well as the gross morphology of BS was evaluated before and after ultrasound pretreatment (**Figure 4.3** and **Figure 4.4**). Untreated BS exhibited droplets of MPLs with an average size of 4000 nm. In native form, MPLs are arranged as liquid-disordered and liquid order complex, having a size of 5000–10,000 nm (Gallier et al., 2010). A reduction of the MPLs droplets was observed at an intensity of $15.58 \pm 1.20 \text{ W cm}^{-2}$ (**Figure 4.3b**). Further increment (31.76 ± 2.46 and $44.56 \pm 3.47 \text{ W cm}^{-2}$) resulted in substantial reduction of the MPLs droplets (**Figure 4.3c** and **Figure 4.4d**). Smaller particles increase the mass transfer (Rosenthal, Pyle, & Niranjana, 1998), where the diffusion of MPLs droplets into the aqueous solvent is enhanced.

The gross morphology of BS before and after ultrasound (**Figure 4.4**) also revealed a significant reduction of the aggregates. Images through scanning electron microscopy from untreated BS displayed numerous heterogeneous porous of variable sizes and shapes

Figure 4.4a). Ultrasound pretreatment seems to fracture the structure of BS, producing small aggregates of proteins and MPLs (**Figure 4.4b-d**).

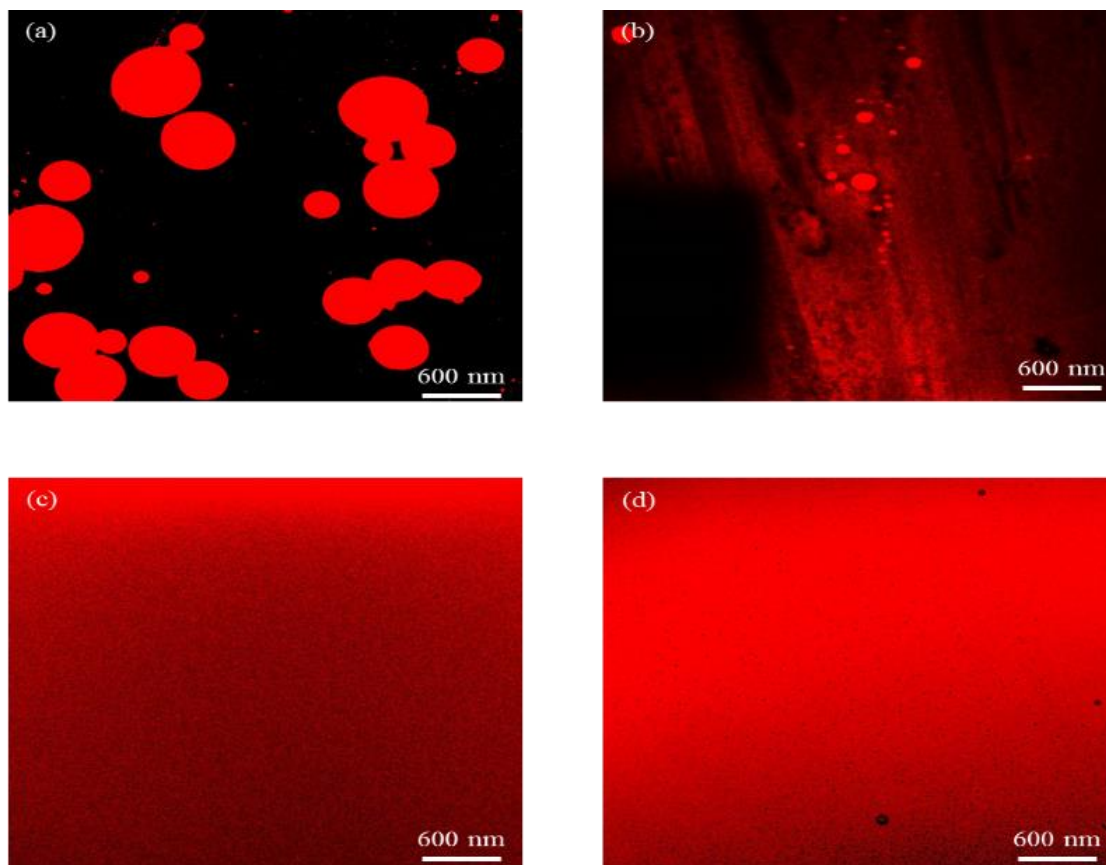


Figure 4.3 Confocal laser scanning microscopy images of beta-serum: (a) untreated beta-serum; (b) ultrasound pretreatment at an acoustic intensity of $15.5 \pm 1.2 \text{ W cm}^{-2}$, (c) ultrasound pretreatment at an acoustic intensity of $31.7 \pm 2.4 \text{ W cm}^{-2}$, and (d) ultrasound pretreatment at an acoustic intensity of $44.5 \pm 3.4 \text{ W cm}^{-2}$.

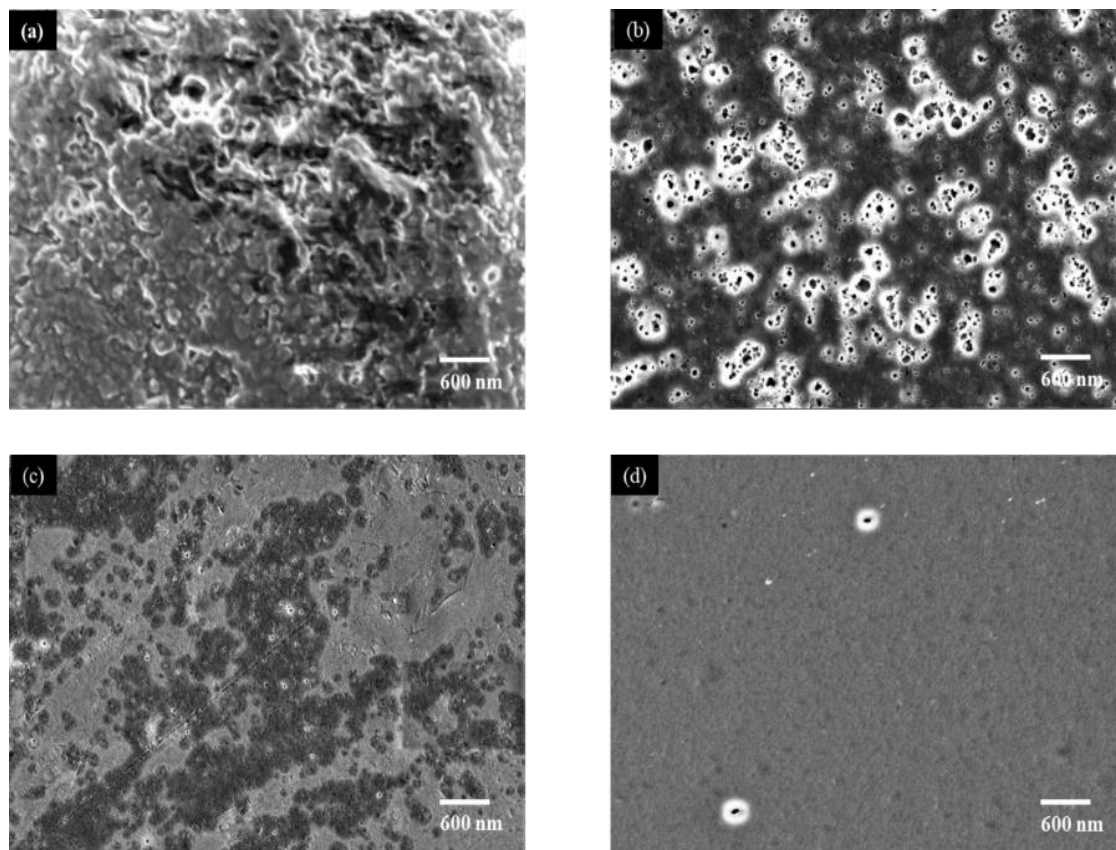


Figure 4.4. Scanning microscopy images of beta-serum: (a) untreated beta-serum; (b) ultrasound pretreatment at an acoustic intensity of $15.5 \pm 1.2 \text{ W cm}^{-2}$, (c) ultrasound pretreatment at an acoustic intensity of $31.7 \pm 2.4 \text{ W cm}^{-2}$, and (d) ultrasound pretreatment at an acoustic intensity of $44.5 \pm 3.4 \text{ W cm}^{-2}$.

4.4.5. Protein profile

The effect of ultrasound pretreatment on the protein profile of BS was evaluated using SDS-page (**Figure 4.5**). Three major classes of proteins were observed in untreated BS, namely milk fat globule membrane (MFGM), caseins, and whey proteins (column 2 in **Figure 4.5**). Milk fat globule membrane included xantane oxidase, butylophilin, and

adipophilin, displaying bands at 100, 75, and 50 kDa, respectively. On the other hand, α -casein and β -casein displayed their characteristics bands at 20 and 25 kDa, respectively. Whey proteins (α -lactalbumin and β -lactoglobulin) showed their bands at molecular weight of 10 and 50 kDa, respectively. Price, Fei, et al. (2020) reported similar protein distribution of untreated BS. Interestingly, the application of ultrasound within the tested acoustic intensities ($15\text{--}44\text{ W cm}^{-2}$) did not significantly impact the primary structure of the proteins. O'Sullivan, Murray, Flynn, and Norton (2016) studied the impact of ultrasound on the structure of dairy proteins. It is thought that ultrasound induces hydrophobic interactions (non-covalent) within the dairy proteins as opposed to peptide cleavage (Chandrapala, Martin, Zisu, Kentish, & Ashokkumar, 2012). This observation agrees with the values of zeta potential (**Table 4.2**) that were within the stability region (-27 and -38 mV) of colloidal suspensions (Czapla, Bart, & Jesberger, 2000).

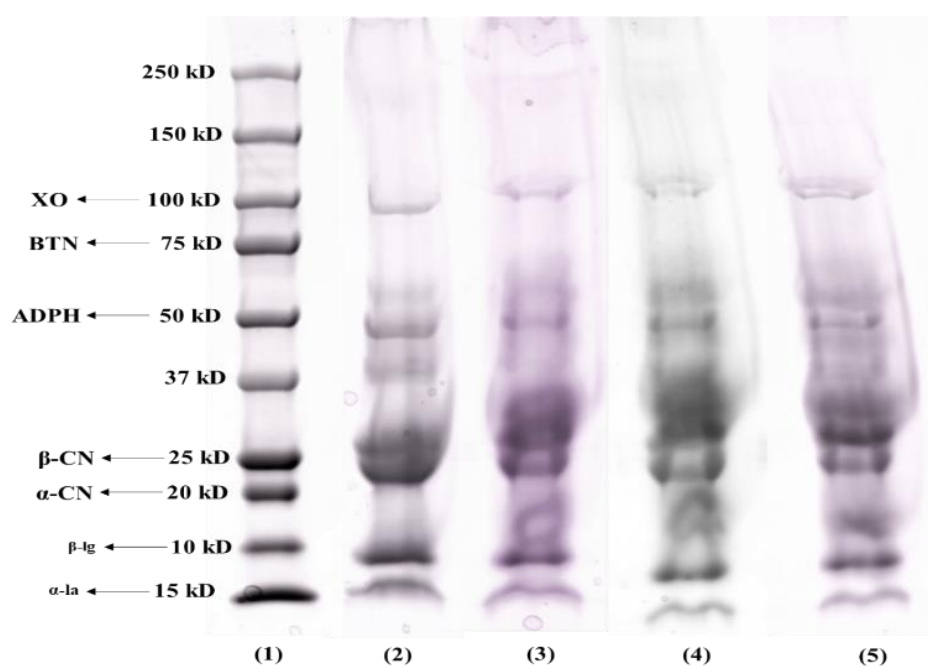


Figure 4.5. SDS-PAGE pattern of Beta serum: (1) Molecular weight standard; (2) untreated beta-serum ;(3) ultrasound pretreatment at $15.5 \pm 1.2\text{ W cm}^{-2}$, (4) ultrasound pretreatment at $31.7 \pm 2.4\text{ W cm}^{-2}$, and (5) ultrasound pretreatment at $44.5 \pm 3.4\text{ W cm}^{-2}$.

4.4.6. Profile of milk phospholipids

Five major classes of MPLs were quantified in the recovered fraction, including PI, PS, PE, PC, and SM (**Table 4.3**). In general, the concentration and relative distribution of MPLs varied considerably according to the extraction method. Extraction without ultrasound resulted in concentrations of 28–63, 25–27, 28–46, 25–63, and 24–55 $\mu\text{g mL}^{-1}$, respectively. Similar profile of MPLs has been reported in from butter serum (Gassi et al., 2016), raw milk (Rombaut, Dewettinck, & Van Camp, 2007), butter milk (Costa et al., 2010), and beta-serum (Price, Fei, et al., 2020). At an acoustic intensity of $15.58 \pm 1.20 \text{ W cm}^{-2}$, the concentration of PI increased about 9.83- to 20.31-fold in comparison with the extraction without ultrasound pretreatment. Further increase in the acoustic intensity (31.76 ± 2.46 and $44.56 \pm 3.47 \text{ W cm}^{-2}$) remarkably improved the concentration of PI by about 15.42- to 46.32-fold, being the highest increment at $31.76 \pm 2.46 \text{ W cm}^{-2}$ prior to CyNMe2-6 extraction. Contrary, the presence of PS was not detected when ultrasound was used prior to CyNMe2 extraction, independently of the intensity. In the case of PE and SM, the greatest improvement in the extraction was obtained at $44.56 \pm 3.47 \text{ W cm}^{-2}$ prior to CyNMe2-12, where the concentration improved by 16.12 and 13.40 fold, respectively.

Figure 4.6 illustrates the relative distribution of MPLs as a function of the extraction method. The MPLs fraction obtained via Folch extraction was made of PI (19%), PS (16%), PE (31%), PC (16%), and SM (16%). In contrast, the recovered fraction of MPLs via ultrasound ($44.56 \pm 3.47 \text{ W cm}^{-2}$) prior to CyNMe2-12 was mainly made of PI (32%), PE (30%), and SM (37%). Such observations exemplify the complexity of the MPLs arrangement within the protein membranes.

Table 4.3. Concentration ($\mu\text{g mL}^{-1}$) of recovered milk phospholipids from beta-serum.

Treatment	Without ultrasound pretreatment					
	PI	PS	PE	PC	SM	Total
Folch	28.71 \pm 2.58	25.19 \pm 2.26	46.75 \pm 4.21	25.42 \pm 2.28	24.67 \pm 2.22	150.76 \pm 13.26
CyNMe2-3	27.62 \pm 2.48	N.D.	N.D.	23.10 \pm 2.07	28.59 \pm 2.57	79.33 \pm 6.98
CyNMe2-6	22.21 \pm 1.99	27.86 \pm 2.46	28.86 \pm 2.59	45.11 \pm 4.05	27.74 \pm 2.49	151.23 \pm 13.31
CyNMe2-12	63.07 \pm 5.67	N.D.	36.82 \pm 3.31	63.92 \pm 5.75	54.96 \pm 4.94	218.79 \pm 19.25
Acoustic intensity 15.38 \pm 1.20 W cm⁻²						
Treatment	PI	PS	PE	PC	SM	Total
CyNMe2-3	271.61 \pm 24.44	N.D.	102.22 \pm 9.21	N.D.	26.21 \pm 2.35	400.05 \pm 35.20
CyNMe2-6	475.81 \pm 42.82	16.78 \pm 1.51	15.08 \pm 1.35	46.51 \pm 4.18	19.19 \pm 1.72	573.39 \pm 50.45
CyNMe2-12	226.54 \pm 20.38	N.D.	214.39 \pm 19.29	N.D.	261.19 \pm 23.51	702.13 \pm 61.78
Acoustic intensity 31.76 \pm 2.46 W cm⁻²						
Treatment	PI	PS	PE	PC	SM	Total
CyNMe2-3	371.01 \pm 33.39	54.62 \pm 4.91	340.76 \pm 30.66	22.88 \pm 2.05	28.62 \pm 2.57	817.81 \pm 71.97
CyNMe2-6	1022.781 \pm 92.07	N.D.	87.67 \pm 7.89	N.D.	27.61 \pm 2.48	1138.01 \pm 100.14
CyNMe2-12	1283.61 \pm 115.52	80.21 \pm 7.21	52.62 \pm 7.21	N.D.	67.16 \pm 6.04	1438.61 \pm 130.55
Acoustic intensity 44.56 \pm 3.47 W cm⁻²						
Treatment	PI	PS	PE	PC	SM	Total
CyNMe2-3	272.14 \pm 24.49	N.D.	49.18 \pm 4.42	226.58 \pm 20.39	281.87 \pm 25.36	829.79 \pm 73.02
CyNMe2-6	445.42 \pm 40.98	N.D.	442.63 \pm 39.83	N.D.	N.D.	898.06 \pm 79.03
CyNMe2-12	638.84 \pm 57.49	N.D.	604.60 \pm 54.41	N.D.	736.56 \pm 66.29	1980.02 \pm 174.24
CyNMe2-3 – extraction with N,N-dimethylcyclohexylamine at a solvent-sample ratio of 3; CyNMe2-6 – extraction with N,N-dimethylcyclohexylamine at a solvent-sample ratio of 6; CyNMe2-12 – extraction with N,N-dimethylcyclohexylamine at a solvent-sample ratio of 12; PI – phosphatidylinositol; PS – phosphatidylserine; PE – phosphatidylethanolamine; PC – phosphatidylcholine; SM – phingomyelin; N.D. – not detected.						

Overall, ultrasound pretreatment produced a higher concentration of PI and PE (**Figure 4.6**) that are located on the inner parts of the membranes. PI and PE might be released to the aqueous medium upon sonication. The relative high concentration of PE is of technological interest since PE has been used for liposomes preparation (Ali et al., 2019). Recovered fractions using ultrasound prior to CyNMe2 extraction were high in SM, which is a desirable MPLs due to its potential health benefits (Ortega-Anaya & Jimenez-Flores, 2019). The concentration and distribution of MPLs can be modulated with the acoustic intensity and solvent to sample ratio. Additional research steps are needed to quantify the oxidation products within the recovered fractions.

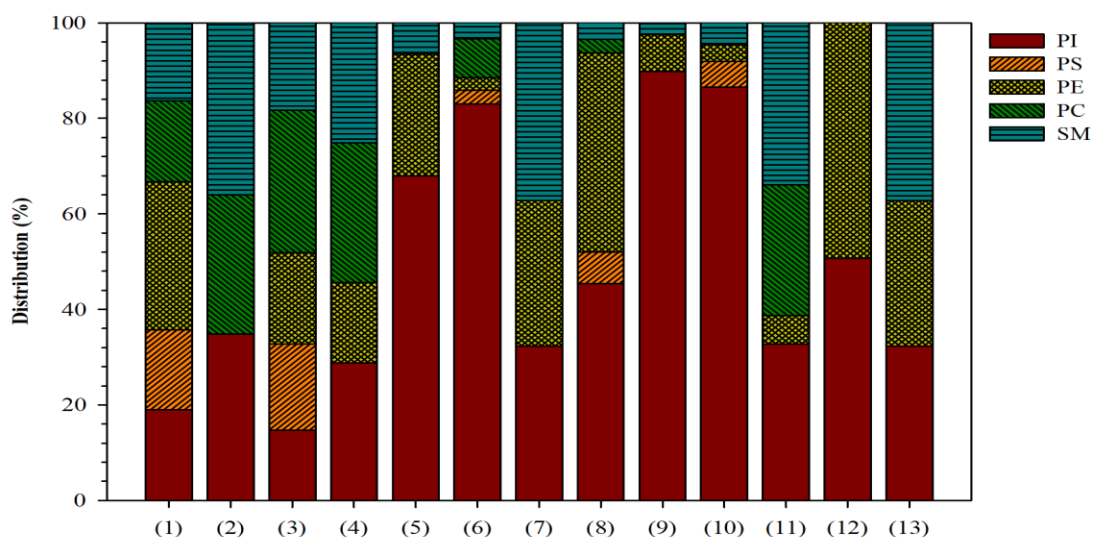


Figure 4.6. Distribution of milk phospholipids after different extraction methods: (1) Folch extraction, (2) CyNMe2 extraction at solvent to sample ratio of 3/1, (3) CyNMe2 extraction at solvent to sample ratio of 6/1, (4) CyNMe2 extraction at solvent to sample ratio of 12/1, (5) ultrasound ($15.5 \pm 1.2 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 3/1; (6) ultrasound ($15.5 \pm 1.2 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 6/1; (7) ultrasound ($15.5 \pm 1.2 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 12/1; (8) ultrasound ($31.7 \pm 2.4 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 3/1; (9) ultrasound ($31.7 \pm 2.4 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 6/1; (10) ultrasound ($31.7 \pm 2.4 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 12/1; (11) ultrasound ($44.5 \pm 3.4 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of 3/1; (12) ultrasound ($44.5 \pm 3.4 \text{ W cm}^{-2}$) prior to CyNMe2 extraction at solvent to sample ratio of

6/1; and (13) ultrasound ($44.5 \pm 3.4 \text{ W cm}^{-2}$) prior to CyNMe₂ extraction at solvent to sample ration of 12/1. PI – phosphatidylinositol; PS – phosphatidylserine; PE – phosphatidylethanolamine; PC – phoshatidylcholine; SM – phingomyelin.

4.5 Conclusions

The application of ultrasound prior to CyNMe₂ extraction improved the extraction yield of MPLs from beta-serum. The analysis of particle size distribution, zeta potential, protein profile, and microstructure suggest that ultrasound can break the complex arrangement between membrane proteins and MPLs, releasing the MPLs into the aqueous medium. In addition, the relative distribution of the major classes of MPLs strongly depended on the extraction method. Upon further separation, MPLs can be used as ingredients for a number of applications, such as infant formula and instant powders. This work demonstrates that ultrasound prior to CyNMe₂ has great potential for producing MPLs fractions from dairy byproducts.

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CHAPTER 5

EFFECT OF TEMPERATURE ON THE SWITCHABLE SOLVENT EXTRACTION
OF PHOSPHOLIPIDS FROM BETA-SERUM⁴**5.1. Abstract**

In this study, the extraction of milk phospholipids(MPLs) from beta-serum is a byproduct obtained from anhydrous milk fat, which contains many phospholipids that go to the aqueous phase during processing. The research aimed to improve the extraction yields of MPLs utilizing temperature assisted N-N,dimethylcyclohexylamine(CyNMe₂) extraction. The extraction conditions such as temperature (25,40,60 °C), time (3,10,18hr) and solvent ratio (3/1 ,10/1, 18/1 mL) were obtained using a factorial design. A temperature of 60°C and a minimized solvent ratio(3/1 mL) and time (3 h) yielded 29.29% ± 0.06% of MPLs, while only 7.57 ± 0.59% (12/1 mL and time 18 h) were recovered without temperature. The extracted lipids were fractionated by Solid-phase extraction, and the individual MPLs of the sample were quantified, and they include (sphingomyelin, phosphatidylethanolamine, serine and choline) from the HPLC-CAD. The fraction of MPLs was made of phosphatidylinositol (59%), phosphatidylethanolamine (35%), and sphingomyelin (4.9%). Further confocal laser scanning images and scanning electron images were determined to reveal the microstructure and how it impacted the protein membranes rupture and released the phospholipids to the aqueous. The outcomes of this study show an alternative approach to extract the phospholipids from dairy by-products.

⁴ A version of this chapter is to be submitted to the *Journal of Food Process Engineering*

5.2. Introduction

Milk phospholipids (MPLs) are quantitatively classified into glycerophospholipids and sphingolipids based on their chemical structure (Avalli and Contarini, 2005). They account for about 0.5- 1%, in which 60-70% are present in the Milk fat globular membrane (MFGM) depending on the season and lactation stage (Contarini and Povolo, 2013a). The PLs choline (PC) and sphingomyelin (SM) are located outside the membrane. At the same time, the Phosphatidylethanolamine (PE), Phosphatidylserine (PS) and Phosphatidylinositol (PI) are present in the inner surface of the membrane (Contarini and Povolo, 2013b). The manufacturing steps greatly influence the PLs components during the process of milk into the byproduct. For a decade, works have been performed to develop methods for concentrating and isolating the milk fat globule membrane (MFGM), especially the PLs. This component has gained a lot of interest in recent times because of health benefits and nutritional aspects. They affect cell functions such as growth, memory processing, stress responses, effectiveness against gastrointestinal functions (Hellhammer et al., 2010, Hellhammer et al., 2014) and improvement in immunological functions (Kuchta-Noctor et al., 2016).

Milk phospholipids (PLs) are considered unique due to the high proportion of Sphingomyelin (SM) and Phosphatidylserine (PS), which accounts for about 24 % and 12 % respectively, compared to the vegetable source such as the soy 0 and 0.5% and the egg yolk which is 1.5 and 0% respectively (Contarini and Povolo, 2013a, Price et al., 2018, Huang et al., 2020b). The absence of SM in soy lecithin makes the milk PLs a potential ingredient formulation, especially in infant formula; this will enhance the nutritional

property of the infant formula and improve the economics of the dairy processing industry (Ortega-Anaya and Jimenez-Flores, 2019).

Beta-serum (BS) is a byproduct obtained during the phase inversion of anhydrous milk fat (AMF), which has a similar composition as that of buttermilk (Catchpole et al., 2008, Price et al., 2019). It has a potential source of PLs and opens up possible ways for extraction and further commercialization (Cheng et al., 2019b, Price et al., 2020). The major challenge in extracting MPLs from the direct byproduct stream involves a multi-step procedure since it is a wet dilute material. It has to be concentrated, mixing of polar solvents, centrifugation, distillation and fractionation resulting in lower extraction yield and easily susceptible to oxidation. Various approaches are currently in use for the production of MPLs concentrates, which involves filtration, evaporation, drying, supercritical carbon-di-oxide, five-stage sequential process ((Costa et al., 2010, Barry et al., 2017a, Price et al., 2018, Huang et al., 2020b, Ubeyitogullari and Rizvi, 2020) all these approaches led to an enriched form of MPLs from 18 to 70%. The commercially available MPLs are from Fonterra is Phospholac 600, which contains more than 70 % of PLs, the process is not disclosed, and they are mostly used in infant formulas.

An innovative alternative approach to extract the phospholipids is the use of tunable or switchable hydrophilicity solvents; the mechanism is based on the reversible reaction of amine, which can change its hydrophilicity by switching “on and off ” from water-immiscible to water-miscible form by simply bubbling with CO₂(Boyd et al., 2012, Alshamrani et al., 2016). A recent investigation by (Cheng et al., 2019c) using N, N, dimethyl cyclohexylamine to extract the phospholipids from different dairy byproducts

reported that 7% of MPLs were extracted from the dilute material of BS and improvements in extraction yield reported that 69 % of MPLs were obtained by using ultrasound treatment prior to the switchable solvent extraction (Rathnakumar et al., 2020).

So far, there is no data available on the cumulative effects of temperature, extraction time, the solvent ratio on the extraction of milk phospholipids from Beta serum using switchable solvents. Hence this study emphasizes the utilization of response surface methodology, especially central composite design, to maximize or minimize the process variables. In this process, time(3,6 and 18hr) was considered as the response on the yield of total PLs. This due to the nature of the process, the reversibility reaction of amine, which is based on the Le-Chatliers principle. The composition of individual phospholipids quantified using HPLC-CAD for all 27 treatments. The conditions which gave a higher yield of total phospholipids was analysed for Scanning electron microscopy (SEM) and Confocal laser scanning microscopy (CLSM) to understand the effect and influence these extraction conditions have on the protein and lipidic material.

5.3. Materials and methods

5.3.1. Materials

The Beta Serum was obtained from a local cheese plant (Valley Queen, Milbank, SD, USA). N, N-dimethyl cyclohexylamine (CyNMe₂, 99%, Sigma Aldrich, St. Louis, MO, USA), hexane (99%, Sigma Aldrich), methanol (99%, Sigma Aldrich), chloroform (99%, Sigma Aldrich), HPLC-grade water (Sigma Aldrich), red Nile (Fisher Scientific), fast green (FCF, Fisher Scientific), Rd-dope (Avanti Polar Lipids Inc., Alabaster, AL, USA).

5.3.2. Compositional Analysis

B-serum was analyzed for total solids, total protein, lactose, fat and phospholipids following the methodology reported (Cheng et al., 2019b). The B-serum's fatty acid profile was determined using methyl esters, and the extracts were analyzed using Gas chromatography, as mentioned in (Mannion et al., 2016).

5.3.3. Extraction of Phospholipids

The CyNMe₂ extraction of MPLs from BS was conducted according to the methodology reported elsewhere (Cheng et al., 2019a). Different extraction conditions were employed such as temperature (25,40, 60° C), time (3,10,18 hr) and solvent ratio (3/1,10/1, 18/1 mL). Each 1 g of BS was mixed with different solvent volumes of CyNMe₂ and kept for 18 h at room temperature under constant agitation using a magnetic stirring (1500 rpm). Then, mL of water as that of the solvent was added into the mixture followed by bubbling CO₂ (Organomation Associates Inc, Berlin, MA, USA) at room temperature for 4 h. Water was added to maintain the stoichiometry of the reaction, amine to salt. At the end of the bubbling, a lipid layer was formed at the top of the vial, and the lipid layer was dissolved in 3 mL hexane and transferred to a test tube for evaporation at 30°C under nitrogen flow. The total lipids were calculated using Equation (5.1), where the weight of dried lipids was divided by the sample weight.

$$\text{Total lipids (\%)} = \frac{\text{weight of dried lipids}}{\text{weight of sample}} \cdot 100 \quad (5.1)$$

The dried lipids recovered after solvent extraction were fractionated using a solid-phase extraction (SPE) column (1 cm x 10 cm) made of activated silica gel. The method of lipid fractionation via SPE can be found elsewhere (Donato et al., 2011). Dried lipids of 100 mg were dissolved in 1 mL of chloroform: methanol solution (95:5, v/v), and flowed through the SPE column, previously conditioned with 10 mL chloroform: methanol solution. Neutral lipids were eluted with 20 mL of the chloroform: methanol solution, while the MPLs were recovered with 10 mL of methanol followed 10 mL of chloroform: methanol: water (5:3:2 v/v/v). Solvents were evaporated at 40°C under vacuum. The recovered fraction was named total PLs calculated using Equation (5.2).

$$\text{Total phospholipids (\%)} = \frac{\text{weight of dried fraction}}{\text{weight of lipids}} \cdot 100 \quad (5.2)$$

5.3.4. Determination of pH

The Orion pH-meter (Versa Star Pro, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the pH at every stage of the extraction process to determine the process of switchable behaviour and its conversion to its salt form (Vanderveen et al., 2014).

5.3.5. Microstructure

The microstructure on the four conditions for the extracted B-serum was determined using CLSM at two different stages i.) SHS +BS(after stirring) ii.) after CO₂ at both the top and bottom phase. Images taken through CLSM were obtained using an Olympus FV1000 inverted confocal laser scanning electron microscope (Olympus America Inc.,

Center Valley, PA). Before the analysis, proteins, fat, and phospholipids were stained with Fast green, Nile red, and Rd-dope, respectively, according to the methodology reported by Gallier et al. (2010b). The dyes were dissolved to final concentration of 0.01% using acetone as a solvent for Fast green and Nile red, and chloroform for Rd-dope. Samples of 20 μ l were transferred to a test tube and mixed with the fluorescent dyes to a ratio 1:100 (v/v) for 20 min in the dark. A droplet of the stained sample was then placed in a microscope slide and dried at atmospheric conditions. The excitation of Fast green, Nile red, and Rd-dope was achieved at 625, 488, and 559 nm, respectively, using emission from a diode laser. All images were acquired at room temperature. SEM images were also obtained for the four extracted conditions using an electron microscope (model S-3400N Hitachi, Ltd. Tokyo, Japan) at the Electrical Engineering and Computer Science Department at South Dakota State University.

5.3.6. Determination of individual phospholipids

The quantification of the individual MPLs was determined using a UHPLC system (Dionex Ultimate 3000, Thermo Scientific) coupled to a charged aerosol detector (CAD, Dionex Corona Veo RS, Thermo Scientific). The methodology reported by (Braun et al., 2010) was followed with modifications. Firstly, the recovered fraction of MPLs after SPE was dissolved in 1 mL of chloroform: methanol solution (9:1, v/v). Then, the MPLs were separated using a binary phase and two identical silica columns (4.6 mm x 250 mm, 5 μ m particle size, Synchronis, Thermo Scientific). Mobile phase A consisted of ammonium acetate at a concentration of 3 g L⁻¹, while acetonitrile-methanol (100+3, v/v) was used as mobile phase B. A gradient run (t) of 52 min was used to separate the major classes of

MPLs. An overall flow rate of 1 mL min⁻¹, and the column oven was kept at 55°C. Fifty µL of resuspended samples were injected. The CAD was set to a power of 1.0, and the data acquisition rate was set at 2 Hz with a filter constant of 3.6. The evaporator temperature was held at 35°C. A corona nitrogen gas generator and air compressor (Peak Scientific Instruments, Billerica, MA, USA) supplied N₂ gas to the detector. A standard curve was generated with the major classes of phospholipids at different concentrations, including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). The peaks corresponding to each standard were integrated using Chromeleon software Version 7 (Thermo Scientific). Each sample was analyzed in duplicates under the same conditions. The five major phospholipids were identified by their retention time and were quantified by comparing their peak area with a standard curve.

5.3.7. Statistical analysis

All extraction conditions were carried out in triplicates, and the mean values for the recovered MPLs were compared using Tukey's test ($p < 0.05$). The statistical analysis was carried out using Design-Expert software (v7.0).

5.4. Results and discussions

5.4.1. Characterization of beta-serum

The chemical characterization of the B-serum, such as total protein, fat, lactose, pH, total solids, has been reported in our previous reports (Cheng et al., 2019b, Rathnakumar et al., 2020). **Table 5.1** shows the BS's fatty acid profile, where the results depicted the

higher presence of C14:0 (myristic), palmitic, oleic and linoleic fatty acids. They are found to be relatively higher in proportion than that of the soybean lecithin (Staňková et al., 2013; Mathiassen et al., 2015). A similar fatty acid profile has been observed for the buttermilk (Gallier et al., 2010b).

5.4.2. Effect of temperature on the extraction of Phospholipids

At 25 °C, **Figure 5.1a.** illustrated that for three different solvent ratios, the solvent ratio (3/1 mL) for the time 18h gave 2.41%±0.44% of PLs (Cheng et al., 2019b) the time 3 h and 10 h gave 0.99 %± 0.28% and 1.47%±0.19 respectively.

Table 5.1. Fatty acid profile of Beta serum

Crude Fat (W/W %)	3.02
Fatty Acid Profile (Expressed as Percent of Total Fat)	
C14:0	11
Myristoleic (9c-14:1)	0.64
C15:0	1.43
C15:1n5	0
Palmitic (16:0)	34.49
Palmitoleic (9c-16:1)	1.81
Margaric (17:0)	0.79
10c-17:1	0.22
Stearic (18:0)	11.23
Elaidic (9t-18:1)	1.76
Oleic (9c-18:1)	17.81
Vaccenic (11c-18:1)	0.64
Linoelaidic (18:2t)	0.26
Linoleic (18:2n6)	3.74
Linolenic (18:3n3)	0.29
g-Linolenic [C18:3n6]	0.03
Stearidonic (18:4n3)	0
Arachidic (20:0)	0.13
Gonodic (20:1n9)	0
C20:2	0.1
Homo-g-linolenic [C20:3n6]	0.33

Homo-a-linolenic(20:3n3)	0
Arachidonic [20:4n6]	0.3
3n-Arachidonic (20:4n3)	0
EPA (20:5n3)	0
C21:0	0.03
Behenoic (22:0)	0.18
Erucic [22:1n9]	0
C21:5n3	0
C22:2n6	0
Adrenic [C22:4n6]	0
Clupanodonic (22:5n3)	0
DHA (22:6n3)	0
C23:0	0.16
Lignoceric (24:0)	0.08
Nervonic (24:1n9)	0

At the solvent ratio (10/1 mL), the highest recovered PLs were $16.23\% \pm 1.26\%$ for the 10 h, while for the solvent ratio (18/1 mL), the recovered PLs for 18 h was $9.89\% \pm 0.56\%$. At 40 °C, **Figure 5.1b** shows the recovered PLs for the solvent ratio (3/1 mL) showed that initially, the PLs content at 3 h was $5.60\% \pm 1.26\%$ further increased to $27.00\% \pm 0.04\%$ at 10 h and decreased again to $5.97\% \pm 3.45\%$. Similarly, for the solvent ratio (18/1 mL) for the time 3 h, the recovered PLs were $11.56\% \pm 0.17\%$, later at 10h it increased to $17.15\% \pm 0.17\%$ and decreased to $4.66\% \pm 0.10\%$. The trend remained the same for solvent ratio (10/1 mL); at 3 h, the recovered PLs were $9.35\% \pm 1.10\%$, while at 10 h, it increased to $20.46\% \pm 0.42\%$ and decreased to $14.25\% \pm 0.11\%$.

At 60 °C, **Figure 5.1c** illustrated that overall higher recovery of PLs was observed at the solvent ratio (3/1 mL) was $29.29\% \pm 0.06\%$ within a 3 h period, then decreased to $8.25\% \pm 1.33\%$ and further decreased to $5.77\% \pm 0.54\%$. Contrary in the case of the solvent ratio (10/1 mL) in the 3 h, the recovered PLs was $4.80\% \pm 1.05\%$, which further increased to $24.09\% \pm 0.00\%$ at 10 h and decreased to $9.00\% \pm 1.26\%$ at 18 h. Likewise, in the case of

solvent ratio (18/mL) at 3 h, the PLs recovered were $3.95\% \pm 0.08\%$, while at 10 h, it increased to $11.58\% \pm 0.54\%$ and again decreased to $9.16\% \pm 0.38\%$.

Table 5.2. Different extraction conditions on the yield of total lipids and total phospholipids recovered from beta-serum

Conditions	Time (h)	Temperature (°C)	Solvent ratio (mL)	Lipids %	PLs%
1	3	60	3	0.634 ± 0.064	29.30 ± 0.064
2	10	40	3	0.084 ± 0.044	27.007 ± 0.044
3	10	60	10	0.346 ± 0.008	24.096 ± 0.008
4	10	40	18	0.912 ± 0.179	17.151 ± 0.179
Mean \pm standard deviation within each column with different letters are significantly different ($p < 0.05$) according to Tukey test.					

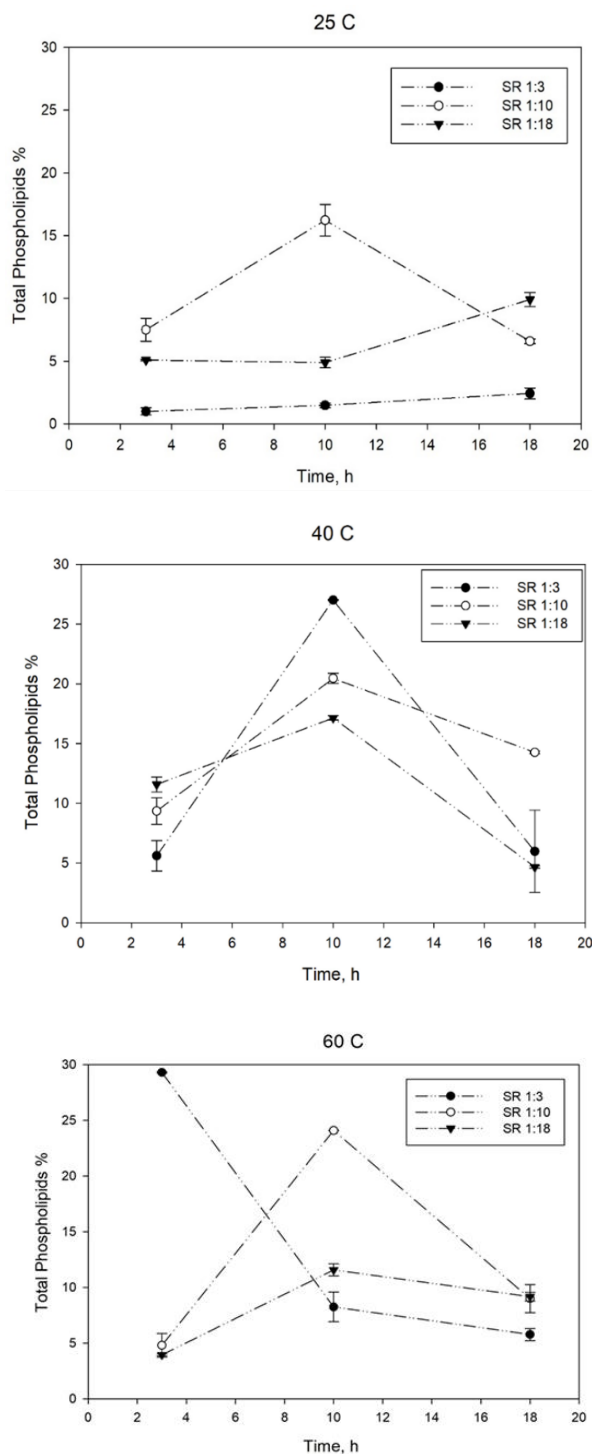


Figure 5.1. Comparison of the different extraction treatments on the yield of total lipids from beta serum. , (a) CyNMe2 extraction at temperature 25 °C, time (3,10, 18 h) solvent to sample ration of 3/1,6/1 and 18/1 mL (b) CyNMe2 extraction at extraction at temperature 40 °C, time (3,10, 18 h) solvent to sample ration of 3/1,6/1 and 18/1 mL (c) CyNMe2 extraction at extraction at temperature 60 °C, time (3,10, 18 h) solvent to sample ration of 3/1,6/1 and 18/1 mL.

Our previous study on BS by Cheng et al. (2019) reported yields of MPLs extracted from BS using Folch and CyNMe₂ (5% and 2-7%, respectively). The effect of temperature had an increment in the amount of PLs up to 29% with a minimized solvent ratio of 3/1 mL and time 3 h. Various reports in the literature reported that a concentrate containing up to 19% of MPLs was obtained using a two-step process that consisted of nanofiltration and supercritical carbon dioxide (Astaire et al., 2003).

5.4.3. Microstructure

Figure 5.2 shows the microstructure images from the scanning electron microscope for four extraction conditions which gave a higher yield of PLs, as presented in **Table 5.2**. The different extraction temperature for the B-serum depicted the presence of numerous porous structures of different shapes and sizes. The effect of temperature has seemed to disrupt the protein-membrane and releasing the phospholipids.

Figure 5.3 illustrates the presence of proteins, lipids and PLs at the different stages. It was also observed the reduction in the aggregated protein-membrane, which eventually caused the release of PLs into the aqueous medium in the presence of temperature and CyNMe₂ solvent. This also favoured the mass transfer between MPLs and the surrounding solvent, thereby causing an improvement in the yield of PLs (Rathnakumar and Martinez-Monteagudo, 2020).

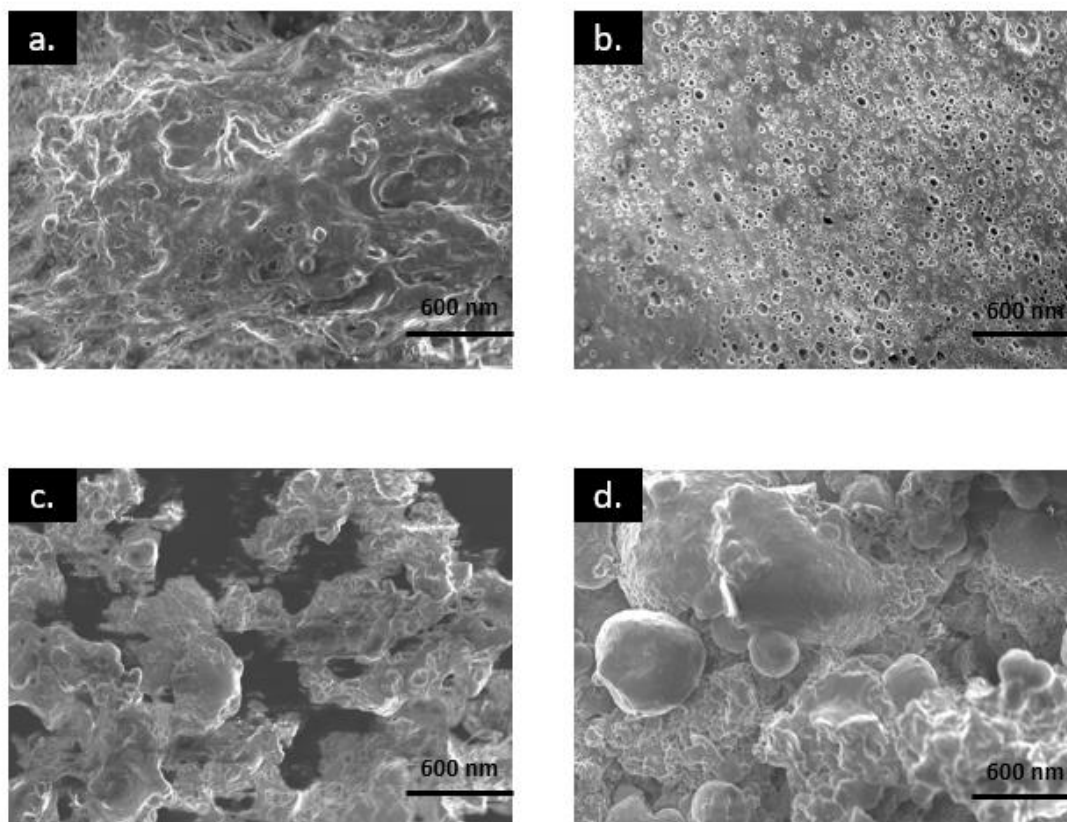
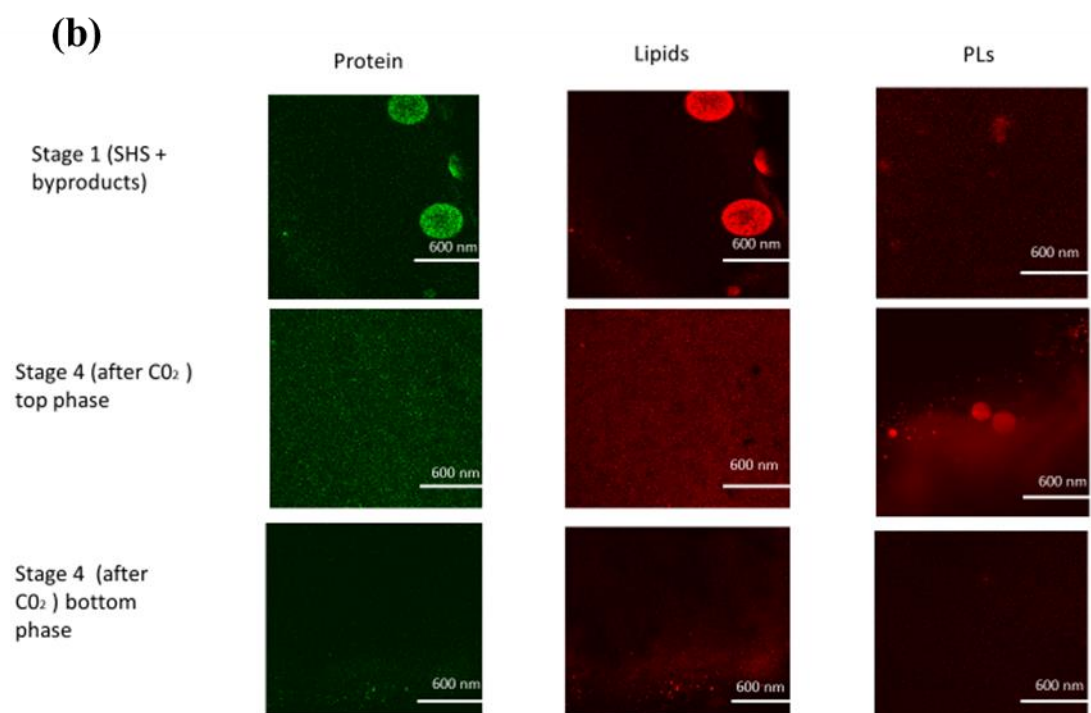
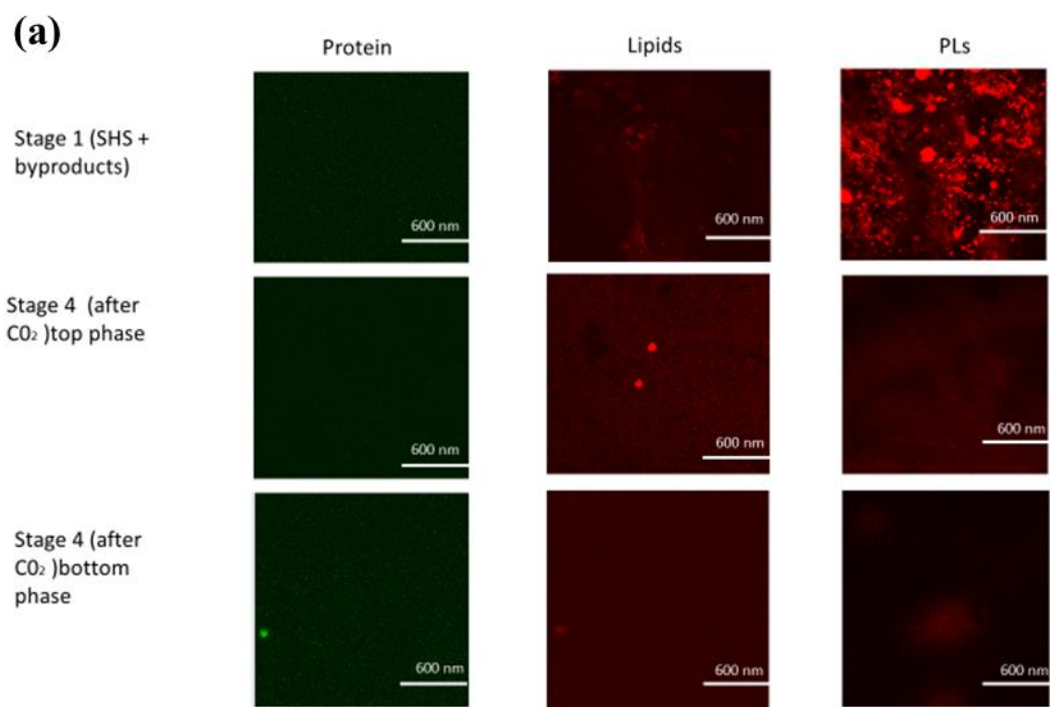


Figure 5.2. Scanning microscopy images of beta-serum: (a) CyNMe₂ extraction condition -1: time (3 h), temperature(60 °C) and solvent ration(3/1 mL) (b) CyNMe₂ extraction condition -2 : time (10 h), temperature(40 °C) and solvent ration(3/1 mL) (c) CyNMe₂ extraction condition - 3 : time (10 h), temperature(60 °C) and solvent ration(10/1 mL) (d) CyNMe₂ extraction condition - 4 : time (10 h), temperature(40 °C) and solvent ration(18/1 mL).



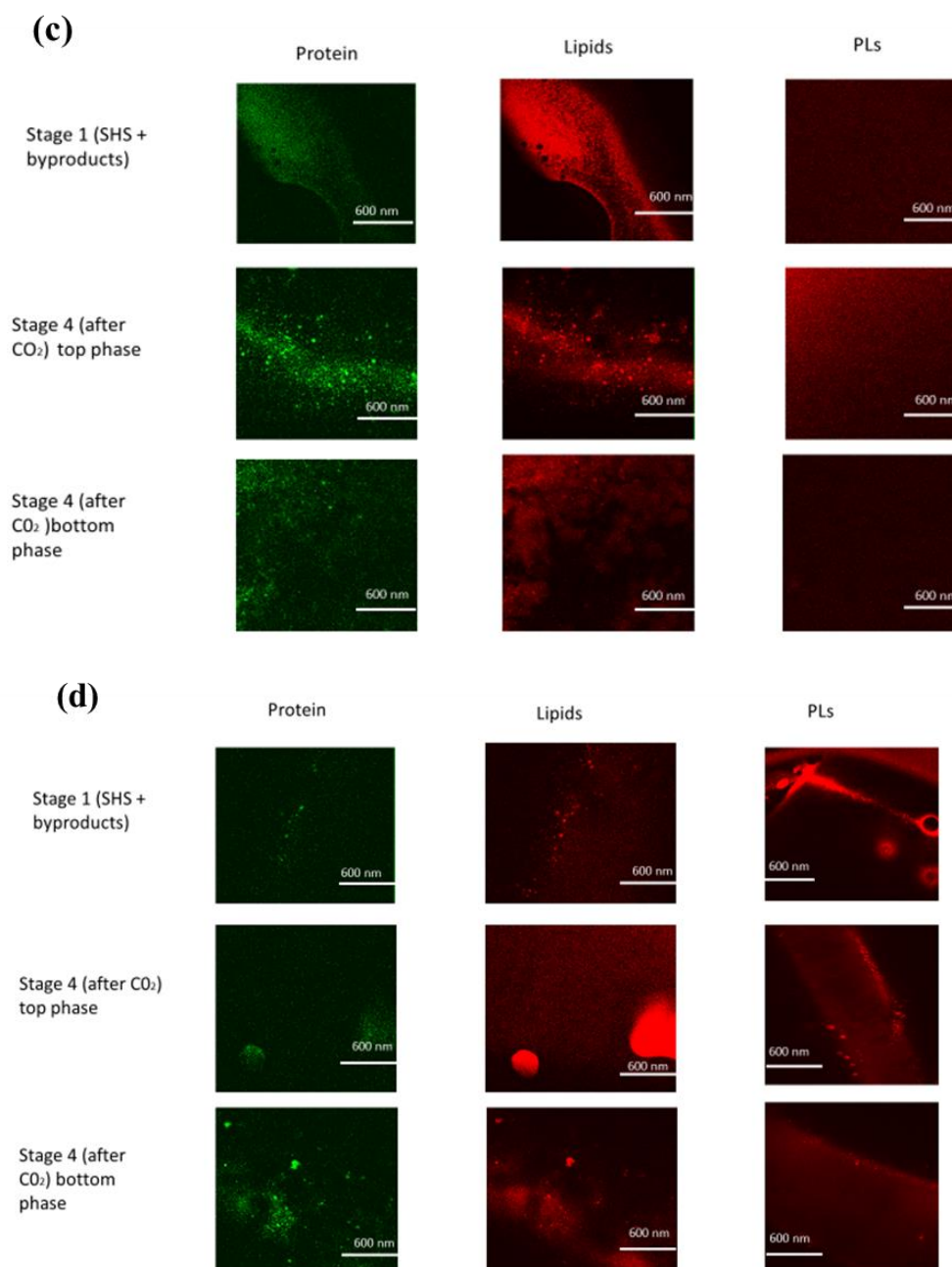


Figure 5.3. Confocal laser scanning microscopy images of beta-serum: at two different points i.) Stage 1(SHS +by-product) ii.) Stage 4 :(After CO₂ - top and bottom phase) for four different higher extraction yield of phospholipids after CyNMe₂ extraction condition (a) time (3 h), temperature(60 °C) and solvent ration(3/1 mL) (b) time (10 h), temperature(40 °C) and solvent ration(3/1 mL) (c) time (10 h), temperature(60 °C) and solvent ration(10/1 mL) (d)time (10 h), temperature(40 °C) and solvent ration(18/1 mL).

5.4.4. Distribution of individual Phospholipids

Five major classes of MPLs were quantified in the recovered fraction, including PI, PS, PE, PC, and SM. The concentration and distribution of the PLs varied according to the temperature, time, and solvent ratio (**Table 5.3**). Overall a higher concentration of PI and SM was observed in all four conditions. Contrarily, the condition which recovered only 17% of PLs had a higher concentration of PI, PS, PE, PC and SM. The PI and SM could have been released into an aqueous medium due to the influence of temperature. This component PI has more technological interests and can be used in liposomes preparation (Ali et al., 2019), while the SM is more significant due to its potential health benefits (Ortega-Anaya and Jimenez-Flores, 2019). The PS and PC were not detected at some conditions because this could have easily degraded due to the temperature, or it might have isomerized to other smaller components of PLs. **Figure 5.4** illustrates the relative distribution of MPLs from the extraction condition presented in **Table 5.3**. A similar profile of MPLs has been reported from butter serum and buttermilk (Costa et al., 2010, Gassi et al., 2016), raw milk (Rombaut and Dewettinck, 2006) and beta serum (Price et al., 2019, Rathnakumar et al., 2020).

Table 5.3. Concentration ($\mu\text{g mL}^{-1}$) of recovered milk phospholipids from beta-serum from different extraction conditions

T (h)	T (°C)	Solvent ratio (mL)	PI	PS	PE	PC	SM
3	60	3	843.2±2.48	N.D	511.9±2.11	N.D	70.4±2.57
10	40	3	428.7±1.99	93.3±1.41	46.8±4.42	194.5±2.07	671±2.33
10	60	10	677.6±3.21	41.3±4.31	40.3±3.84	477.2±1.05	548±5.82
10	40	18	1514.6±2.68	201.8±2.34	657±2.01	701.8±2.03	699.4±6.01

PI – phosphatidylinositol; PS – phosphatidylserine; PE – phosphatidylethanolamine; PC - phosphatidylcholine; SM –Sphingomyelin; N.D. – not detected.

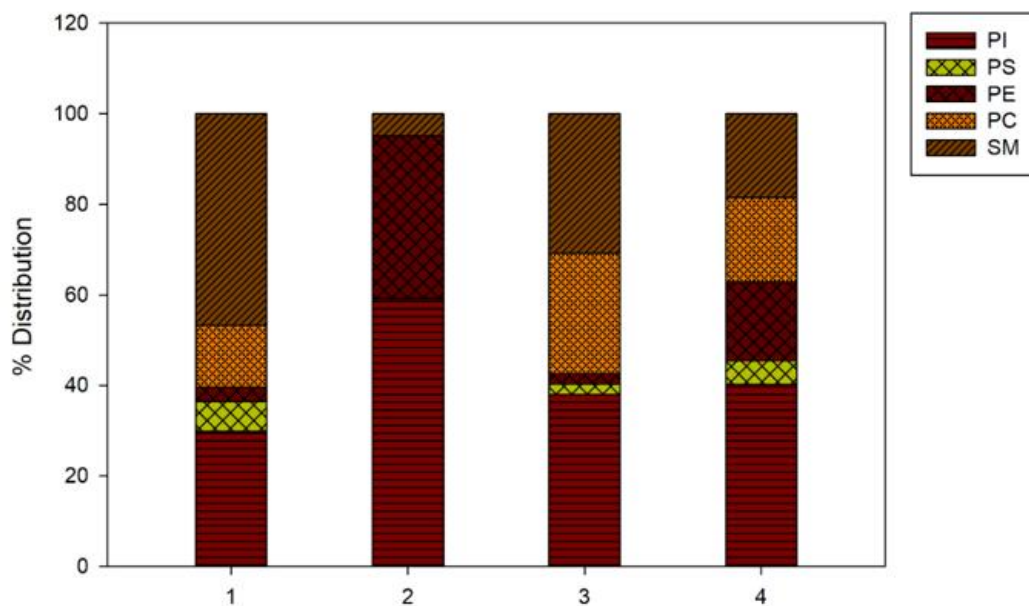


Figure 5.4. Distribution of milk phospholipids after different CyNMe₂ extraction condition(1) : time (3 h), temperature(60 °C) and solvent ration(3/1 mL) (2) time (10 h), temperature(40 °C) and solvent ration(3/1 mL) (3) time (10 h), temperature(60 °C) and solvent ration(10/1 mL) (4)time (10 h), temperature(40 °C) and solvent ration(18/1 mL)

5.4.5. Conversion of Switchable behavior

The switchable solvent mechanism is based on its conversion to a hydrophilic bicarbonate salt in the presence of CO₂. A solvent switchability can be determined in the following way that by its appearance, when a mixture of organic compound and water form two phases before the addition of CO₂ and one phase after CO₂, then it is called a switchable solvent(Vanderveen et al., 2014). During the conversion, the separation of lipids from the solids and water phase is a critical step. Therefore, in dairy byproducts, some components such as minerals, caseins, and smaller peptides might affect the overall equilibrium of the reaction and could result in incomplete conversion to its salt form. This can affect the overestimation of the lipid content and also the presence of the amine residue. To determine

whether the salt form's conversion is complete, pH was determined at every stage until it reaches a pH around 8 shows that the resulting solution indicates the bicarbonate salt (Durelle et al., 2014). **Table 5.4** depicts the pH at different stage points for the four extraction conditions, and a pH of around 8 was obtained for all. It was observed that with a lesser solvent ratio (3/1 mL), the conversion was quicker, while for a higher solvent ratio such as 18/1 mL, the conversion took around 6 hrs, which seems to be a time-consuming process. (Boyd et al., 2012) reported incomplete conversion of the salt (18–24% of residual CyNMe₂) during the lipid extraction from microalgae.

Table 5.4. pH at different stage points during the N,N-dimethylcyclohexylamine (CyNMe₂) extraction

pH stage points	Condition 1	Condition 2	Condition 3	Condition 4
SHS+B-serum(Before stirring)	10.97±0.000	11±0.000	10.76±0.005	10.58±0.005
SHS+ B-serum(after stirring 10 hrs)	10.22±0.000	10.87±0.000	10.33±0.000	10.40±0.005
After addition of water (before CO ₂)	11.11±0.005	11.46±0.005	11.32±0.000	11.49±0.000
CO ₂ bubbling after 3hrs	8.15±0.005	8.15±0.000	8.20±0.011	9.26±0.000
After 4 h CO ₂ bubbling	8.05±0.000	8.04±0.005	8.1±0.000	8.95±0.000
After 4 and ½ h CO ₂ bubbling				8.57±0.005
After 5 h CO ₂ bubbling				8.42±0.000
After 5 and ½ h CO ₂ bubbling				8.21±0.005
After 6 h				8.10±0.005

5.5. Conclusions

A fraction containing up to 29.29% ± 0.06% of MPLs was obtained when switchable solvents were subjected to a temperature of 60 °C, at minimized time and solvent ratio (3 h and 3/1 mL respectively). The microstructural analysis SEM and CLSM show the impact of temperature on the membrane proteins disintegration and PLs and the release of PLs.

The relative distribution of the major classes of MPLs shows the predominance of PI and SM. This gives more insights for further separation and utilizes it in various ingredient formulations. This study provides a road map for the temperature assisted CyNMe₂ extraction of MPLs from beta-serum. Further research investigation can be carried out on determining the isomerization or degradation of the individual PLs.

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CHAPTER 6
UNDERSTANDING THE SWITCHABLE SOLVENT EXTRACTION OF
PHOSPHOLIPIDS FROM DAIRY BYPRODUCTS⁵

6.1. Abstract

Dairy by-products represent a rich source of phospholipids with the potential for isolation and further commercialization. In this study, we extracted phospholipids from two dairy byproducts (buttermilk and beta-serum) using a tertiary amine (N,N-dimethylcyclohexylamine, CyNMe₂) as a switchable hydrophilicity solvent. For comparison, the phospholipids were extracted via Folch (chloroform: methanol). CyNMe₂ extraction resulted in recovery values of 98.66 ± 0.89 and $7.67 \pm 0.51\%$ for buttermilk and beta-serum, respectively. Insights into the extraction mechanism were obtained by the analysis of protein profile, particle size, zeta potential, and microstructure. CyNMe₂ generated microfractures and hollow openings in the solid matrix through ion pair formation that releases the phospholipids from the solid matrix. In comparison to Folch, CyNMe₂ resulted in higher concentration of phospholipids (up to 9-fold increment), and it provided a different relative distribution, where phosphatidylcholine and phosphatidylinositol were the predominant phospholipids. The outcomes of this study help to gain insights into the extraction mechanism by which CyNMe₂ acts and develop extraction strategies for dairy byproducts.

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6.2. Introduction

Phospholipids are a group of lipids containing phosphorus in their structure and found as bilayers in all the membranes of living organisms (Ali et al., 2019). They are classified based on their chemical structure, glycerophospholipids and sphingolipids. The phosphorus group in the glycerophospholipids is linked to a polar group and two fatty acids esterified to a glycerol backbone (Avalli and Contarini, 2005). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) are classified as glycerophospholipids. Sphingolipids are made of sphingoid bases linked to fatty acid. Sphingomyelin (SM) is the dominant species within the sphingolipids (Contarini and Povolo, 2013).

Phospholipids are relevant to human nutrition since they provide health benefits beyond their basic nutrition (Küllenberg et al., 2012). The ability of milk PLs (MPLs) to scavenge free radicals has been demonstrated in vitro by Huang et al. (2020a). Several other health claims have been investigated over the past years, including anti-inflammatory effect (Hartmann et al., 2009), protecting effect against gastrointestinal infections (Hellhammer et al., 2010), and improved immunological functions (Kuchta-Noctor et al., 2016). Most of the health claims of PLs have been related to PS and SM, whose concentration is relatively high in milk fat (12 and 24% of the total PLs, respectively) (Huang et al., 2020b).

The frequent association between consumption of phospholipids and the reduced risk of several malignancies (Küllenberg et al., 2012) has driven the dairy industry to develop technological approaches to recover and further utilize MPLs (Huang et al., 2020a).

Buttermilk (BM) and whey (BS) are two common dairy streams (Huang et al., 2020b). Buttermilk is the byproduct of the manufacture of butter, and it consists of the remaining liquid after churning (Guggisberg et al., 2012). On the other hand, BS is the aqueous product (serum) produced after the phase inversion during the manufacture of anhydrous milk fat (Fletcher et al., 2013). The worldwide generation of BM was estimated to be 5.2 million tons in 2013 (Barry et al., 2017). Conventional uses of BM and BS (ingredient for cheese, ice-cream, and yogurt) are not enough to expand the market of dairy byproducts. Thus, the extraction of MPLs from BM and BS offers opportunities for improving the current value of such industrial byproducts.

One major challenge regarding the direct extraction of MPLs is the low efficiency due to the multiple steps within the entire process. In general, the extraction of MPLs from byproducts involves concentration, extraction, distillation, and fractionation (Huang et al., 2020a; Cheng et al., 2019). A more viable approach is the production of MPLs concentrates through membrane filtration, concentration, and drying, where concentrates ranging from 20 to 70% of MPLs are commercially available. The state-of-the-art regarding the production of MPLs concentrates has been reviewed elsewhere (Huang et al., 2020b).

In a recent investigation, Cheng et al. (2019) extracted about 99 and 8% of the MPLs from BM and BS, respectively, using a tertiary amine (N,N-dimethylcyclohexylamine, CyNMe₂) as a switchable hydrophilicity solvent. This new type of solvents can switch from a hydrophobic to a hydrophilic form by adding or removing CO₂ (Jessop et al., 2011). The extraction yields from BM and BS using CyNMe₂ were quite different, which raises

the intriguing question on the governing mechanisms. Understanding the CyNMe₂ extraction mechanism is essential for process development and further optimization. In this work, the extraction mechanisms of CyNMe₂ were elucidated in the context of the yield of MPLs, protein profile, particle size, confocal laser scanning microscopy, and scanning electron microscopy.

6.3. Materials and Methods

6.3.1. Materials

N,N-dimethylcyclohexylamine (CyNMe₂, 99%, Sigma Aldrich, St. Louis, MO, USA), hexane (99%, Sigma Aldrich), methanol (99%, Sigma Aldrich), chloroform (99%, Sigma Aldrich), acetone (99%, Fisher Scientific, Hampton, NH), HPLC-grade water (Sigma Aldrich), phospholipid mixture (Soybean, P3817-1VL, Sigma Aldrich), activated silica gel (Silica gel 60 G, EMD Millipore), red Nile (Fisher Scientific), fast green (FCF, Fisher Scientific), Rd-dope (Avanti Polar Lipids Inc., Alabaster, AL, USA), Laemmli buffer (Bio-Rad, Hercules, CA, USA), Trisacrylamide gels (4-15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad), Tris/Glycine/SDS Buffer (Bio-Rad), ProteinTM Kaleidoscope standards (Bio-Rad), and 2-mercaptoethanol (Fisher Scientific) were purchased from a commercial supplier. Liquid buttermilk was obtained after churned raw cream at 4 °C using a laboratory-scale churner (TM 31 Thermomix, Vorwerk LLC, Thousand Oaks, CA, USA). Beta-serum was obtained from a local cheese plant (Valley Queen, Milbank, SD, USA).

6.3.2. Proximate analysis

Buttermilk and BS were analyzed for pH, total solids, protein content, fat, and lactose. The pH was measured in 200mL of the sample using an Orion pH-meter (Versa Star Pro, Thermo Fisher Scientific, Waltham, MA, USA). Total solids were determined gravimetrically, according to the Association of Official Analytical Chemists (AOCA) method 990.20 (AOAC, 2000). Total protein was quantified by the Kjeldahl method (AOCA) method 990.20, while the Mojonnier extraction was used to quantify the fat content (AOCA) method 991.20 (AOAC, 2000). Lactose content was determined by HPLC following the methodology reported elsewhere (Amamcharla and Metzger, 2011).

6.3.3. Extraction methods

The CyNMe₂ extraction of MPLs from BM and BS was conducted according to the methodology reported elsewhere (Cheng et al., 2019). Briefly, 1 g of either BM or BS was mixed with 12mL of CyNMe₂, and kept for 18h at room temperature under constant agitation using a magnetic stirring (1500 rpm). Then, 12mL of water were added into the mixture followed by bubbling CO₂ (Organomation Associates Inc, Berlin, MA, USA) at room temperature for 4h. Water was added to maintain the stoichiometry of the reaction, amine to salt. At the end of the bubbling, a lipid layer was formed at the top of the vial, while the bottom layer corresponded to the CyNMe₂ salt dissolved in water. The lipid layer was dissolved in 3mL hexane and transferred to a test tube for evaporation at 30 °C under nitrogen flow. The extraction of MPLs from BM and BS was also carried out using the Folch method, following the protocol reported elsewhere (Cheng et al., 2019). Twenty mL of chloroform: methanol solution (2:1, v/v) were mixed with 1 g of either BM or BS. After

vortexed for 3min, the mixture was centrifugated at room temperature (4200 x g) for 5min. The upper phase was discarded, while the lower phase was transferred to a test tube for evaporation of chloroform at 45 °C under nitrogen flow. The total lipids were calculated using Eq. (6.1), where the weight of dried lipids was divided by the sample weight.

$$Total\ lipids\ (\%) = \frac{weight\ of\ dried\ lipids}{weight\ of\ sample} \cdot 100 \quad (6.1)$$

The dried lipids recovered after solvent extraction were fractionated using a solid-phase extraction (SPE) column (1cm× 10cm) made of activated silica gel. Guidelines of lipid fractionation via SPE can be found elsewhere (Donato et al., 2011). One hundred mg of dried lipids were dissolved in 1mL of chloroform: methanol solution (95:5, v/v), and flowed through the SPE column, previously conditioned with 10mL of the chloroform:methanol solution. Neutral lipids were eluted with 20mL of the chloroform:methanol solution, while the MPLs were recovered with 10mL of methanol followed 10mL of chloroform: methanol: water (5:3:2 v/v/v). Solvents were evaporated at 40 °C under vacuum. The recovered fraction was named total PLs calculated using Eq. (6.2).

$$Total\ phospholipids\ (\%) = \frac{weight\ of\ dried\ fraction}{weight\ of\ lipids} \cdot 100 \quad (6.2)$$

6.3.3. Microstructure

The microstructure of BM and BS was determined before and after the CyNMe₂ and Folch extraction by confocal laser scanning microscopy (CLSM) and scanning electron

microscopy (SEM). Images taken from untreated BM and BS were denominated as before extraction. Images after extraction were taken prior to the CyNMe₂ switched to its corresponding salt, after 18h of extraction. In the case of the Folch extraction, the images were obtained after centrifugation of the mixture, sample and chloroform: methanol. Images taken by CLSM were obtained using an Olympus FV1000 inverted confocal laser scanning electron microscope (Olympus America Inc., Center Valley, PA). Before the analysis, proteins, fat, and phospholipids were stained with Fast green, Nile red, and Rddope, respectively, according to the methodology reported by Gallier et al. (2010a). The dyes were dissolved to final concentration of 0.01% using acetone as a solvent for Fast green and Nile red, and chloroform for Rddope. Samples of 20l were transferred to a test tube and mixed with the fluorescent dyes to a ratio 1:100 (v/v) for 20min in the dark. Then, a droplet of the stained sample was placed in a microscope slide and dried at atmospheric conditions. The excitation of Fast green, Nile red, and Rddope was achieved at 625, 488, and 559nm, respectively, using emission from a diode laser. All images were acquired at room temperature. Scanning electron images (SEM) were obtained using an electron microscope (model S-3400 N Hitachi, Ltd. Tokyo, Japan) at the Electrical Engineering and Computer Science Department at South Dakota State University.

6.3.4. Analytical determinations

6.3.4.1. Protein profile

The protein profile was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Details on the methodology can be found elsewhere (Meletharayil et al., 2015). Two mL of sample were mixed with 20mL

of a chloroform: methanol solution (2:1, v/v). Then, the mixture was placed in a freezer for 1h at -18°C , and then, centrifugated (Jouan CR412, Jouan Inc., Winchester, VA, USA) for 15min at 3600 rpm at 0°C . After centrifugation, the supernatant was discarded, while the pellets were dissolved with phosphate buffer (3mL, 0.1 N). Five L of the dissolved pellets were transferred into a test tube followed by the addition of 4.75L of 2x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), and 0.25L of 4% 2-mercaptoethanol (Fisher Scientific, Hampton, NH, USA). Then, the tested tubes were capped and heated for 5min at 90°C . Afterwards, an aliquot of 10L was loaded into Tris-acrylamide gels (4-15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad), and the gels were run for 1h at 200 V using Tris/Glycine/SDS Buffer (Bio-Rad). Then, the gels were removed from the cassettes and stained with Bio-safe Coomassie G-250 stain (Bio-Rad). A molecular weight standard (Bio-Rad, USA; Precision Plus ProteinTM Kaleido scope Standards) was used as reference. Finally, the gels were de-stained with a de-staining solution containing 100mL of acetic acid, 300mL of methanol, and 600mL of distilled water. The gels were scanned using Bio-5000 Microtek (Microtek, Taiwan).

6.3.4.2. Particle size distribution and zeta potential

The particle size distribution and zeta potential were determined by using a ZetaSizer Nano ZS (Malvern Instruments Ltd, Cambridge, UK), according to the guidelines provided elsewhere (Ma et al., 2018). Before the analysis, the samples were brought to room temperature (25°C) and equilibrated for 20min. An aliquot of 10L was transferred to disposable cuvette (DTS 0012, Sigma-Aldrich, St Louis, MO, USA), and diluted 100x with deionized water to prevent multiple scattering. Then, the cuvettes were placed in the

measuring chamber, where the samples were equilibrated for 120 s at 25 °C. For particle distribution, the analysis was conducted at a scattering angle of 173° and a refractive index of 1.46. The average size and distribution of particles were obtained in the percentage of volume as a function of droplet diameter in the range of 0.6–6000nm. In the case of zeta potential, deionized water was used as a diluent and placed inside a disposable polycarbonate cuvette (ATA scientific, DTS1061). Measurements were repeated a minimum of 10 times per run with a minimum of 7 runs.

6.3.5. Determination of individual phospholipids

The quantification of the individual MPLs was determined using a UHPLC system (Dionex Ultimate 3000, Thermo Scientific) coupled to a charged aerosol detector (CAD, Dionex Corona Veo RS, Thermo Scientific). The methodology reported by Braun et al. (2010) was followed with modifications. Firstly, the recovered fraction of MPLs after SPE was dissolved in 1mL of a chloroform: methanol solution (9:1, v/v). The, the MPLs were separated using a binary phase and two identical silica columns (4.6mmx 250mm, 5m particle size, Synchronis, Thermo Scientific). The mobile phase A consisted of ammonium acetate at a concentration of 3 g L⁻¹, while acetonitrile-methanol (100 + 3, v/v) was used as mobile phase B. A gradient run (t) of 52min was used to separate the major classes of MPLs using the following conditions: 5% A and 95% B at t = 0min; 5% A and 95% B at t = 2min; 25% A and 75% B at t = 35min; 25% A and 75% B at t = 40min; 5% A and 95% B at t = 41min; 5% A and 95% B at t = 52min. An overall flow rate of 1mL min⁻¹, and the column oven was kept at 55 °C. Fifty L of resuspended samples were injected. The CAD was set to a power of 1.0, and the data acquisition rate was set at 2 Hz with a filter

constant of 3.6. The evaporator temperature was held at 35°C. A corona nitrogen gas generator and air compressor (Peak Scientific Instruments, Billerica, MA, USA) supplied N₂ gas to the detector. A standard curve was generated with the major classes of phospholipids at different concentrations, including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). The peaks corresponding to each standard were integrated using Chromeleon software Version 7 (Thermo Scientific). Each sample was analyzed in duplicates under the same conditions. The five major phospholipids were identified by their retention time and were quantified by comparing their peak area with standard curve.

6.4. Results

6.4.1. Extraction of total lipids and total phospholipids

Table 6.1 presents the composition of BM and BS prior to the extraction. The fat content in both byproducts was relatively high, representing about 42 and 49% d.b. for BM and BS, respectively. Lactose was the second most predominant compound, accounting for about 41 and 37% d.b. in BM and BS, respectively. On the other hand, the protein content was between 3 and 2% d.b. for both byproducts. Overall, the composition for BM reported in the literature varied between 6-18% for fat, 3- 31% for protein, and 40-50% for lactose on dry basis (Huang et al., 2020a; Astaire et al., 2003; Costa et al., 2010).

Table 6.1. Composition of buttermilk and beta-serum

Parameter	Butter Milk	Beta-serum
Protein (% d.b.)	3.43 ± 0.01	2.20 ± 0.01
Fat (% d.b.)	42.47 ± 4.26	49.57 ± 0.49

Ash (% d.b.)	9.73 ± 0.83	12.73 ± 1.77
Lactose (% d.b.)	41.33 ± 4.05	36.47 ± 1.88
Total Solids (%)	9.63 ± 0.03	8.17 ± 0.34
pH	6.38 ± 0.01	6.74 ± 0.01

Total lipids extracted from BM and BS using Folch and CyNMe2 extraction are given in **Figure 6.1a**. Mojonnier extraction reported by Cheng et al. (2019) was included in the graph for comparison. The recovered lipids from BM were significantly higher with Folch than CyNMe2, where values of about 29 and 8% d.b. were obtained, respectively. Similar trend and values were obtained for BS (37 and 15% d.b., respectively). Interestingly, Mojonnier extraction resulted in higher content of total lipids regardless of the byproduct. **Figure 6.1b** shows the total MPLs recovered from BM and BS using Folch and CyNMe2 extraction. In the case of BM, only $5.34 \pm 0.05\%$ of MPLs were recovered using Folch, while the CyNMe2 extraction resulted in $98.55 \pm 0.78\%$ of the total MPLs. The CyNMe2 extraction yielded higher values than Folch for BS (7.61 ± 0.51 and $5.31 \pm 0.65\%$, respectively). Phospholipids extracted via Mojonnier showed relatively low values (2.66 ± 0.66 and $3.92 \pm 0.33\%$ for BM and BS, respectively).

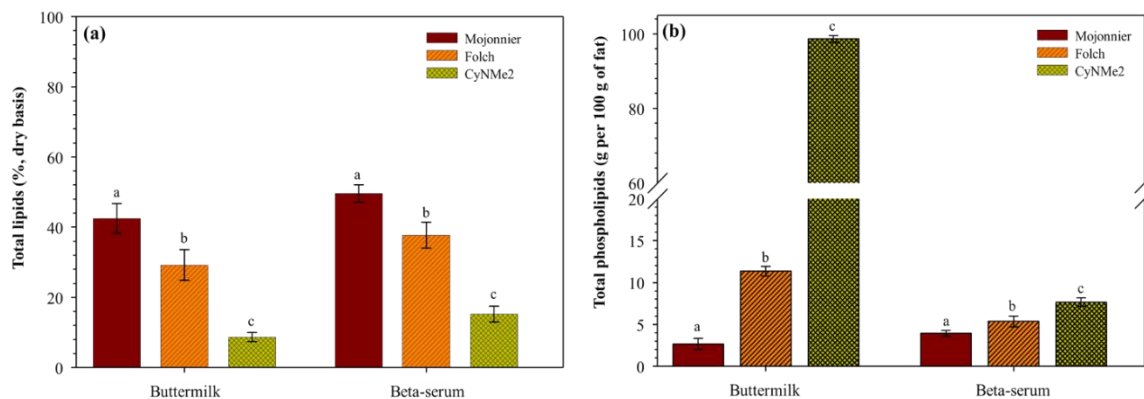


Figure 6.1. Total lipids (a) and total phospholipids (b) recovered from buttermilk and beta-serum after Folch and N,N-dimethylcyclohexylamine (CyNMe2) extraction. Mean \pm standard deviation within each column with different letters are significantly different ($p < 0.05$) according to Tukey test. The Mojonnier extraction was added for reference.

6.4.2. Protein profile

The protein distribution of BM and BS before and after Folch and CyNMe2 extraction was evaluated using SDS-PAGE (**Figure 6.2**). The initial stream of BM and BS (column 2 and 4 in **Figure 6.2**) displayed three major classes of proteins whey proteins (α -lactalbumin and β -lactoglobulin at 10 and 50 kDa, respectively), caseins (α -casein and β -casein at 20 and 25 kDa, respectively) and milk fat globule membrane (MFGM) at >50 kDa (adipophilin, butylophilin, and xanthane oxidase). Similar protein profile has been reported elsewhere (Price et al., 2020; Rombaut et al., 2007). Changes in the protein profile due to Folch extraction were marginal, judging by the similarities of the bands for BM and BS. Contrary, the bands corresponding to the MFGM were markedly different after CyNMe2 extraction.

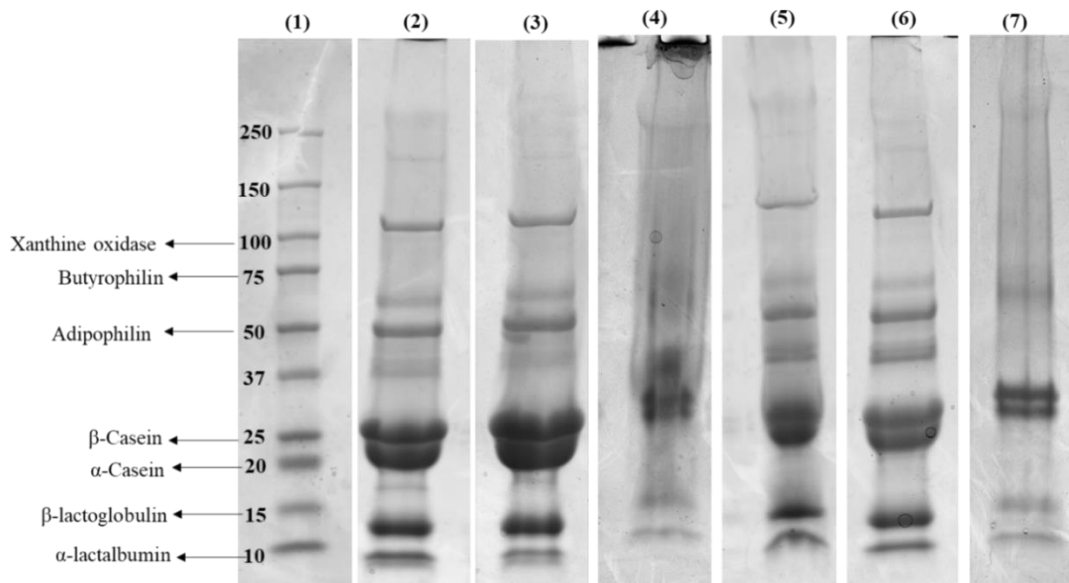


Figure 6.2. SDS-PAGE patterns of dairy byproducts. (1) Molecular weight standard, (2) buttermilk, (3) buttermilk after Folch extraction, (4) buttermilk after CyNMe2 extraction, (5) beta-serum, (6) beta-serum after Folch extraction, (7) beta-serum after CyNMe2 extraction.

6.4.3. Particle size distribution and zeta potential

Figure 6.3 shows the effect of extraction method on the particle size distribution of BM and BS. Untreated BM (**Figure 6.3a**) showed a distribution of particles characterized by the presence of two distinctive peaks. The first peak spanned from 200 to 550nm, and it corresponds to about 83% of the total particles. The second peak spanned from 40 to 150nm, accounting for the remaining of the particles. Untreated BS (**Figure 6.3b**) also exhibited two peaks (from 130 to 300nm and 20–100nm), accounting for about 80 and 20% of the total particles, respectively. Folch extraction produced a broader distribution of particles (40–1200nm) in BM. Similar behavior but less pronounced was observed in BS treated by Folch extraction, where a peak spanned from 120 to 1000nm was observed. On the other hand, CyNMe2 for BM resulted in a broader and smaller distribution of particles than that obtained in Folch extraction. Interestingly, BM and BS exhibited similar

distribution of particles, a single peak spanned from about 80–500nm.

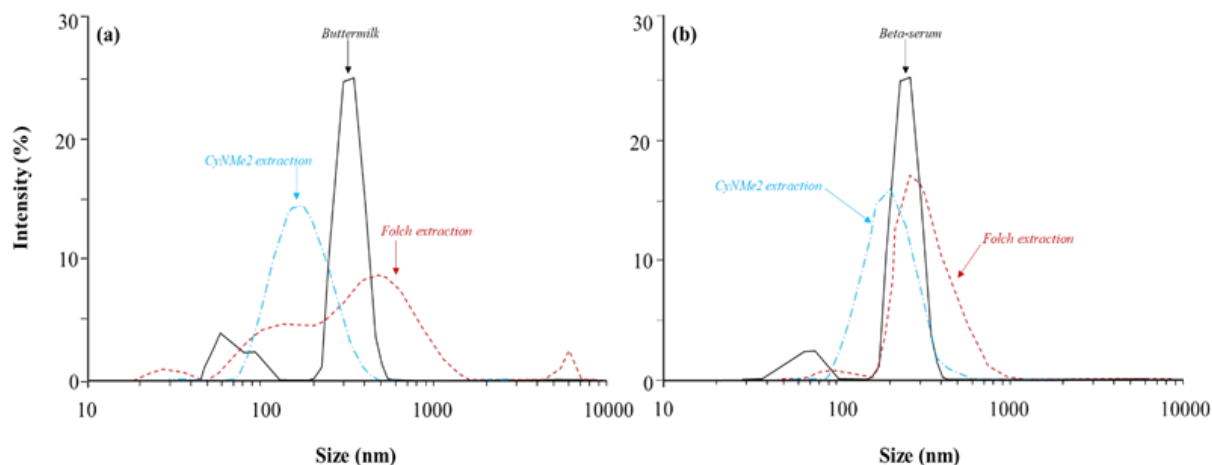


Figure 6.3. Particle size distribution before and after Folch and N,N-dimethylcyclohexylamine (CyNMe2) extraction for buttermilk (a) and beta-serum (b).

Table 6.2 shows the zeta potential for BM and BS before and after Folch and CyNMe2 extraction. Before extraction, BM and BS presented values of -29.5 ± 5.2 and -34.2 ± 6.1 mV, respectively. For Folch extraction, the values for zeta potential decreased to -61.8 ± 11.5 and -72.9 ± 10.3 mV for BM and BS, respectively. CyMNe2 yielded large negative values (-81.1 ± 8.7 and -82.2 ± 10.3 mV, respectively).

Table 6.2. Zeta potential of buttermilk and beta-serum before and after Folch and N,N-dimethylcyclohexylamine (CyNMe2) extraction.

Extraction	Byproduct	
	Buttermilk	Beta-serum
Untreated	-29.5 ± 5.2 mV	-34.2 ± 6.1 mV
Folch	-61.8 ± 11.5 mV	-72.9 ± 10.3 mV
CyNMe2	-81.1 ± 8.7 mV	-82.2 ± 10.7 mV

6.4.4. Microstructure

The microstructure of BM and BS before and after Folch and CyNMe₂ extraction was analyzed through CLSM and SEM (**Figure 6.4** and **Figure 6.5**, respectively). Images through CLSM illustrated droplets of MPLs, while SEM images showed the gross morphology of the materials. Before extraction, droplets of MPLs were clearly visible for BM and BS (**Figure 6.4a** and **Figure 6.4d**). The MPLs in native or minimally disturbed MFGM are organized as liquid-disordered phase coexisting with liquid-order phase, having a size of 5000–10000nm (Gallier et al., 2010b). Folch extraction reduced the MPLs droplets (**Figure 6.4b** and **Figure 6.4e**), while CyNMe₂ showed a greater reduction in the droplets of MPLs (**Figure 6.4a** and **Figure 6.4f**).

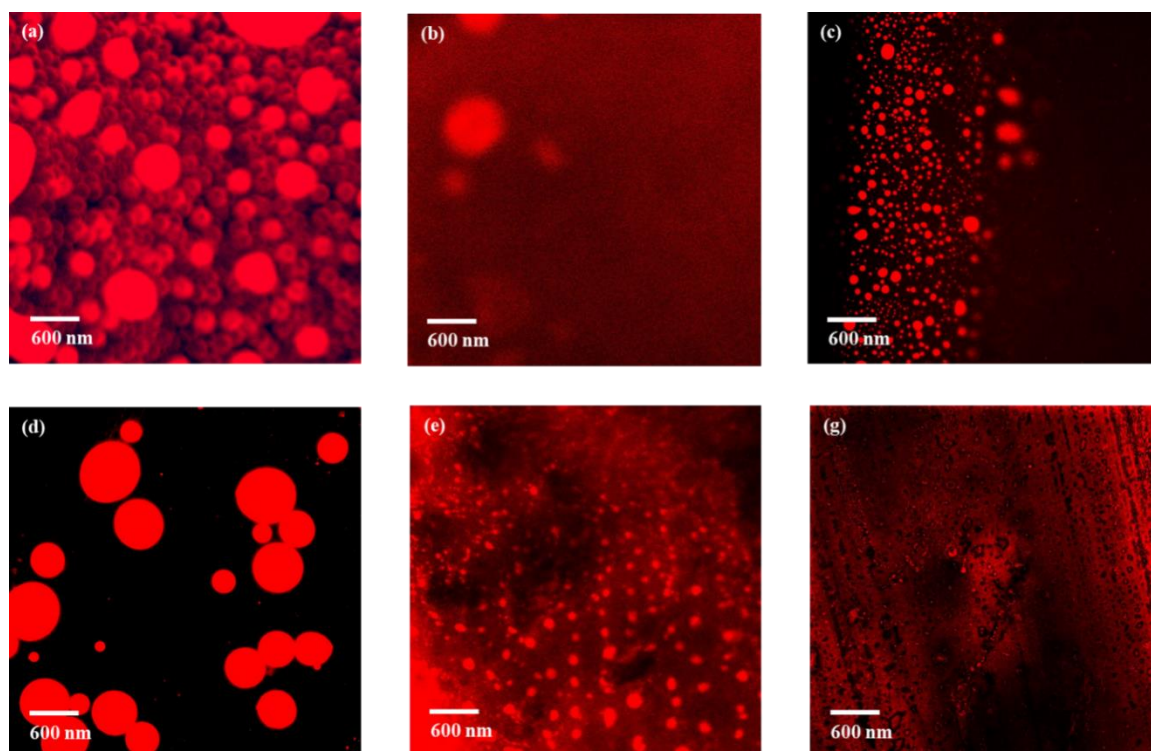


Figure 6.4. CLSM images of the microstructure of phospholipid droplets before and after extraction from dairy byproducts: (a) buttermilk; (b) buttermilk after Folch extraction; (c) buttermilk after N,N-dimethylcyclohexylamine (CyNMe₂) extraction; (d) beta-serum; (e) beta-serum after Folch extraction; (f) beta-serum after N,N-dimethylcyclohexylamine.

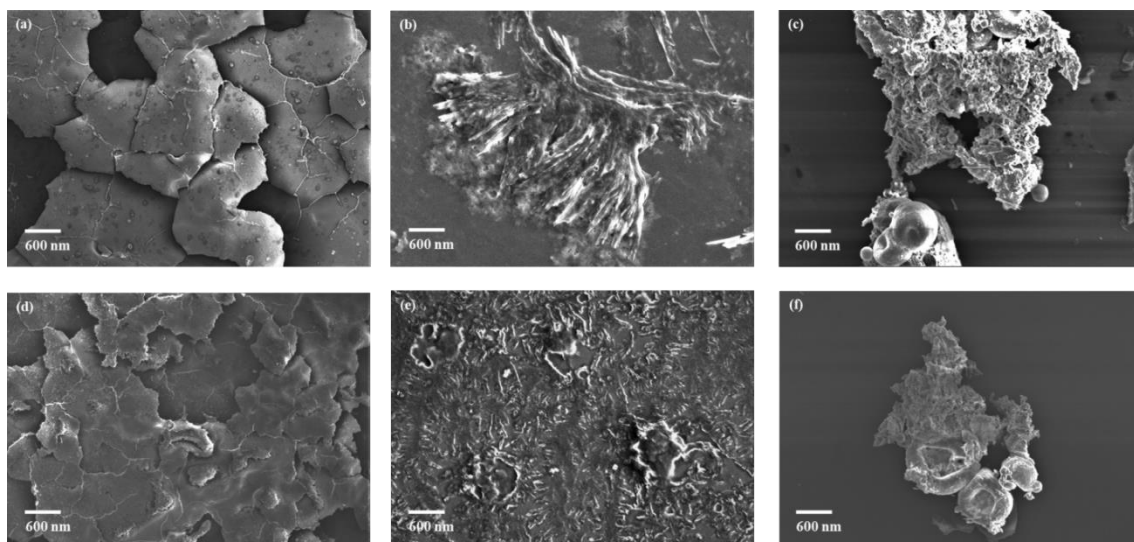


Figure 6.5. SEM images of the microstructure of phospholipid droplets before and after extraction from dairy byproducts: (a) buttermilk; (b) buttermilk after Folch extraction; (c) buttermilk after N,N-dimethylcyclohexylamine (CyNMe2) extraction; (d) beta-serum; (e) beta-serum after Folch extraction; (f) beta-serum after N,N-dimethylcyclohexylamine (CyNMe2) extraction.

6.4.5. Profile of milk phospholipids

The recovered fraction after solid-phase extraction was analyzed by HPLC-CAD to quantify the major classes of MPLs. Five major classes of MPLs were quantified, including PI, PS, PE, PC, and SM (**Table 6.3**). Overall, CyNMe2 extraction yielded higher concentration for all MPLs compared with Folch extraction. Among the MPLs, PS and SM are regarded as highly bioactive lipids due to their health claims (Huang et al., 2020b). Slightly higher concentration of PS was obtained in BM after Folch than that obtained for CyNMe2 (73.8 ± 3.6 and 68.1 ± 4.1 g mL⁻¹, respectively). In BS, CyNMe2 resulted in 2.1-fold increment in the concentration of PS than that obtained for Folch.

Table 6.3. Concentration of recovered milk phospholipids from buttermilk and beta-serum.

Phospholipid ($\mu\text{g mL}^{-1}$)	Buttermilk after Folch	Buttermilk after CyNMe2	Beta-serum after Folch	Beta-serum after CyNMe2
PI	62.6 ± 4.1	718.2 ± 35.9	22.2 ± 1.7	960.1 ± 48.1
PS	73.8 ± 3.6	68.1 ± 4.1	45.5 ± 4.1	98.8 ± 4.9
PE	41.8 ± 4.1	158.8 ± 10.1	34.5 ± 1.7	484.1 ± 24.5
PC	69.1 ± 7.4	541.8 ± 30.1	445.9 ± 29.5	669.8 ± 33.9
SM	47.1 ± 8.4	410.6 ± 20.5	560.6 ± 30.1	584.9 ± 29.2

PI – phosphatidylinositol; PS – phosphatidylserine; PE – phosphatidylethanolamine; PC – phosphatidylcholine; SM – sphingomyelin

The recovery of SM from BS was remarkably higher in CyNMe2 extraction compared with Folch (410.6 ± 20.5 and $47.1 \pm 8.4 \text{ g mL}^{-1}$, respectively), while similar concentration of SM was obtained for both extraction methods (584.9 ± 29.2 and $560.6 \pm 30.1 \text{ g mL}^{-1}$, respectively). The extraction via CyNMe2 resulted in 11.4 and 43.2 fold increment in the concentration PI for BM and BS, respectively. Similar behavior but less pronounced was observed for PE in BM and BS (3.7 and 14.1 fold, respectively).

6.5. Discussion

An efficient extraction of MPLs strongly depends on the composition of the byproducts. In this investigation, we used BM and BS as industrial byproducts obtained from the manufacture of butter and anhydrous milk fat, respectively. The composition reported in the literature for BM is quite diverse due to the wide range of protocols used during the manufacture of butter.

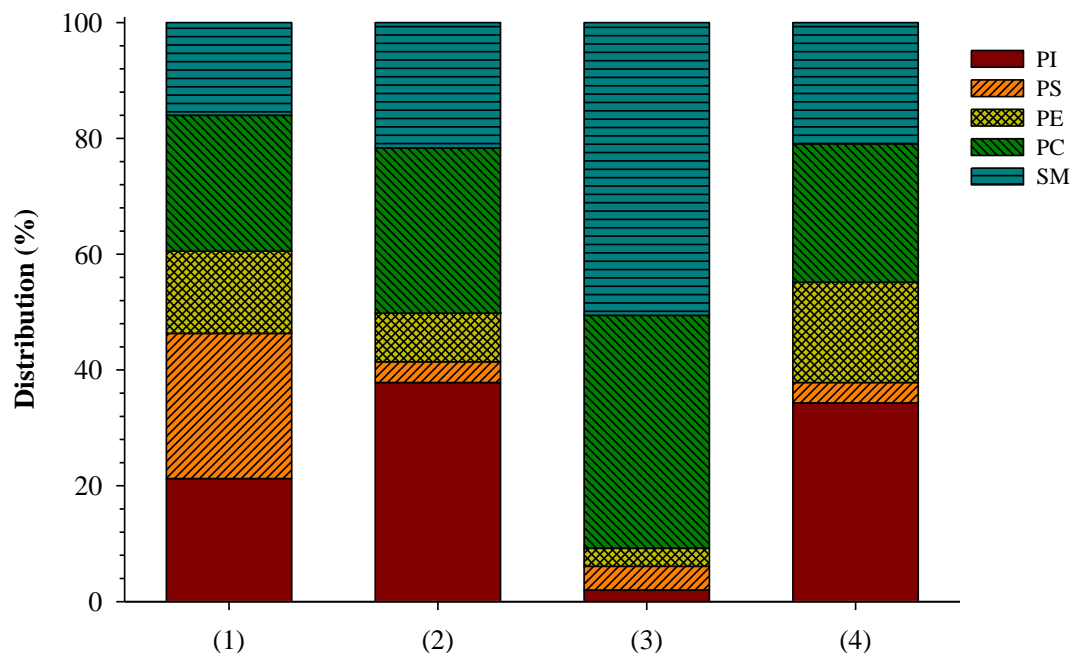


Figure 6.6. Distribution of milk phospholipids after Folch and N,N-dimethylcyclohexylamine (CyNMe2) extraction from dairy byproducts. (1) buttermilk after Folch extraction, (2) buttermilk after N,N-dimethylcyclohexylamine (CyNMe2) extraction, (3) beta-serum after Folch extraction; (4) beta-serum after N,N-dimethylcyclohexylamine (CyNMe2) extraction. PI – phosphatidylinositol, PS – phosphatidylserine, PE – phosphatidylethanolamine, PC – phosphatidylcholine, and SM – sphingomyelin.

The working principles of the evaluated extraction methods are quite different. For instance, Mojonier extraction involves the digestion of proteins by the action of a strong base (NH₄OH) followed by the dissolution of lipids using a combination of organic solvents (Gallier et al., 2010a). Folch extraction combines an organic solvent, chloroform, and a polar alcohol, methanol. The alcohol destabilizes the proteins allowing the organic solvent to dissolve the lipids (Du et al., 2015). On the other hand, CyNMe₂ is an organic base having strong basicity and medium polarity. Studies dealing with the physical and chemical characteristics of CyNMe₂ can be found elsewhere (Vanderveen et al., 2014; Zhao et al., 2020a; Zhao et al., 2020b; Xu et al., 2020).

Over the past decades, a number of technological approaches have been developed to concentrate the MPLs in BM, BS, and whey protein phospholipid concentrate. Astaire et al. (2006) developed a two-step process to concentrate MPLs up to 19% of the total lipids. The process consisted of microfiltration of BM followed by supercritical CO₂ extraction. Similarly, Costa et al. (2010) concentrated BM using the two-step process following by spray drying, obtaining a powder up to 61% of MPLs. Another investigation on the use of enzymatic treatment followed by ultrafiltration showed an 8.5-fold increase in the MPLs content. Moreover, these authors further concentrated the BM with supercritical CO₂ to obtain a product containing up to 56% of MPLs. More recently, Price et al. (2018) concentrated up to 58% of the MPLs in whey protein phospholipid concentrate after five stages of sequential precipitation of proteins with ethanol. Similarly, Price et al. (2020) studied a set of pretreatments (pH changes, temperature, and concentration of salts) prior to ethanol extraction for improving the recovery of MPLs from whey protein phospholipid concentrate. These authors concentrated between 24-38% of MPLs under optimal conditions (pH = 6.5, 30°C, and 25mM of zinc acetate). Sprick et al. (2019) removed neutral lipids from whey protein phospholipid concentrate by supercritical CO₂, while the fraction containing the polar lipids was further concentrated (up to 26% of MPLs) using ethanol (10-20%) as a co-solvent during supercritical CO₂. The recovered MPLs from BM using CyNMe₂ is unprecedented, and it raises some intriguing questions on the mechanism by which CyNMe₂ enhanced the recovery of MPLs. The first consideration is the conversion of the CyNMe₂ into its respective salt, bicarbonate, in the presence of CO₂:



Table 6.4. Nitrogen content during the N,N-dimethylcyclohexylamine (CyNMe2) extraction.

Sample point	Nitrogen content (%)
CyNMe2+Byproduct	1.44 ± 0.07
Lipid phase	0.37 ± 0.10
After solid phase extraction	No detected

This is a critical step that allows the separation of lipids from the solids and water phase. The conversion of a neutral group to a protonated state by CO₂-responsive species have been critically reviewed by Alshamrani et al. (2016). In the case of dairy byproducts, the presence of minerals and other components might affect the overall equilibrium of the salt formation. This is an important consideration since the total MPLs were quantified gravimetrically, and residual CyNMe2 in the lipid phase can lead to overestimation of the MPLs. Additional set of experiments were conducted to measure the nitrogen content at the beginning of the extraction (byproduct +CyNMe2), after the salt conversion (lipid phase), and after solid phase extraction (MPLs), **Table 6.4**. As expected, the highest nitrogen content was detected at the beginning of the extraction (1.44 ± 0.07%). Nitrogen content was also detected after the addition of CO₂, corresponding to 25% of the initial nitrogen. Two possible scenarios can explain the outcome: 1) an incomplete conversion of the salt, and 2) fragments or residual proteins presence in the lipid phase. Boyd et al. (2012) reported incomplete conversion of the salt (18–24% of residual CyNMe2) during the lipid

extraction from microalgae. Interestingly, nitrogen content was not detected in the recovered MPLs after solid phase extraction. Therefore, the quantification of MPLs was not impacted by the incomplete conversion of the salt.

Results from gel electrophoresis, particle size, zeta potential, and microstructure revealed insights into the mechanism by which CyNMe₂ extracts MPLs. The disappearance of the bands corresponding to the membrane proteins (**Figure 6.2** columns 4 and 7) indicates severe protein modification by CyNMe₂, while the whey proteins and caseins remain in the solid matrix. This observation helps to explain the large amount of PE and PI (Error! Reference source not found.) recovered by CyNMe₂ since these MPLs are primarily located at the inner parts of the membrane. On the other hand, the protein bands remained visible for the Folch extraction, suggesting a moderate modification of the membrane proteins and lower concentration of PE and PI (Error! Reference source not found.). The disappearance of the protein bands could also be a result of dissolution of the membrane proteins during the addition of CyNMe₂. The extraction using CyNMe₂ not only yielded higher concentration of MPLs (**Figure 6.6**) but also a different relative distribution. A graphical representation of such observation is given in . Folch extraction resulted in a relative distribution of MPLs comparable to that reported elsewhere (Price et al., 2020; Costa et al., 2010; Spence et al., 2009). The relative distribution of MPLs for CyNMe₂ was noticeable higher in PI and PE, which are mainly located on the inner parts of the membranes. Importantly, the final distribution of MPLs can be optimized considering the extraction time, temperature, and solvent to sample ratio.

The ability of CyNMe₂ to modify the solid matrix is also evident by the analysis of the particle size distribution. Overall, lower particles favored the diffusion into the solvent and enhanced the extraction. Rosenthal et al. (1998) related the extraction yields with the degree of cells disruption. However, the numerical relation between recovered MPLs and disruption of the membrane proteins will require time-dependent experiments, which is beyond the scope of this work. The zeta potential offers insights into the interaction between the membrane proteins and CyNMe₂. Untreated samples, BM and BS, presented values of zeta potential (-29 and -34mV) within the stability region of colloidal suspensions (Czapla et al., 2000). After extraction, the values of zeta potential suggested some degree of ionization, being greater for CyNMe₂. In the presence of CyNMe₂, the carboxyl groups of the proteins become negatively charge (-COO⁻) and the amino groups become neutral (-NH₂), which could result in the formation of ion pairs between CyNMe₂ and the carboxyl group. The formation of ion pairs may reduce the interaction between MPLs and the solid matrix, exposing the MPLs located on the inner parts of the membrane.

Important changes were also observed in the gross morphology of BM and BS (**Figure 6.5**). In the Folch extraction, a disruption of the morphology was observed that exposes the MPLs and enhanced solvent percolation. Such a disruption needs to be substantial considering that the MFGM thickness is about 20nm (Gallier et al., 2010a). It appears that the mechanisms governed the Folch extraction involves the diffusion of MPLs into the solvent, whereas proteins and lactose remain in the solid matrix. After CyNMe₂ extraction, the surface of BM and BS was greatly destroyed, where microfractures and hollow openings were generated. During the fracture or rupture process, the solid matrix became

porous and soluble compounds within the membrane proteins were dissolved by CyNMe₂. Thus, disruption of the protein membranes by CyNMe₂ favors the mass transfer between MPLs and the surrounding solvent, improving the extraction of the MPLs. This process is fundamentally different from the Folch extraction, which relies on the diffusion of the solvent into the solid matrix and solubilization of MPLs. Upon solubilization, the MPLs droplets are surrounded by solvent, destabilizing the electrostatic forces and creating a reduction in the droplet size (**Figure 6.4**).

CyNMe₂ can be used as a protein destabilizing agent and it can be potentially pair with different solvents to shorten the extraction time. The repeated use of the CyNMe₂ need to be studied for further development. The analysis of the basicity and ionic strength of the CyNMe₂ and byproduct will provide essential information to select suitable extraction conditions.

6.6. Conclusions

Extraction of MPLs from dairy byproducts via CyNMe₂ resulted in high yields (up to 98%). The high efficiency was attributed to a rupture of the protein membranes through ion pair formation, followed by a release of the MPLs from the solid matrix. Microstructure and charged particle analysis seem to support the proposed mechanism. Compared with Folch extraction, CyNMe₂ resulted not only in higher concentration of MPLs (up to 9-fold increment) but also a different relative distribution, where PI and PC were the predominant MPLs. The outcomes of this work will help to design novel extraction strategies for the recovery and isolation of MPLs.

6.7. References

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CHAPTER 7

APPLICATION OF BETA-SERUM IN ICE-CREAM MANUFACTURING⁶**7.1. Abstract**

Beta-serum (BS) is the aqueous product (serum) produced after the phase inversion during the manufacture of anhydrous milk fat. Although its gross composition resembles non-fat dry milk (NFDM), BS contains about 6-8% of phospholipids (PLs) on a dry basis. Such a concentration of PLs may improve the emulsification during icecream. The objective of this work was to evaluate the effect of BS on selected quality parameters of ice cream. A secondary objective was to monitor the presence of PLs within the serum phase at different processing steps of icecream manufacture, including mixing, pasteurization, freezing, and melting. Icecream formulated with NFDM was used as a control treatment. Icecream mix formulated with and without BS was centrifugated (4000 rpm for 30 min) to separate the serum phase (upper phase) and solid phase (lower phase), which were analyzed for total lipids and total phospholipids. Additionally, the presence of PLs in both phases was monitored through confocal laser scanning microscopy. Overall, the majority of PLs were found at the bottom phase during mixing, pasteurization, and melting. The particle size and zeta potential measurements were observed to be 474.6 ± 13.21 and 564.8 ± 12.7 nm, -37.9 ± 5.90 and -43.1 ± 4.90 mV for the control icecream and B-serum icecream, respectively. The PLs reported after meltdown using a sieve (0.833mm) for IC control and B-serum were 58.03 ± 4.10 and $63.47 \pm 3.02\%$, respectively, while before meltdown it was reported 4.04 ± 1.49 and $11.27 \pm 0.56\%$, respectively. The flow curve indicated a shear-thinning behavior for both samples. Therefore, results document the

⁶ A version of this chapter is to be submitted to the *International Dairy Journal*

presence of PLs in the manufacturing of ice cream and significant quality attributes with the addition of Beta-serum.

7.2. Introduction

Icecream is the most popular frozen dessert consumed all over the world. According to the International dairy federation, 2020, icecream consumption per person each year in the US alone is around 2L. Ice cream global sales represent over USD 73.8 Billion per year, with annual growth of close to 5 % (Velásquez-Cock et al., 2019) and are projected to increase around USD 97 billion by 2023. Icecream is a complex food-generated emulsion (oil in water) containing fat globules, air bubbles, ice-crystals, and unfrozen viscous serum(Akbari, Eskandari, & Davoudi, 2019) along with proteins, mineral salts, polysaccharides and water. Regular ice cream contains fat 10-16% from dairy or non-dairy sources, an essential component in the icecream, non-fat milk solids, sweeteners, stabilizers, and flavouring components. Cow's milk serving as the main ingredient for the icecream, contains a wide variety of high-quality nutrients for human health, such as protein, vitamins, and minerals(Agrawal, Karkhele, Karthikeyan, Shrivastava, & Geetesh, 2016).

In recent times, the dairy industry has been paying attention to dairy by-products such as buttermilk, pro-cream, beta-serum. Dairy by-products are low-cost materials that are low in fat and have excellent technological and functional properties that benefit human health. Dairy by-products represent 80% of the total milk and, therefore, generate high disposal costs (Panesar & Kennedy, 2012)discard their by-products in the environment, causing intense pollution due to the high concentration of organic matter, i.e.) high

chemical (COD) and biochemical oxygen demands(BOD) (Barbosa de Meneses et al., 2020). These by-products can cause a serious impact on the environment. The dairy industry and researchers are interested in applying the valuable components to a lower cost, sustainable dairy product, and icecream is a potential vehicle for reusing the dairy by-products.

The beta serum is a by-product obtained from the phase inversion of anhydrous milk fat (AMF). The starting material for beta serum is cream, which is further processed to AMF. And they contain a significant quantity of fat and protein and a high level of phospholipids that has a composition similar to buttermilk and butter serum (Price, Fei, Clark, & Wang, 2019). The beta-serum contains a higher protein content and a different protein profile when compared to the whey-by-products. The health benefits of consuming milk phospholipids have been discussed extensively in various reports (Ali et al., 2019; Shouyun Cheng, Rathnakumar, & Martínez-Monteagudo, 2019; Ortega-Anaya & Jimenez-Flores, 2019). Therefore, because of its health benefits and functionality (Rebouillat & Ortega-Requena, 2015), it can be used in a variety of foods such as frozen desserts, bakery, pumping hams and other meats. They are often referred to as powerful natural emulsifiers, oxidation stability, milky taste, and a good stabilizer that makes it possible for ingredient formulation(Rebouillat & Ortega-Requena, 2015), an active health ingredient, especially in icecreams.

Recent works have focused on the effect of dairy by-products such as ricotta whey (RW), cheese whey (CW), and butter whey (BUW) as replacers of whole milk (WM) by (de Meneses et al., 2020; Meneses, Silva, Monteiro, Rocha-Leão, & Conte-Junior, 2020),

the stability and rheological properties (Barbosa de Meneses et al., 2020) and sensory characteristics in chocolate icecream (de Meneses, Guerra Monteiro, dos Santos, da Rocha-Leão, & Conte-Junior, 2021) all portray the potential milk replacing by the dairy by-products. Delactosed whey permeate (DLP) and pro-cream, a by-product of microfiltration during whey protein isolate production, were made and assessed for application in ice creams as a source of protein fat (Bund & Hartel, 2013). All these studies showed the potential application of dairy by-products in icecream. In this context, the study aimed to investigate the effect of the icecream's quality attributes with non-fat dry milk and freeze-dried beta-serum powder. Further, to map the phospholipid presence at each stage (Mixing, pasteurization, Freezing and Hardening) using both quantitative and quality measurements.

7.3. Materials and Methods

7.3.1. Materials

Methanol (99.9%, Sigma Aldrich), chloroform (99.9%, Sigma Aldrich), hexane (99.9%, Sigma Aldrich), HPLC-grade water (Sigma Aldrich), activated silica gel (Silica gel 60 G, EMD Millipore), phospholipid mixture (Soybean, P3817-1VL, Sigma Aldrich), Rd-dope (Avanti Polar Lipids Inc., Alabaster, AL, USA), Laemmli buffer (Bio-Rad, Hercules, CA, USA), Tris-acrylamide gels (4–15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad), Tris/Glycine/SDS Buffer (Bio-Rad), Protein™ Kaleidoscope standards (Bio-Rad), and 2-mercaptoethanol (Fisher Scientific) were purchased from commercial suppliers. Beta-serum was obtained from a cheese plant (Valley Queen, Millbank, SD) and was freeze-dried using a laboratory freeze drier (Harvest right) to obtain the B-serum powder.

7.3.2. Ice cream processing

The formulation used in this study is exhibited in **Table 7.1**. The ingredients were purchased from a local market in Las Cruces, New Mexico, USA. The blend of stabilizers consisted of guar gum, locust bean gum, carrageenan, polysorbate 80, and mono- and diglycerides obtained from(Continental Colloids, Inc., West Chicago, IL). Dry ingredients were dissolved in skim milk and heavy cream at 45 °C and blended using a laboratory blender (Polytron, CH-6010,60Hz, Switzerland) for 15min at room temperature followed by pasteurization (FT74 UHT/HTST processing system) at 80 °C for 30 s, further the mix was aged at (4 C for 12 h) and a final step the freezing of ice cream was done in a batch type icecream maker (Breville, Smart Scoop). The draw temperatures during the freezing were measured using a data logger (Omega Engineering Inc., Stamford, CT) connected to a K-type thermocouple mounted inside the freezer. Then, the icecream was transferred to plastic cups left for hardening at -40 C for 8 hrs and then was kept in a conventional freezer (-18 C) until further analysis.

Table 7.1. Ice cream formulations with Non-fat dry milk and Beta-serum powder

Ingredient	Formulation	
	Control (%)	B-serum(%)
Heavy cream	35	26
Skim milk	40.72	46.72
Beta serum	-	10
Non-fat dry milk(NFDM)	6	-
Sweeteners	18	17
Stabilizers	0.28	0.28

7.3.3. Physiochemical analysis

The samples control and B-serum were tested for total solids, pH and titratable acidity (TA). The pH was directly measured using a digital pH meter (Fisher Scientific, AB 15). The titratable acidity (TA) was measured by titration with 0.1 N NaOH, and results were expressed as per cent lactic acid, and the ash content was measured using a muffle furnace(Thermo scientific, Lindberg Blue M) according to the Association of Analytical Chemists (AOAC International, 2012). The total solids were determined gravimetrically at 103 ° C for 15 h using a laboratory oven (Thelco laboratory Oven, NM, USA), and protein was determined using the Kjeldahl method (AOCA) method 990.20.

7.3.4. Quality analysis of Ice-cream

7.3.4.1. Fat destabilization

Fat destabilization measurements were calculated as the percentage of turbidity in ice cream compared to the turbidity in ice cream described elsewhere (Adapa, Dingeldein, Schmidt, & Herald, 2000). Turbidity measurements were made with the UV-spectrophotometer(Thermo scientific Genesys 10 UV-VIS mode # 840-208200) set at 540 nm. The formula of fat destabilization was expressed as

$$(\textit{Turbidity of the ice – cream})/(\textit{turbidity of the mix}) \times 100 \quad (7.1)$$

7.3.4.2. Particle size distribution

ZetaSizer Nano ZS (Malvern Instruments Ltd, Cambridge, UK) was used to determine the particle size distribution of the ice cream mix and ice cream samples, according to the methodology reported elsewhere (H. Goff, Verespej, & Smith, 1999). The dilution of the

samples at 1:1000 was done using deionized water before placing the sample chamber and further transferred to a disposable cuvette (DTS 0012, Sigma-Aldrich, St Louis, MO, USA). Diluted samples were transferred to the measuring chamber and equilibrated at room temperature for 120 s. A scattering angle of 170° with a refractive index of 1.46 was used to determine the average size.

7.3.4.3. Zeta Potential

Ice cream mix and ice cream samples were analyzed for the zeta-potential using a Zetasizer Nano (Malvern Instruments Ltd., Cambridge, UK). Diluted samples (1000x) were transferred to a disposable polycarbonate cuvette (ATA scientific, DTS1061). Measurements have repeated a minimum of 10 times per run with a minimum of 7 runs.

7.3.4.4. Desorption index

The ice cream samples (control and B-serum) were observed for desorption according to the methodology reported by (Meneses et al., 2020) with slight modifications. A 50 mL graduated cylinder was filled with melted ice cream was slightly sealed and observed at a controlled temperature of 29 ± 1 °C for 2- 3 hrs. Observation of phase separation, the volume of the serum fraction formed in each sample was obtained. The desorption index was determined using the following equation:

$$\left(\frac{H_{\text{serum fraction}}}{H_{\text{melted ice cream}}} \right) \times 100 \quad (7.2)$$

Where $H_{\text{serum fraction}}$ = volume of the whey layer formed (mL); $H_{\text{melted ice cream}}$ = total volume of the melted ice cream (mL).

7.3.4.5. Gel electrophoresis

The protein profile of the ice cream mixes, icecream and the melted ice cream was determined using Sodium Dodecyl Sulfate- Polyacrylamide gel (SDS-PAGE) under reducing conditions as reported elsewhere (Rathnakumar, Ortega-Anaya, Jimenez-Flores, Reineke, & Martínez-Montegudo, 2020). Briefly, 1:4 volume of sample to cold acetone(-20 °C) was mixed for the precipitation of the proteins, the mixture was placed in a freezer at -4 °C for 1 h and centrifuged (3600 rpm) (Jouan CR412, Jouan Inc., Winchester, VA, USA) for 20 min at 0°C. The supernatant was discarded, and the pellets were dissolved in 1x phosphate buffer saline(PBS). Then, 5 µL of dissolved pellets were transferred into a vial containing 4.75 µL of 2x Laemmli sample buffer (Bio-Rad, Hercules, CA) and 0.25 µL of 4% 2-mercaptoethanol (Fisher Scientific, Hampton, NH). Dissolved pellets were heated at 90 °C for 5 min. Upon cooling, 10 µL of the preparation was loaded into Tris-acrylamide gels (4–15% Mini-Protean TGX precast gels with 10 wells, Bio-Rad). Gels were run for 1 h at 200 V using Tris/Glycine/SDS buffer (Bio-Rad). Then, the gels were removed and stained using Bio-safe Coomassie G-250 stain (Bio-Rad) and destined to get the protein pattern. The individual proteins were estimated based on the molecular weight using the standard from BIO-RAD (Precision plus protein standards, 161–0375).

7.3.4.6. Overrun

Overrun measurements were taken by comparing the weight of ice cream mix and ice cream in a fixed 100 mL volume container (Muse & Hartel, 2004) calculated using the formula:

$$\text{Overrun} = \frac{\text{weight of icecream mix} - \text{weight of icecream}}{\text{weight of ice-cream}} \times 100 \quad (7.3)$$

7.3.4.7. Steady shear measurements

The icecream mixes flow curve was determined in a rheometer (Discovery Hybrid rheometer, HR 30, TA instruments) equipped with a 20 mm parallel plate and a peltier steel plate (108783). Flow sweep measurements were carried out in the shear rate range from 100 1/s to 1 1/s at 4°C. The flow behavior was characterized by the Herschel-Buckley model in equation (7.4):

$$\sigma = \sigma_0 + K \dot{\gamma}^n \quad (7.4)$$

where σ_0 represents yield stress (Pa), κ is consistency index (Pa.sn), $\dot{\gamma}$ is the shear rate (s⁻¹), and n is a dimensionless number known as flow behavior index ($n = 1$ for Newtonian fluids, $n < 1$ for pseudoplastic, and $n > 1$ for dilatant).

7.3.4.8. Melting behaviour

Icecream samples were evaluated for melting rate using oscillatory analysis with an MCR92 225 rotational rheometer (Anton Paar USA Inc.) coupled with a Peltier chamber

with a plate-plate 226 geometry (25 mm diameter). Guidelines of the methodology can be found elsewhere (Eisner, Wildmoser, & Windhab, 2005; Wildmoser, Scheiwiler, & Windhab, 2004). The oscillatory analysis was conducted within a temperature range of 20 to 10°C, at a heating rate of 0.5°C min⁻¹, a deformation amplitude of 0.5%, a frequency of 10 Hz, and a gap width between plates of 3 mm. The G' and G'' were recorded continuously during the analysis.

7.3.5. Meltdown

The meltdown studies were carried by following the method described elsewhere ((Mahdian, MAZAHERI, & Nobahari, 2012; Muse & Hartel, 2004; Velásquez-Cock et al., 2019) with slight modifications. The frozen ice cream samples of around 70 g were carefully removed from plastic cups and placed in a sieve (0.833 mm) which was mounted on a beaker at a controlled temperature ($25 \pm 1^\circ\text{C}$). The empty weight of the beaker was taken, and the time(min) from the beginning to end of the melting was measured. The meltdown icecream samples were further analyzed for total lipids, phospholipids and individual phospholipids, and the samples were also subjected to confocal laser scanning microscopy to determine the presence of phospholipids after the meltdown. The melting rate of the ice cream with and without Bserum was determined by taking the dropping weight and was measured every 5 min for 1 hr.

7.3.6. Mapping of PLs

The samples of the ice cream mix formulated with and without BS were collected at the different stages of the ice cream manufacturing process such as (mixing, pasteurization

and freezing) were centrifugated (4000 rpm for 30 min, at -9°C) to separate the serum phase (upper phase) and solid phase (lower phase). In order to map the presence of phospholipids, the samples were analyzed for microstructure using Confocal Laser Scanning Microscopy (CLSM), and the total lipids & phospholipids were quantified using the Folch method.

7.3.6.1. Confocal laser scanning microscopy

The serum phase and solid phase of all the samples at different processing stages, along with the samples obtained after the meltdown, were subjected to a confocal microscope (Leica TCS SP5 II, Leica, Wetzlar, Germany) to examine where the PLs are present during the ice-manufacturing process. According to the methodology described elsewhere, the samples were stained using Rd-dope (Gallier, Gragson, Cabral, Jimenez-Flores, & Everett, 2010; Rathnakumar & Martinez-Monteaudo, 2020). A 50 μl of the test sample and 100 μl Rd -dope were slightly vortexed and kept in the dark for 20 min. Images were captured at 10x magnification, and the excitation of the Rd-dope was achieved at 559 nm using emission from a diode laser.

7.3.6.2. Total lipids and phospholipids

A Folch extraction was carried to determine the amount of total lipids present at different stages of the ice cream manufacturing process (mixing, pasteurization, freezing and hardening), 2 g of samples were mixed with 20 mL of chloroform: methanol solution (2:1, v/v). The mixture was vortexed for 3 min, followed by centrifugation at room

temperature (4200×g) for 20 min. The upper phase was then discarded, while the lower phase was transferred to a test tube, where the chloroform was removed at 45 °C using a vacuum oven.

$$\text{Total lipids(\%)} = \frac{\text{weight of dried lipids}}{\text{weight of sample}} \cdot 100 \quad (7.5)$$

The extracted lipids were fractionated to recover the MPLs using solid-phase extraction (SPE). An activated silica gel column (1 cm × 10 cm) was used for the separation, following the methodology reported elsewhere (S. Cheng, Rathnakumar, & Martinez-Monteagudo, 2019). Dried lipids around 0.1 g were dissolved in 1 mL of chloroform: methanol solution (95:5, v/v) and run through the SPE column previously conditioned with 10 mL of the chloroform: methanol solution. The MPLs were recovered with 10 mL of methanol, followed by 10 mL of chloroform: methanol: water (5:3:2 v/v/v). Finally, solvents were evaporated at 40 °C under vacuum, and the MPLs fractions were calculated using Equation (7.6). The extracted MPLs were stored at -20 °C until further analysis.

$$\text{Total phospholipids(\%)} = \frac{\text{weight of dried fraction}}{\text{weight of lipids}} \cdot 100 \quad (7.6)$$

7.3.6.3. Quantification of Individual phospholipids

The quantification of the individual MPLs was determined using a UHPLC system (Dionex Ultimate 3000, Thermo Scientific) coupled to a charged aerosol detector (CAD, DionexCorona Veo RS, Thermo Scientific). The methodology was reported elsewhere

(Rathnakumar, Ortega-Anaya, Jimenez-Flores, & Martínez-Monteagudo, 2021b). Each sample was analyzed in duplicates under the same conditions. The five major phospholipids were identified by their retention time and were quantified by comparing their peak area with a standard curve.

7.3.7. Statistical analysis

All analysis was carried out in triplicates, and the mean values for the total lipids and recovered MPLs were compared using Tukey's test ($p < 0.05$). The statistical analysis was carried out using Sigma plot software V11 (SPSS Inc., Chicago, IL, USA).

7.4. Results and discussion

7.4.1. Compositional analysis

Table 7.2 shows the composition of the ICM resembled that of the typical icecream where fat and total solids were about 11% and 40-43%, respectively, similar to studies by (Ruger, Baer, & Kasperson, 2002). The pH of an ice cream mix can be used as an indicator of mix quality. The pH of an ice cream mix and ice cream was around 6.5 -6.6 and is related to the solid content; as the solid portion of a mix increases, the normal acidity is elevated, and the pH is lowered (Holcomb, 1991). The titrable acidity(TA) and ash % depicted was 0.11 to 0.18 % (Haque & Ji, 2003) and 0.77-0.89%, respectively, which were in agreement with results from (Meneses et al., 2020), who utilized different dairy by-products in icecream. The protein % of icecream was $0.48 \pm 0.00\%$ and $0.52 \pm 0.06\%$ for the control and B-serum icecream, respectively.

Table 7.2. Compositional analysis of Ice-cream

Samples	pH	Titrateable acidity(TA%)	Ash(%)	Total solids(%)	Fat %
Ice cream mix (control)	6.63 ± 0.01	0.18±0.04	0.89 ±0.00	43.47±0.06	11.33±3.35
Ice cream mix (B-serum)	6.65 ±0.07	0.13±0.00	0.86 ± 0.01	42.95±0.06	8.92±1.88
Ice cream (control)	6.53±0.01	0.12±0.00	0.81± 0.01	42.11±0.03	32.49±1.49
Ice cream (B-serum)	6.53±0.02	0.11±0.00	0.77 ±0.01	40.33±0.15	19.39±0.56

7.4.2. Quality analysis of ice cream mix and Ice cream

7.4.2.1. Particle size distribution and Zeta potential

The zeta potential measurements are a tool to determine whether the constant stability of the icecream emulsions. According to **Table 7.3**, it was observed that the zeta potential increased along with the particle size for both the control and B-serum mix and ice cream, respectively. This behaviour doesn't prevent the aggregation of droplets present in the ice cream mixes. The average particle size of the control mix and icecream were 411.6 ± 11.24 and 474.6 ± 13.31 , respectively, and they were found to be lesser compared to the particle size of the b-serum mix and icecream which was found to 685.7 ± 14.13 and 564.8 ± 12.7 , respectively. Similar results on the particle sizes were observed by (Barbosa de Meneses et al., 2020), who utilized the dairy by-products cheese whey and ricotta whey in chocolate flavour icecream 684.43 ± 16.65 nm and 523.10 ± 18.42 nm, respectively. The zeta potential results also correlated with the results (Barbosa de Meneses et al., 2020). The

particles with zeta potential greater than + 30mV or less than - 30mV usually are considered stable (Malvern, 2004). In our study, both the ice cream mixes control and b-serum was found to be stable. The zeta potential values of both the ice cream were above -30 mV, similar results were found in research carried out by (J. Cheng, Ma, Li, Yan, & Cui, 2015), in which the researchers evaluated the stability of various ice cream using different protein-polysaccharide interactions showed a zeta-potential value range of - 30 to - 50 mV). Zeta potential often determines the surface charge density and interaction between the emulsions.

Table 7.3. Zeta potential and average particle size of Ice cream mix and control

Samples	Average particle size (nm)	Zeta Potential(mV)
Ice cream mix (control)	411.6 ±11.24	-24.3±4.3
Ice cream mix (B-serum)	685.7±14.13	-27.1±5.1
Ice cream (control)	474.6±13.31	-37.9±5.9
Ice cream (B-serum)	564.8±12.7	-43.1±4.9

7.4.2.2. Desorption index

Desorption is a relevant quality parameter for ice creams, wherein the absence of phase separation during and after melting indicates a good ice cream (Bodyfelt et al., 1988). In our study, the ice cream did not present any syneresis, and, therefore, it was not possible to quantify the whey fraction. The proteins strong influence the phase separation after melting. Similar results were observed by (Syed, Anwar, Shukat, & Zahoor, 2018). There was no syneresis by replacing dry milk to dry whey protein in non-fat ice cream, and (Meneses et al., 2020) study on different dairy whey's in ice cream showed no syneresis.

7.4.2.3. Flow behavior of Ice-cream mix

Figure 7.1 exhibits the flow curves on ice cream mix samples. All ICM exhibited shear thinning behavior, typical behavior for regular ice cream mixes, where the viscosity decreased with the shear rate (Regand & Goff, 2003). This due to the breakage of the entangled network as the shear is exerted over the mix (Cavender & Kerr, 2020). The n values was found to be 0.7 for the B-serum mix and 0.9 for the control mix where the K values increase exponentially for the B-serum mix were 4.52 and 1.82 respectively. The highest viscosity observed was 16.0 Pa.s for the B-serum mix, while it was 0.96 Pa.s for the control mix at the shear rate 1 s^{-1} and decreased as the shear rate increased. Overall the ICM with B-serum was more viscous compared to the control. This could be because of the presence of large molecules present in the beta serum powder, large molecules tend to have a higher viscosity, as they have stronger intermolecular forces attracting to one another, and there is a greater strength that hinders molecular flow, hence more viscous (Pon, Lee, & Chong, 2015). The changes in the viscosity during ice cream manufacturing affects melting and overrun (Ismail, Hameed, Refaey, Sayqal, & Aly, 2020).

7.4.2.4. Fat destabilization and Over-run

The fat destabilization for the ice cream control was $77.18 \pm 1.66 \%$, while the ice cream with B-serum was $63.10 \pm 2.10\%$, as in **Table 7.4**. Often the extent of fat destabilization depends on many factors such as viscosity, emulsifier type, shear forces, ice-crystals, air cells, total solids and fat content (Amador, Hartel, & Rankin, 2017; Hartel, Rankin, & Bradley Jr, 2017). The overrun of the IC with B-serum and without were 13.48 ± 0.67 and

14.11 ± 0.16 , respectively. A low range of over-run was presented in this study is because freezing was done using batch freezing, and the air wasn't incorporated into the mix.

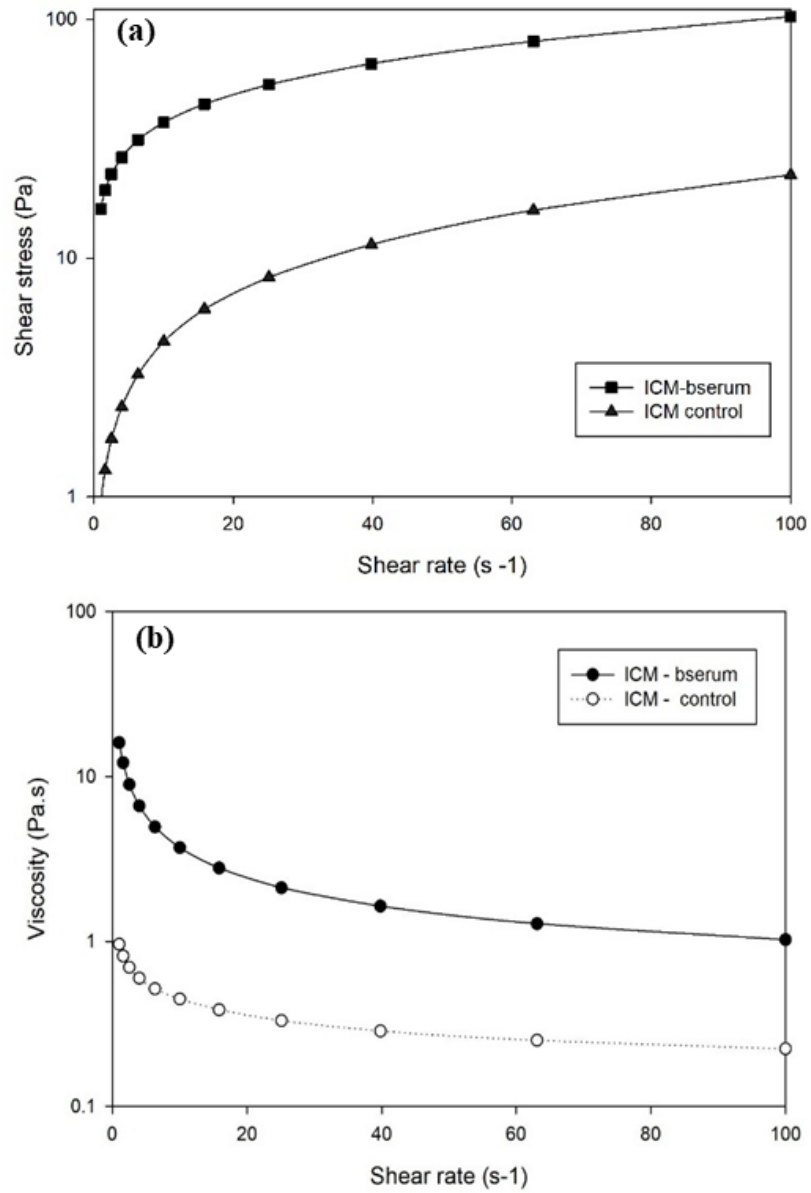


Figure 7.1. Flow curve of ice cream mixes control and b-serum.

Table 7.4. Fat destabilization and overrun of ice cream

Samples	Fat destabilization %	Overrun %
IC - NFDM	77.18±1.66	14.11±0.16
IC- B-serum	63.10±2.10	13.48±0.67

7.4.2.5. Melting behavior of Ice-cream

The two ice cream samples' melting behaviour was evaluated using an oscillatory test with a temperature sweep. A melting curve is given in **Figure 7.2a**, where G' and G'' are plotted against temperature. Additionally, the melting curve was also evaluated using the loss tangent ($\tan(\delta)$), b. The storage modulus (G') values illustrate the solid body like behavior of the material in terms of elastically stored deformation energy, while the loss modulus (G'') values represent the viscous fluid behavior corresponding to the loss of deformation energy. As observed in **Figure 7.2a**, both G' and G'' values decreased as temperature increased. As proposed by (Wildmoser et al., 2004), the melting curve resembled a sigmoidal shape similar to the typical melting curve of hard ice cream (Freire, Wu, & Hartel, 2020; Tekin, Sahin, & Sumnu, 2017). G' and G'' curves were divided into three zones and the $\tan \delta$ (G'/G'') behavior, which is an important indicator of the phase melting during the test (Wildmoser et al., 2004). Zone 1 that exists at a temperature range of -20 °C to -10 °C explains the scoopability and rigidity. It was observed that the ice creams with B serum had higher G' and G'' values compared to the control ice cream without changing the overall behavior of the melting curve. This shows that b serum powder in ice cream contributed to more rigid structures in the ice cream.

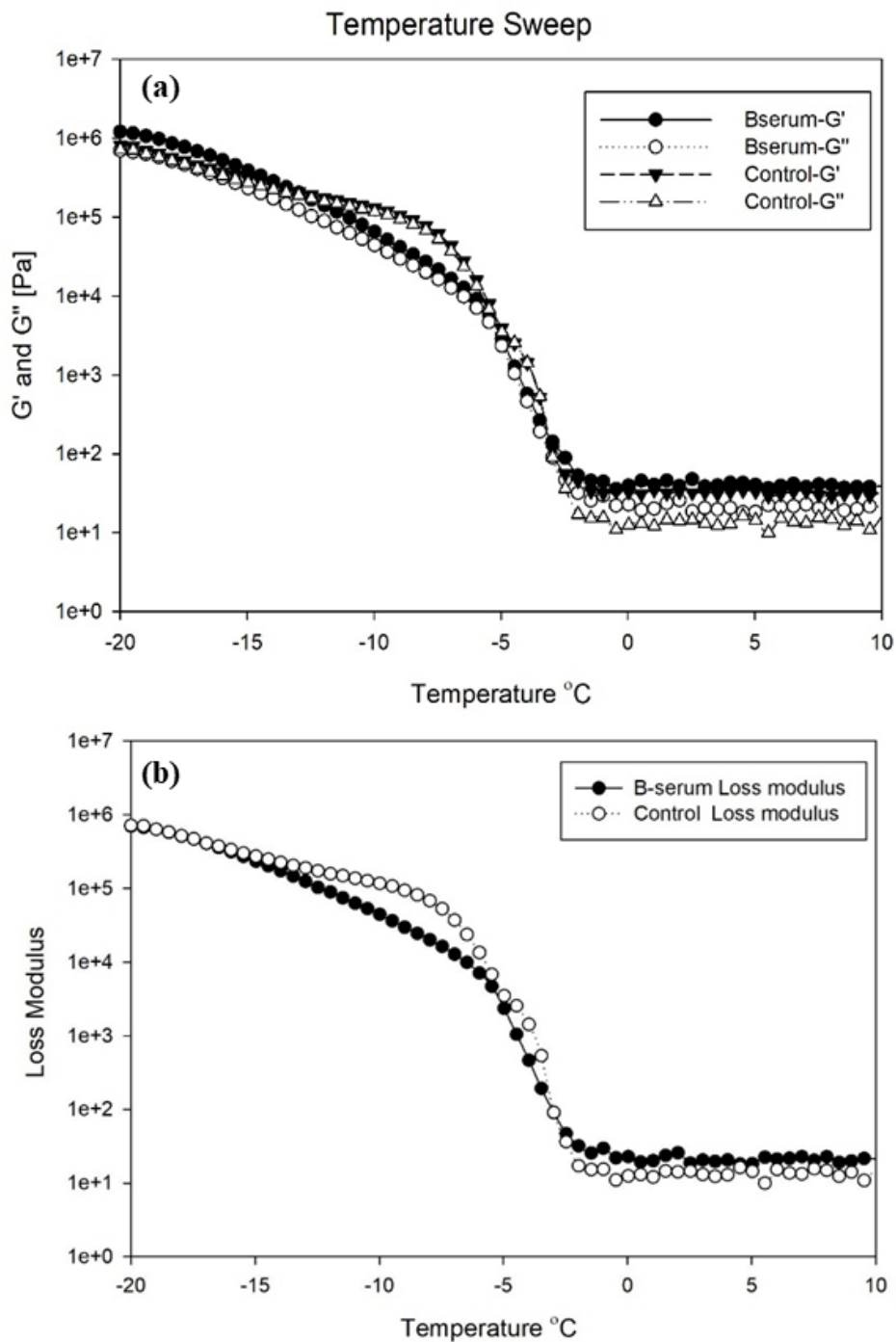


Figure 7.2. Melting curve of ice-creams control and B serum : (a) thermal variation of storage module (G') and loss module (G'') and (b) thermal variation of loss modulus ($\tan(\delta)$).

Further heating of the ice cream (zone II) -10 °C to 0 °C produced a sharp decrease of G' and G'' were observed for both the ice cream samples. The zone explains that a steep slope is related to the sensorial impression of coldness. The steeper slopes were observed in the B-serum ice cream compared to the control ice cream, which showed a dominance of icy structure. Further, zone 3, the temperature range between 0 °C and 10 °C, has a lower plateau level. All ice is melted in this temperature range and governed by the dispersed air and fat phase, which impacts the rheological characteristics (Wildmoser et al., 2004). In this zone, all samples exhibited G' values higher than G'' , with higher G' values, the B-serum ice cream showed higher creaminess than the control.

7.4.2.6. Protein Profile

The ice cream protein contributes to its structure, emulsification and water holding capacity, enhanced viscosity that provides a beneficial body to the ice cream, which increases the meltdown time and contributes to iciness (H. D. Goff, 1997). The protein pattern for both the mixes and ice cream were depicted in **Figure 7.3**. Therefore, three major classes of proteins were observed: milk fat globule membrane (MFGM), caseins, and whey proteins. Milk fat globule membrane included xanthine oxidase, butylophilin, and adipophilin, displaying bands at 100, 75, and 50 kDa, respectively. On the other hand, α -casein and β -casein revealed their characteristics bands at 20 and 25 kDa, respectively. Whey proteins (α -lactalbumin and β -lactoglobulin) showed their bands at a molecular weight of 10 and 15 kDa, respectively. They were similar to the bands present in beta-serum (Price et al., 2019) and ultrasound treated beta-serum (Rathnakumar, Ortega-Anaya,

Jimenez-Flores, & Martínez-Monteaagudo, 2021a). The SDS-page also provides insights into the proteins present in the mix and ice cream.

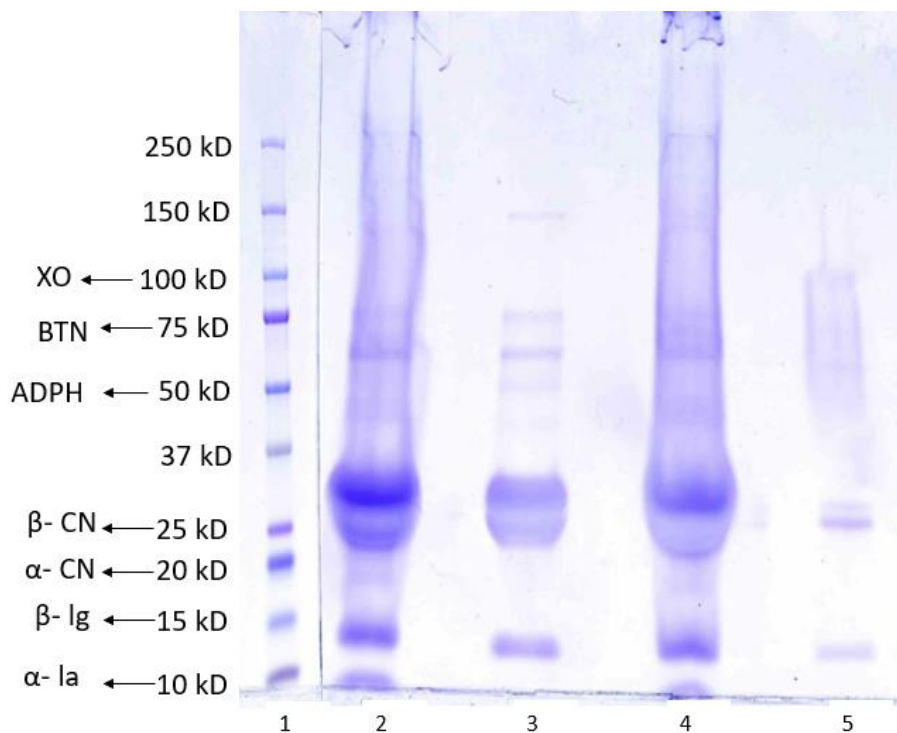


Figure 7.3. SDS -page patterns of mix and ice cream. (1) molecular weight standard (2) ice cream mix without b-serum (3) Ice cream mix with b-serum (4) ice cream without B-serum (5) Ice cream with b-serum.

7.4.3. Meltdown

The meltdown curve of the control and the b-serum was sigmoidal in shape, as shown in **Figure 7.4**, and the onset of the meltdown was at 10 minutes for the control ice cream and 15 minutes for the B-serum ice cream. The meltdown's maximum values for the control and B serum were 70.2% and 55.6%, respectively. The meltdown of ice cream is influenced by several factors such as air cell, stabilizer concentration and melted ice-crystals (Muse & Hartel, 2004). The ice cream with low over-run melted quickly (Sakurai, 1996), as

observed in our study. Air cells present in the ice cream structure were attributed to the slower meltdown rate, thereby increasing over-runs.

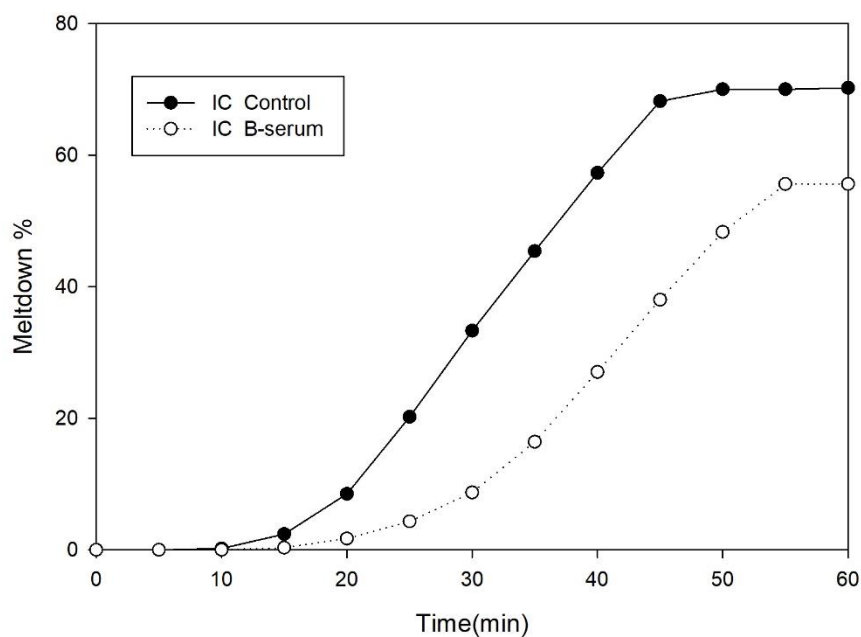


Figure 7.4.Meltdown of ice cream control and B-serum

7.4.3.1. Total Lipids and phospholipids before and after the meltdown

Figure 7.5 represents the amount of total lipids and PLs in the ice cream before and after the meltdown. The total lipids for the ice cream control and B-serum were 32.49 ± 1.49 and 19.39 ± 0.56 , respectively, while after the meltdown, the total lipids were 13.05 ± 4.1 and 24.55 ± 3.02 , respectively. In the case of the total PLs, the control and B-serum ice cream before melting were found to be $4.04 \pm 1.49\%$ and $11.27 \pm 0.56\%$, respectively, while after the meltdown, the samples were $58.03 \pm 4.1\%$ and $63.47 \pm 3.02\%$, respectively. From the quantitative results, both the samples had higher lipids before the meltdown and a higher amount of PLs at the melted phase. During melting, the conversion of ice to liquid,

water-soluble PLs is easily carried away to the melted side, resulting in a higher amount of PLs for both samples.

The microstructure is shown in **Figure 7.6** also supports the presence of PLs obtained after the meltdown in beta serum and the control Ice-cream. The SDS-page also provides insights into the proteins after the ice cream meltdown, as shown in **Figure 7.7**. The presence of α -lactalbumin, β -lactoglobulin, caseins, and other whey protein was present after the meltdown, which was also present in the ice cream before the melting.

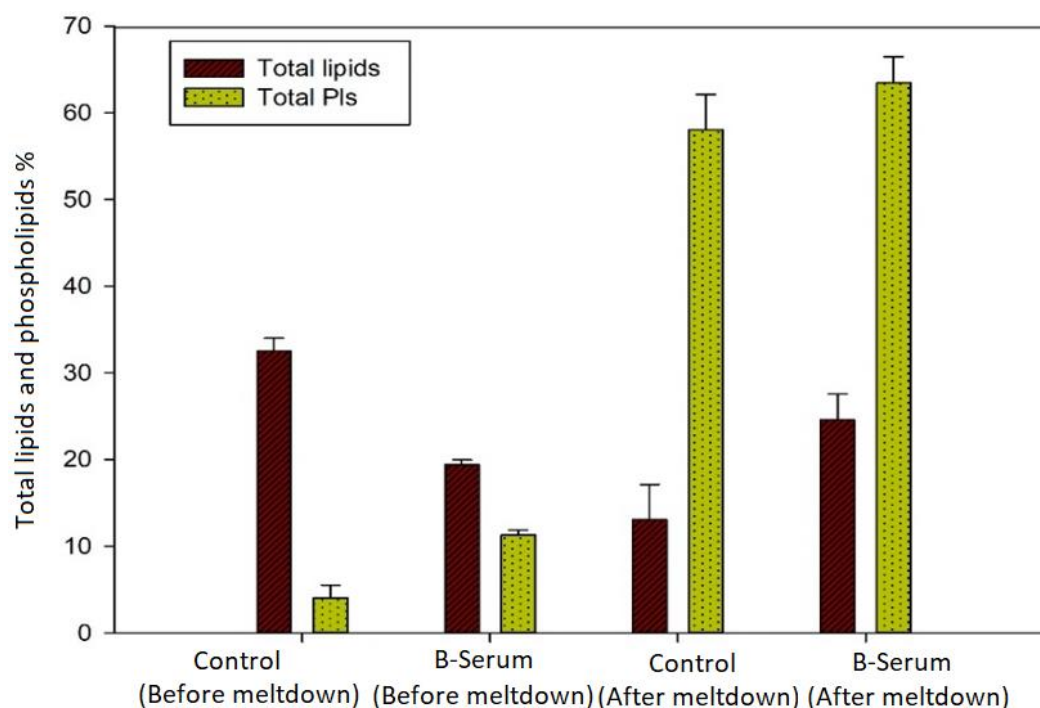


Figure 7.5.Total lipids and phospholipids for control and ice cream before and after the meltdown.

7.4.4. Mapping of PLs

Figure 7.8 and **Figure 7.9** illustrate the recovered total lipids and phospholipids at every stage of the ice cream manufacturing process, such as [mixing (1), pasteurization (2)

and freezing (3)]. In typical ice cream manufacturing, ingredients are pre-blended before pasteurization. This pasteurization process destroys the pathogenic bacteria addition and helps the fat melt for proper homogenization (Goff, Verespej, & Smith, 1999). The homogenization process in ice cream begins the process of fat structure formation. During homogenization, the newly formed fat globule is practically devoid of any membranous material due to its tremendous increase in surface area and readily adsorbs amphiphilic molecules from the solution. They positively influence the physical/chemical condition of proteins, phospholipids, and any added emulsifiers. This creates a uniform fat-in-water emulsion preconditioned to “de-emulsify” (i.e., partial fat agglomeration) as needed.

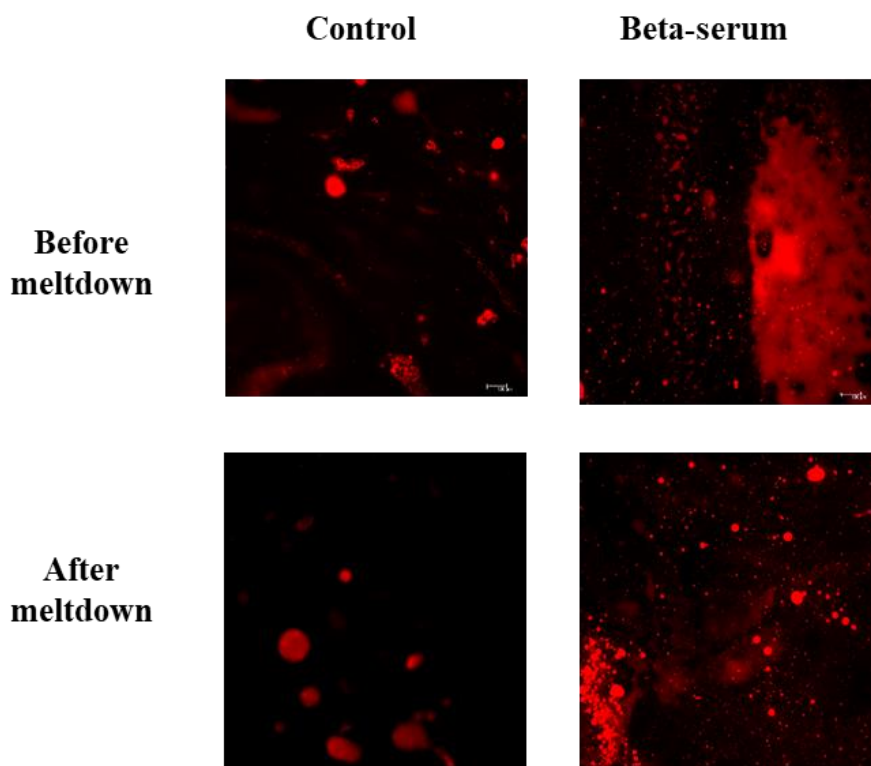


Figure 7.6. Confocal micrographs of the Ice-cream (control and B-serum) before and after a meltdown.

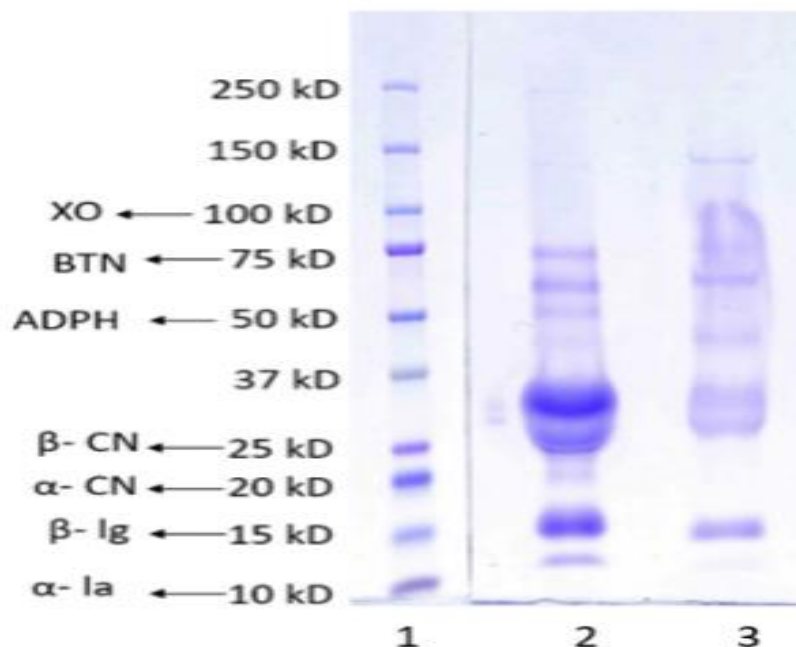


Figure 7.7. SDS- page patterns of melted ice cream. (1) molecular weight standard (2) control (3) B-serum.

The centrifugation resulted in two-phase the top phase, the serum phase and the lower phase, the solid phase. The total lipids recovered from all the stages depicted a higher percentage in the upper phase. However, there was a difference in the amount of lipids from the samples with and without beta serum. From the three different stages 1, 2 and 3, the total lipids observed for the control samples at the upper phase were $55.54 \pm 9.26\%$, $47.69 \pm 2.67\%$ and $51.36 \pm 9.8\%$ respectively, while for the samples that contain beta serum were $30.08 \pm 10.69\%$, 50.82 ± 5.45 and $31 \pm 0.39\%$, respectively. In the case of the lower phase of three stages (1,2,3), the total lipids obtained for the control mixes were $13.71 \pm 4.06\%$, $8.96 \pm 0.12\%$ and $13.6 \pm 2.64\%$ respectively, the beta serum samples contained $7.59 \pm 0.14\%$, $10.26 \pm 0.48\%$ and $15.11 \pm 1.76\%$ respectively.

It was evident that the fat portion remains on the top during the centrifugation resulting in higher lipids; however, there was a difference in the lipidic content for the samples with and without b-serum. The ice cream mix control had comparatively higher lipids in the upper phase in all three stages than the samples with b-serum. The solid phase consists of unabsorbed casein micelles in suspension in a freeze-concentrated solution of sugars, unabsorbed whey proteins, salts and high molecular mass polysaccharides. The fat phase undergoes partial coalescence during the freezing process resulting in an agglomerated fat that partially surrounds the air bubbles giving rise to a solid-like structure. (H. D. Goff, 1997).

Furthermore, through solid-phase extraction, the total phospholipids were obtained. **Figure 7.9** illustrates the amount of phospholipids from the three different stages. It was evident that the ice cream mix with Beta serum contained a higher amount of PLs, and it was noted a higher level of PLs was confined to the lower phase. In the ice cream mix with b-serum, the total PLs at the lower phase for three stages (1,2,3) were $39.48 \pm 0.14\%$, $31.88 \pm 0.48\%$ and $10.14 \pm 1.76\%$, respectively. Simultaneously, for the samples without b-serum, it was observed to be $16.64 \pm 4.06\%$, $17.73 \pm 0.12\%$ and $16.64 \pm 2.64\%$, respectively, the higher PLs of the beta serum mix was depicted after pasteurization, and it was lowered during the freezing. In the upper phase of the mixes containing beta serum for three different stages, the PLs content were $3.10 \pm 10.69\%$, $6.15 \pm 5.45\%$ and $4.62 \pm 0.39\%$ respectively, and the samples without b-serum were $2.28 \pm 9.26\%$, $5.24 \pm 2.67\%$ and $3.74 \pm 9.8\%$ for the three stages respectively.

To support the presence of PLs, the microstructure of the icecream mixes at three different stages and for two phases were evaluated using confocal laser scanning microscopy (**Figure 7.10**). The staining for the PLs using Rd-dope (Gallier et al., 2010) revealed its presence at every stage. The phospholipids droplets have been dispersed within both phases, depicted through the images presented in (**Figure 7.10**). Compared to the control samples, the samples with B-serum showed more droplets of PLs emulsified within the ice cream matrix in both the serum and solid phase. It was also observed that the distribution was not even, this could be because of the lack of homogenization step during the manufacturing process that contributes to the development of the structure(H. D. Goff, 1997).

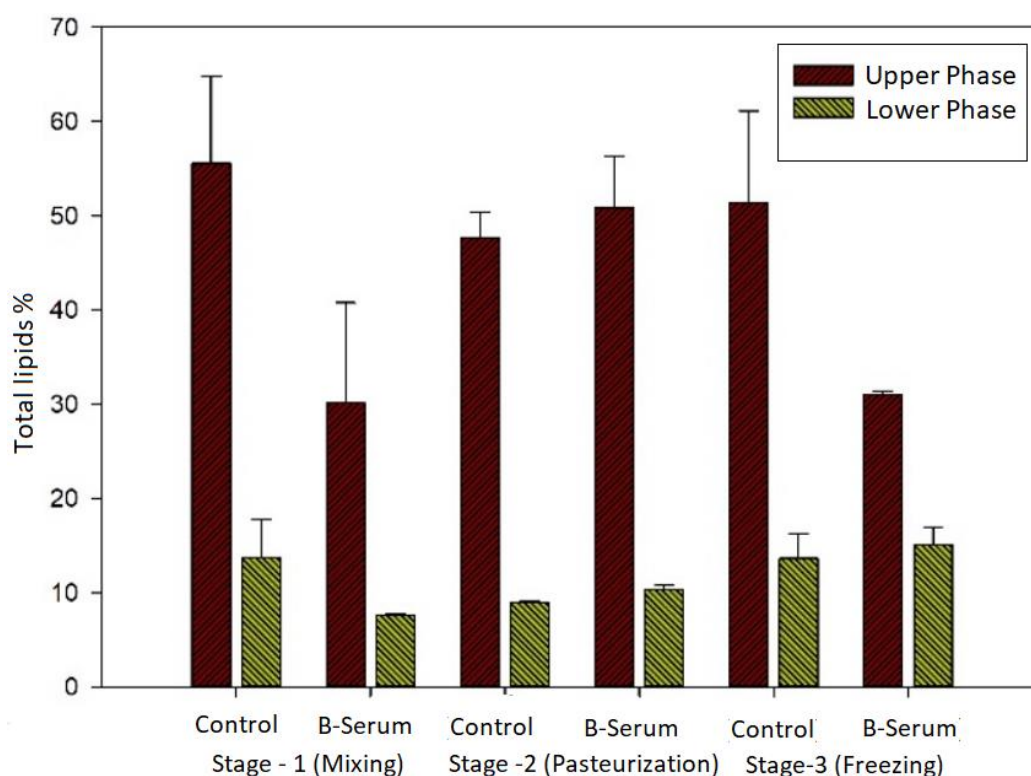


Figure 7.8. Total lipids (wt %) on the three-stage of the ice cream manufacturing process for both the samples control and b-serum.

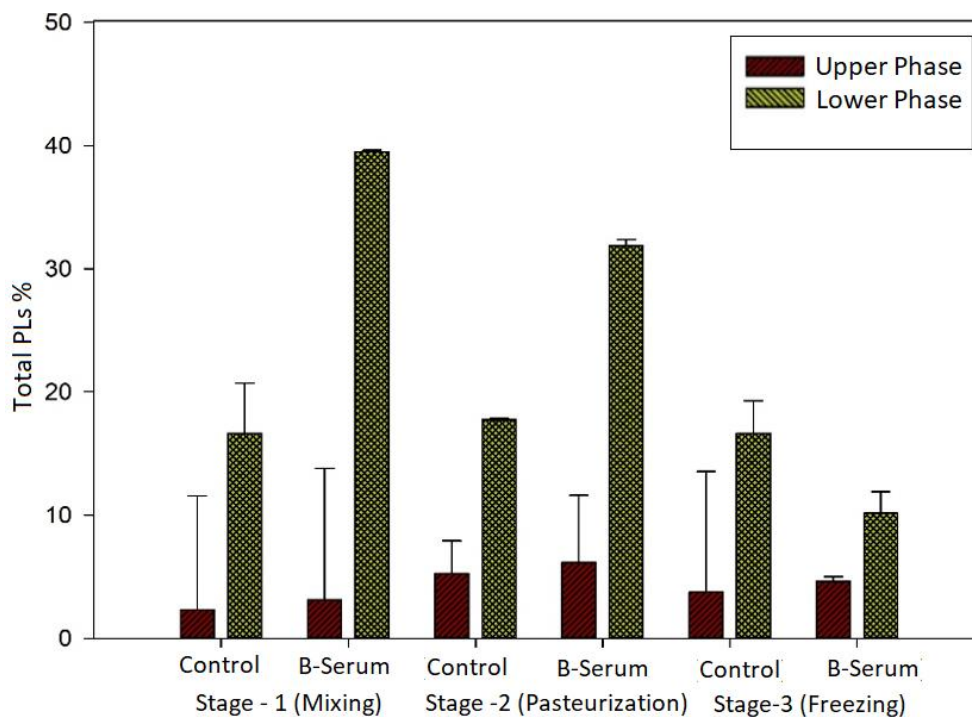


Figure 7.9. Total phospholipids(%) from two samples (1) Mixing (2) Pasteurization (3) Freezing.

7.5. Conclusions

In the present study, Beta-serum powder was assessed for the first time for application in ice creams, one of the most commonly consumed frozen desserts. The quality attributes of the ice cream with non-fat dry milk and beta serum were evaluated. The rheological behavior for both the samples showed predominant pseudoplastic character, and the zeta potential values were below -30 mV for both the ice-cream mixes showed that they were stable. Further studies on the strain sweep measurements will provide more detailed information on the stability of the mix. Fat destabilization was lowest for the ice-cream containing Beta serum and had the lowest melting rate than the ice-cream with NFDM. The microstructure studies show the presence of phospholipids at every stage of the ice-cream manufacturing process . It was also depicted that the PLs were at the lower phase of and

after the meltdown, the presence of PLs was quantified at the melted phase in both the ice-cream samples. This mapping can provide insights into where the PLs is during the ice-cream manufacturing process. Further scale-up will require to study of the sensorial description of the product as well as consumer acceptance.

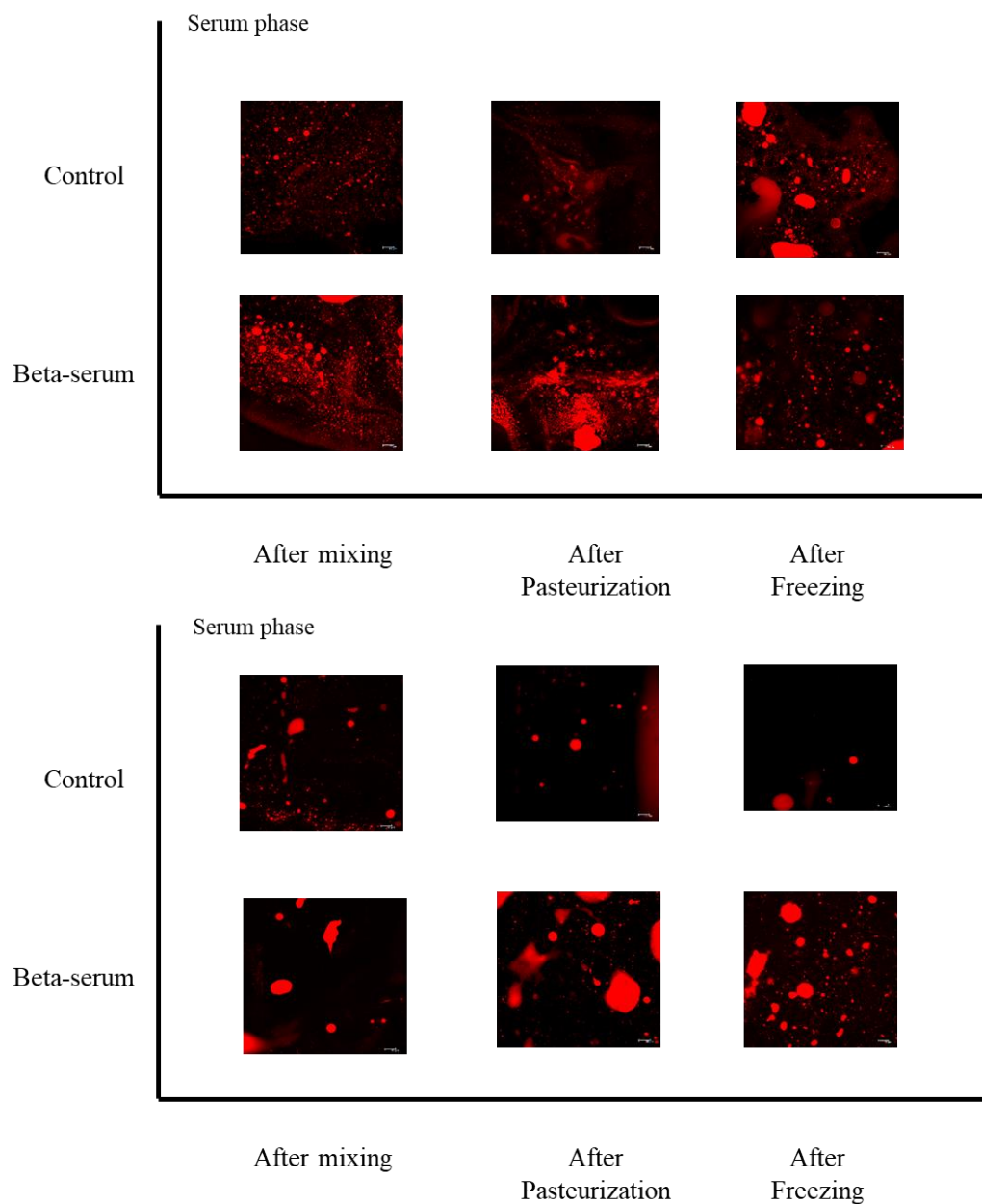


Figure 7.10. Confocal laser scanning microscopy (CLSM) of the different stages of the ice cream manufacturing process.

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CHAPTER 8

OVERALL CONCLUSION

Buttermilk, concentrated buttermilk and Beta-serum, are the low-value dairy by-products that contain large amounts of milk fat globules where PLs are in their membranes. The extraction and separation of the dairy by-products were successfully achieved using the N, N dimethyl cyclohexylamine. For the first time, the feasibility of utilizing CyNMe₂ as a switchable hydrophilicity solvent was an effective way to produce milk PLs from the buttermilk, resulting in a 99% extraction yield when compared to the traditional methods such as the Folch and Mojonnier method. The extraction value of switchable solvents depended on the type of matrix used. The concentrated buttermilk was 77%, and B serum was 7%.

Beta-serum was used in future studies because of its potential interest from the industries. This work demonstrated that ultrasound pretreatment prior to switchable solvent extraction has a great potential for producing valuable PLs from the beta-serum or other similar dairy processing by-products. The application of ultrasound can disrupt the fat globules and enhanced the separation process. The phospholipid recovery was 70%, ten times higher compared to yield with pretreatment and a 31-fold increment in the concentration of individual PLs phosphatidylinositol (PI) was obtained compared to the untreated one.

Optimization for extraction of phospholipids from the beta serum involving different conditions of time, temperature and solvent ratio were utilized. The optimization of the

extraction process aims at maximizing the amount of PLs while minimizing the use of solvent. The different combinations of temperature, solvent ratio and time is an approach to optimize the process and as well as to study the effect of temperature in combination with the switchable solvents. Therefore, manipulating these conditions may lead to obtaining valuable milk phospholipids and understanding the effects these extraction conditions have on the protein and the lipidic material. The temperature did not cause much impact in the process while the time was relevant since the entire relies on the reversibility of amine-based on the Le-chatliers principle. The distribution profile on the individual PLs seemed entirely different. The maximum amount of PLs obtained at minimized solvent ratio (3/1 mL), temperature (60 °C) and time (3 h) yielded $29.29\% \pm 0.06\%$ of MPLs, while only $7.57 \pm 0.59\%$ (12/1 mL and time 18 h). Though this process did not provide a higher extraction yield of the PLs from Beta-serum as the ultrasound process, this work was a proof of concept. The experiments may provide insights for future work with practical or theoretical importance.

An understanding on the mechanism of switchable solvents was investigated to know how the SHS accounted for a higher yield of PLs without the use of polar alcohols. The various analytical measurements obtained revealed a reduction in PLs droplets' size, which favored the diffusion into the solvent and enhanced the extraction. This study will help design novel extraction strategies for the recovery and isolation of phospholipids and other protein matrices.

The beta-serum application in ice cream manufacturing provides insights that dairy by-product beta-serum can be used as replacers of non-fat dry milk replacers ice creams for sustainable and healthy markets. This work can help to effectively utilize the low-value by-products and convert them into a high-value end product. However, evaluation of the sensory studies will be required for consumer acceptance.

The technology utilized in these studies will allow the dairy industry to utilize dairy by-products more effectively and provide methodologies appropriate for industrial scale-up. Further optimization to concentrate on valuable PLs classes, such as sphingomyelin and phosphatidylserine, can better understand these lipidic fractions and processing applications. Additionally, understanding the economics associated with each technological process and its associated costs will provide industrial scale-up insights. Other relevant aspects need further investigation, such as amine recovery, reusability, and percentage recovery of valuable components from reusable solvents can be determined. Further, evaluating the feasibility of different primary and secondary amines in the extraction process of the dairy by-products can be researched. Studies involving the utilization of milk PLs in the agglomeration process as a replacer of soy lecithin can be investigated in the future. The concentration of PLs is a complex procedure, and more alternative methods are required to bridge the gap existing between academia and industry. This technology can be used for low dairy-byproducts to produce dairy lecithin at a very low cost in the future years.