2022

Production of Calcium Reduced Micellar Casein Concentrate Powders and Their Use in Process Cheese Products

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PRODUCTION OF CALCIUM REDUCED MICELLAR CASEIN CONCENTRATE POWDERS AND THEIR USE IN PROCESS CHEESE PRODUCTS

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biological Sciences

Specialization in Dairy Science

South Dakota State University

2022

DISSERTATION ACCEPTANCE PAGE

Anil Kommineni

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 dissertation is dedicated to my loving parents, my wife Prathima, my son Sriram, my loving brother and sister.

Thank you for your love and sacrifice

ACKNOWLEDGEMENTS

I am so thankful to my advisor, mentor, and one of the best persons I have ever known, Dr. Lloyd E Metzger. His guidance, support, and encouragement have made this academic journey such a rewarding experience. In addition, I want to thank my committee members Dr. Vikram V. Mistry, Dr. Joseph Cassady, and Dr. Muthukumarappan Kasiviswanathan, for their help and support.

I am thankful to Dr. Venkat Sunkesula, for your time in reviewing my work. The constructive criticism and timely help in improving my results and manuscript have been precious to this research work. Thank you, Dr. Chenchaiah Marella, who was my teacher mentor and became a lifelong friend who helped me learn and grow. Without his encouragement and guidance, I might not have considered coming to the US for higher studies. Special thanks to Steven Beckman and Dr. Vijay Sundaram for their help with pilot trials and sample analysis. Special thanks to Ahmed Hammam, Achyut Mishra, Suresh Sutariya, Ananya Biswas, John Haberkorn, Akimoto Ichinomiya, Dr. Haridas Meletherayil, Kumar Tammineedi, and Dr. Jayendra Amamacharla for their help and support.

My sincere thanks to my company Whitewave Foods, and Danone North America, which provided tuition assistance. I want to extend my special thanks to my colleagues and supervisors, Dr. Wendy Behr, Keith Johnson, Mary Malone, Pradeep Suriya, and Rosie McGovern, who continuously encouraged me to complete my Ph.D. Finally, I am grateful to the SDSU Dairy and Food Science department, Midwest Dairy Foods Research Center, and Dairy Management Inc for supporting my research project funding.

CONTENTS

ABBREVIATIONS

CGE	Capillary gel electrophoresis
CF	Concentration factor
C-MCC	Control Micellar Casein
CN	Casein
DSR	Dynamic stress rheometer
GP	Graded permeability
MCC	Micellar casein concentrate
MPC	Milk protein concentrate
NCN	Non-casein nitrogen
NFDM	Non-fat Dry Milk
NPN	Non-protein nitrogen
PC	Process cheese
PCP	Process cheese products
RC-MCC	Reduced Calcium micellar casein
RVA	Rapid viso analyzer
SP	Serum protein
SW	Spiral wound
TN	Total nitrogen
TP	True protein
TPA	Texture profile analysis
UF	Ultrafiltration
UTP	Uniform transmembrane pressure
WPI	Whey protein isolate
β-CN	β-casein
αS1-CN	αS1 casein

αS2-CN	αS2 casein
κ-CN	κ-casein
γ-CN	γ-casein
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
tan δ	Tan delta

ABSTRACT

PRODUCTION OF CALCIUM REDUCED MICELLAR CASEIN CONCENTRATE POWDERS AND THEIR USE IN PROCESS CHEESE PRODUCTS

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Protein is an essential dietary component, and sufficient intake is vital in a healthy and balanced diet. Consumers are becoming increasingly aware of and knowledgeable about the role of protein in the diet. Two of the next-generation dairy protein ingredients isolated from milk are micellar casein (MCC) and milk-derived whey protein, isolated from skim milk using microfiltration (**MF**). Membrane filtration has been used extensively by the dairy industry to produce a variety of dairy ingredients from milk. MCC manufactured from freshly pasteurized milk can be directly consumed or as a supplement to fortify and enhance nutritional qualities in processed food products. However, the use of MCC as an ingredient in food applications is sometimes limited due to some of its poor functional properties. Therefore, several researchers studied different ways to improve the functionality of the MCC. One of them is the acidification of milk to solubilize colloidal calcium.

The first objective of this study was to develop a novel filtration method to manufacture MCC with higher soluble casein factions while effectively removing the calcium from acidified skim milk. Although some of the previous studies achieved a 50% reduction in calcium, there might be a loss of soluble caseins through MF permeate when MF-DF is directly applied to acidified skim milk. In addition, acidification of milk with any acid salts is nonreversible, whereas acidification of milk with CO_2 is reversible. Hence an alternate strategy for improving casein functional properties via shifts in protein and mineral distributions is manufacturing modified milk protein ingredients by acidifying the milk

through CO_2 injection, giving a clean label functional MCC. In this study, we have evaluated the novel process of producing calcium reduced micellar casein concentrate powders (RC-MCC) using MF, UF-DF, and injecting the CO_2 into the liquid Micellar casein to lower the pH to 5.7 and maintain the same pH during the UF-DF process. This novel production process resulted in an RC-MCC with 30% less calcium than the C-MCC, retaining higher soluble casein fractions; otherwise, it would have permeated through the MF when only MF is used to produce MCC powders. Furthermore, the retention of serum casein in the resultant RC-MCCs was established quantitively by comparing the ζ-potential and particle size distribution values of RC-MCC and C-MCC powders. We conclude that an additional UF-DF and CO_2 injection step to the current standard only MF process could produce reduced calcium MCCs without losing a lot of serum casein fractions generating because of pH adjustment to solubilize calcium.

The objective of the second study was to evaluate the physicochemical and functional properties of 30% reduced calcium MCCs (RCMCC) produced in the first study to confirm improved functionality of RCMCC powders with higher soluble caseins lower calcium content. This study evaluated the pilot-scale production of calcium-reduced MCC 80 powders using a novel Microfiltration-CO_2 injection-Ultrafiltration process and the effect of the calcium reduction on the physicochemical and functional properties of the RC-MCC powders and dispersions, respectively. In addition, control micellular casein powders (C-MCC) without CO_2 injection were also compared with RC-MCC. This study confirmed significantly improved instant solubility and heat stability of the RC-MCC powders. In addition, reducing calcium was observed to improve foam capacity; however, the emulsions stability and foam stability were lower than control powder dispersions. This could be attributed to smaller particle size and not enough viscosity to retard the coalescence of smaller oil droplets or foam bubbles.

The objective of the third study was to determine if a process cheese product (PCP) could be produced with less emulsifying salts if 30% reduced calcium RCMCC is utilized in the formulation and its impact on the functional properties of PCP. PCP formula made with reduced calcium MCC at 25% less emulsifying salt than control PCP had improved the functional characteristics. Using reduced calcium MCC, PCP manufactured with 25% less emulsifying salts showed a significant decrease in hardness, improved meltability, and optimal viscosity, confirming improved emulsification in the process cheese products. Consequently, this study concluded a 30% reduced calcium MCC powder could be used to partially replace emulsifying salt up to 25% in PCP manufacture.

CHAPTER I

REVIEW OF LITERATURE

1.1 Composition of milk

Milk is a complex heterogeneous colloidal suspension containing many constituents in various forms, such as fat in an emulsion, proteins in colloidal dispersion, and lactose and minerals in the soluble phase. It is an essential source of macronutrients, such as fat, proteins, lactose, and essential micronutrients such as minerals (Haug et al., 2007). Each milk component has its own biological, nutritional, and technological significance. Cow milk contains ~3.25 -3.5 % protein, with about 80% in the colloidal state (Whitney, 1988). Traditionally, bovine milk is consumed as liquid milk and products made from milk such as cheese, yogurt, or ice cream. In addition, bovine milk is also a valuable source for milk-derived ingredients such as milk protein concentrate and whey protein concentrate that are in global demand.

1.2 Milk proteins

Because of their high nutritional value, milk proteins are used in various foods (Morr et al., 1967; Kinsella and Whitehead, 1989). Their functional properties include water binding, viscosity, gelation, emulsifying and foaming, etc. The functional properties of milk proteins in food products are the result of the intrinsic properties of native proteins (amino acid composition and sequence, molecular weight, genetic variation, charge, hydrophobicity, and protein configuration) and extrinsic factors (temperature, pH, salts, and concentration) (Kinsella and Morr, 1984; Henning et al., 2006). Additionally, processing techniques such as heating and drying affect the

functionality of milk proteins in foods. The milk protein fraction consists of two major fractions: caseins and whey proteins. Skimmed milk has Casein (**CN**) and whey protein (**WP**) in the ratio of 80:20. The individual CN fractions (αS1-CN: αS2-CN: β-CN: κ-CN: γ-CN) are in the proportion of 4:1:4:1:0.4, whereas major individual WP (α-lactalbumin (**α-LA**) and β-lactoglobulin (**β-LG**)) are in the ratio of 35:65 (α-LA: β-LG) (Walstra and Jenness, 1984; Swaisgood, 1992; Fox et al., 1998). The typical quantity and molecular weight of protein fractions are shown in Table 1.1. All CNs have a molecular weight (**MW**) of about 20 to 25 kDa, whereas most WP has an MW below 20 kDa (Walstra and Jenness, 1984; Swaisgood, 1992; Fox et al., 1998; Farrell Jr et al., 2004).

Understanding the factors affecting the functional properties of milk proteins has led to the production of milk protein products tailored for specific applications. Accordingly, there are three developmental approaches in milk protein-enriched ingredient manufacturing which can be classified into three major groups: 1) ingredients with a typical ratio of milk constituents (SMP and NFDM, which are devoid of fat, WMP); 2) ingredients with WP only (WPC, WPI); and 3) ingredients with CN only (rennet casein, acid casein, MPC, MCC). In addition, the other categories like fat (cream, AMF, butter oil) and lactose only (lactose powder, deproteinized whey powder) products are also available. The major underlying principles for manufacturing these products involve separation or fractionation, concentration, and drying. Most of these specialized ingredients are dried to preserve functionality and increase shelf life. Added advantages include ease of use and a smaller required quantity to get the desired functionality in the formulation.

1.3 Caseins and properties of caseins

Initially, casein proteins were defined as the fraction of protein that precipitated at a pH of 4.6 and a temperature of 20°C (Jenness et al., 1956). Each protein has distinct physicochemical properties and structure but shares some standard features. All casein proteins have many nonpolar residues that suggest a low aqueous solubility. However, high phosphoryl groups, and low sulfur-containing amino acids counterbalance the nonpolar amino acid residues (McSweeney and Fox, 1998). The tertiary structures are deficient in α-helix or β-sheet structures, making them readily available for proteolysis. Their secondary structure may be intrinsically unstructured (Farrell Jr et al., 2006). Limited secondary and tertiary structure and low sulfhydryl content render casein proteins resistant to thermal denaturation (Fox and McSweeney, 2013). The high proline content in CN leads to a lack of organized secondary and tertiary structures of CN. As a result, CN is heat stable (Huppertz et al., 2004). Due to the high content of CN in MCC, MCC is heat stable at 110°C and pH >6.9 (Sauer and Moraru, 2012). The average size of CN micelles is 0.1 μm, which is around 100 times larger than the SP size. The CN and SP could be separated by different methods based on their sizes. In the prior 1960s, CN was manufactured for industrial applications (e.g., plastic, paints, and glues). In the 1960s, Australia and New Zealand initiated the production of CN for use as a food ingredient; however, CN is widely used nowadays as a functional food ingredient (Huppertz et al., 2004). The characteristics of CN (e.g., amphiphilic, open, and flexible structures) have been used in food systems to provide foaming, emulsifying, and water binding properties (Rollema and Muir, 2009). In addition, these functional properties also provide necessary amino acids to the human body, such as valine, leucine, isoleucine, phenylalanine,

tyrosine, and proline (Pritchard et al., 2010). Also, CN micelles provide the body with calcium, which is essential for bone development. Rennet caseins, acid caseins, caseinates, co-precipitates, and milk protein concentrate are some commercial casein products available today. The production and characteristics of CN products have been reviewed (Rollema and Muir, 2009; Fox and McSweeney, 2013). In addition to these casein products, microfiltration (MF) has been recently utilized to produce a novel casein ingredient called MCC.

1.4 Membrane filtration

The membrane process is among the leading separation techniques nowadays. The dairy industry has seen a surge in membrane technology in the last few decades. Membrane-mediated fractionation separates a stream into two fractions based on the molecular or particulate size exclusion principle. Membrane filtration processes are pressure-driven molecular separation processes to clarify, fractionate, and concentrate milk by forcing milk to stream through the membrane. Larger components retained are called retentate; those which pass through the membrane are called permeate. The separation is determined by the membrane characteristics and the molecular size of the individual components present in the liquid. Membrane filtration changes the volume and/or the composition of a liquid, as the feed is divided into two new liquids of altered chemical/microbiological composition. With the advent of new technologies, protein fractionation and purification are becoming popular. Both processors and consumers are demanding novel ingredients with specific properties and functionality.

The major separation technologies employed in the dairy industry are reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), and microfiltration (MF)

(Henning et al., 2006). RO retains all components except water; UF retains macromolecules, and MF retains micron particles. NF uses charged membranes with pores slightly larger than RO but does not allow the permeation of organic compounds such as sugar (Cheryan, 1998). As a purely physical process, membrane processing can be used for minimally processed foods (Goulas and Grandison, 2008) or as a non-thermal process. Membrane filtration techniques for the manufacture of protein-enriched retentates and powders have provided new ingredients for functionality. Membrane separation technology is currently being used on a large scale to produce a variety of high-protein concentrates. Concentrates are typically used in liquid form or dried for extended shelf life (Schuck, 2009).

Various membrane elements are used in the dairy industry and are broadly classified as those made of polymers (organic) or ceramics (inorganic). Organic membranes are typically spiral-wound elements, and ceramic membranes are typically made as tubular elements. The ceramic element can be operated with permeate back-pressure to achieve a low Transmembrane pressure (TMP), which is crucial for successful results. The TMP is the driving pressure, the pressure difference between the mean pressure on the retentate side (high) and the mean pressure on the permeate side (low or zero). The membrane elements used are of various diameters and lengths. An element designated with the term "3840" means 3.8" diameter and 40" long. The elements can also be divided according to the height of the spacer net, which is designated in "mil" (1/1000 of an inch). Since an increase in protein concentration leads to increased viscosity, the spacer height must be selected accordingly.

UF and MF are widely used in the dairy industry to fractionate, separate, or concentrate skim milk. In UF and MF, only some components are concentrated. The major products manufactured using UF and MF and diafiltration (DF) are MPC and MCC. UF produces MPC with the same ratio of CN and WP as in skim milk, while MF selectively retains CN depending on the membrane selectivity. However, there are changes in the mineral ratios. Proteins are retained, but free ions transmit through the membrane, increasing the CN volume fraction and decreasing the ratio between soluble and colloidal minerals, altering the interaction between the CN micelles (Dalgleish and Corredig, 2012). However, the nativity of the CN micelle is maintained during the processing. Ferrer et al. (2011) reported minimal changes in CN micelles during concentration. However, the amount of CCP remains unchanged unless extensive diafiltration with water or changes in pH occurs. Hence, the CN or WP obtained from UF and MF processes are in their native and original state.

Since CN micelle integrity is maintained, these CNs are different from CN or WP Since CN micelle integrity is maintained, these CNs are different from CN or WP obtained from other processes like rennet or acid coagulation. The CN obtained is a soluble protein with different functional and technological behavior. The ionic strength and water activity remain constant, and protein conformation is almost unaltered (Walstra and Jenness, 1984). Some physical changes like viscosity increase with the concentration factor and become limiting factors in UF or MF. However, diafiltration (DF) is a procedure that involves adding water to the retentate during the membrane filtration process to eliminate, replace, or reduce the content of salts and other soluble elements.

With DF, milk serum components such as lactose and minerals can be removed quickly and conveniently.

There are two major types of DF techniques. Continuous DF involves washing out the salts or other low MW compounds in the retentate as water is added in proportion to the filtrate generated. As a result, the volume of retentate and the concentration of the product do not alter during the DF process. DF via successive dilution begins with diluting the milk to a predetermined volume with water. Membranes are then used to concentrate the diluted material back to its original volume. This DF process is repeated until the unwanted salts or low MW substances are removed. Additional dilution removes more of the small molecules. In the discontinuous DF, the sample is concentrated to a predetermined volume first and then diluted back to its original volume with water and is repeated until the soluble salts and low MW substances are removed. Any additional concentration and dilution remove more of the small molecule. However, DF increases permeate volume, dilutes the components in permeate, and lengthens the processing time.

Membrane separation technologies and selective fractionation affect the total nitrogen (TN) to total solids (TS) ratio and also the CN and WP ratios, which has a profound effect on the functional and rheological properties of the products made (Marella et al., 2011). On the other hand, the concentration of micellar systems by evaporation, UF, or MF does not harm micelle integrity. The native properties of milk components remain largely unaffected by moderate drying conditions (Schuck, 2009).

1.5 Manufacturing of MCC

1.5.1 Microfiltration of milk

MF cross-flow systems have led to the development of novel applications, the removal of microorganisms, clarification, fat removal, and protein separations (Goulas and Grandison, 2008). This membrane technology is used in milk reception, cheese making, whey protein concentration, fractionation of proteins, and effluent treatment (Gesan-Guiziou, 2007). When only bacteria are removed, no fractionation takes place. However, the membrane may partially reject aggregated protein particles/micelles and large fat globules. The production of serum protein (SP) and micellar CN from skim milk can be accomplished using MF. Potential commercial applications exist for both SP and micellar CN. In protein fractionation processes using membranes with 0.1 to 0.2-micron pore size, large proteins (CN micelles) are separated from small soluble proteins (WP). This will concentrate the casein micelles, which may have applications in the production of cheese, fermented products, and beverages. The fractionation process produces 96% CN on the retentate side. The protein concentrate produced using MF has a reduced level of whey protein (10% of the total protein) relative to MPC (Metzger, 2007; Marella et al., 2011) an increased amount of unhydrolyzed or intact CN, which is also called insoluble protein. Whey removed is called native whey and contains GMP attached to κ-CN. The permeate from the MF of SM is free of rennet, culture, color, or lactic acid, which leads to different functional and sensory characteristics (Karasu et al., 2010). It is sterile, and proteins are in native form (Nelson and Barbano, 2005).

1.6 MCC composition and physiochemical properties

There is no standard of identity for MCC. Typically, it is expressed as a function of serum protein elimination, which accounts for around 20% of total protein. (Beckman et al., 2010). As a consequence of membrane separation, ~70 to 90% of serum protein, lactose, and minerals are removed (Hurt et al., 2010). The rejection profile will vary based on membrane composition, diafiltration protocol, and operational parameters. This removal may further contribute to changes in physicochemical properties. Holt et al. has referred the casein micelle as a "functional aggregate." (Holt et al., 2013b). Casein micelles tend to form fibril, planar, and polygonal aggregates. (Glantz et al., 2010, Holt and Carver, 2012). This may be a contributing factor to the high film-forming capacity of MCC. Additionally, apparent viscosity is inversely correlated with serum protein content even at equivalent casein concentration. (Sauer et al., 2012). Sauer suggests that the soluble components (SP, lactose, NPN, and minerals) collectively interfere with casein-casein interactions. This interference causes the inverse correlation, as casein has been indicated as the primary contributor to viscosity. Furthermore, MCC has been purported to reversibly gel when protein concentrations are ≥ 16% (Lu et al., 2015b). The temperature of cold-gelation has been inversely correlated to protein concentration. Gelation of MCC could occur at 16%, 17%, 20%, and 23% protein at 5°, 7°, 28°, and 38 °C, respectively.

Compared to other dairy ingredients (i.e., WPC, sodium caseinate), MCC has a higher ratio of bound water (Schuck et al., 1998). The desorption curve of the bound water, (β) as defined by the slope of the sigmoidal part at the inflection point, is also more significant (Schuck et al., 1998). This indicates that water is slower to be released

from the casein micelle than from globular proteins or when the micelle has been solvated. This is likely due to a film formation caused by a rapid release of water and subsequent tightening of the protein network. Serum proteins, particularly beta-lactoglobulin, have been responsible for the reduced heat stability of milk (Singh and Fox, 1987, Oldfield et al., 1998). The removal of most serum proteins has been suggested to increase the heat stability of the resultant MCC. However, modern high thermal treatments and drying still result in aggregation (Sauer and Moraru, 2012). It is postulated that an increase in casein concentration would correspond with an increased whiteness. The whiteness associated with milk can be attributed primarily to light diffraction by the casein micelle (Kaliappan and Lucey, 2011).

The mineral composition of milk-based systems can dramatically affect viscosity and heat stability. In turn, the casein micelle structure can affect mineral solubility(Bienvenue et al., 2003). Mineral salts in milk are most often phosphates, citrates, sulfates, carbonates, and bicarbonates, with the primary elements associated with them being: sodium, potassium, calcium, and magnesium (Fox and McSweeney, 1998). Other trace elements do exist.

Calcium and inorganic phosphate are critical to the stability of the casein micelle. (Bienvenue et al., 2003) There is a quasi-equilibrium between colloidal calcium phosphate (CCP) nanoclusters and soluble states. This partition is purported to be influenced by changes in pH and temperature. Lower pH and temperatures will shift the equilibrium to a higher soluble phase concentration (Holt et al., 2013a) due to the dissolution of the CCP as it acts as an anchor point for the micelle structure. There is

some resistance to dissolution due to hydrophobic interactions of the casein proteins (Dalgleish and Corredig, 2012).

1.7 Functional properties of MCC

In food applications, ingredient functionality is defined as any attribute other than nutritional characteristics that affect that ingredient's usage in food. Functional qualities of proteins refer to the physicochemical properties of proteins that enable them to contribute to the desirable characteristics of food. Apart from its nutritional importance, caseins are incorporated into food systems due to their diverse functional qualities. Hydrophobicity (as a result of aliphatic and aromatic side chains) and an open, amphipathic structure (as a result of a high proline content) of caseins are critical in determining functional properties such as viscosity, gelation, swelling, foaming, and emulsification, all of which have significant technological implications in the food product. Caseins are excellent film-formers and are used in whipping and foaming applications and fat or oil emulsions. Sodium caseinate is more effective as an emulsifier, thickening, and foaming agent than calcium caseinate and more effectively absorbs water in the food system.

1.8 Methods to modify casein functionality

Caseins and caseinates (sodium, potassium, or calcium salts) are predominantly used in the food sector due to their exceptional functional qualities. Caseinates can be used to enhance the viscosity, structure, and texture of gels and the emulsifying and foaming qualities of a variety of food products (Fox and Mulvihill 1983; Rollema 2003; Dickinson 2006). On the other hand, the functional features of casein micelles have

received less attention and are not widely used in industry. However, it is critical to understand the relationship between their structures and functions to develop new and novel dairy products. Complex interactions exist between the structural and functional features of pure casein molecules or micelles. They can, however, be illustrated schematically in Fig. 2 using intrinsic (casein) and extrinsic (environment) components. All changes to internal or extrinsic factors can alter the functional characteristics of caseins. These alterations were utilized to create a variety of dairy products or to decipher the structure-function correlations of casein molecules in casein micelles. Additional activities or Physico-chemical circumstances can modify the structure and function of proteins (Foegeding and Davis 2011; Chobert 2012). This is particularly true of casein molecules and micelles (Gaucheron 2004a, b; Augustin and Udabage 2007; Dalgleish and Corredig 2012).

Calcium, magnesium, sodium, and potassium are the major cations in the aqueous milk phase, whereas inorganic phosphate, citrate, and chloride are the major anions. Together, these ions produce salts such as calcium phosphate, calcium citrate, and sodium chloride. A portion of them is also free in the ionic state. Considering these various associations and free forms, the aqueous phase of milk at its native pH of 6.6–6.7 is saturated with calcium phosphate. It has an ionic strength of approximately 70 mM. (Holt 1997; Gaucheron 2004a, b; 2005). Casein micelles and the aqueous phase are in dynamic equilibrium. Depending on the physicochemical circumstances, they can interchange casein molecules, calcium, inorganic phosphate, and water with the aqueous phase (Fig. 1). In some instances, ions and casein molecules can be transported from the

micellar to the aqueous phase. Alternatively, these components can be incorporated into casein micelles.

Colloidal calcium removal through acidification is one of the several methods for modifying casein's functionality. The effect of acidifying milk to pH 6.0 and below, where changes in mineral balance are apparent, is well documented in the literature. The pH value affects the structure and charge of casein molecules. Reduced ionization and alterations in intra- and intermolecular interactions are caused by lowering pH. The phosphoryl residues and carboxyl groups change their ionization state during the acidification of caseins because they have an affinity for protons. Their protonation relies on their pKa values. Casein molecules are negatively charged at neutral pH. Hence, Caseins bind to protons during acidification. Casein molecules grow less and less negatively charged until they reach neutrality at their isoelectric pH. Casein molecules cluster at this pH, and their solubilities are negligible. Several hundred publications detail the various changes that occur as pH decreases (De Kruif, 1997; McMahon et al., 2009). Recently several attempts have been made to manufacture micellar casein with lower calcium content, and investigations on the effects of demineralization on the rehydration behavior of casein powders (Schokker et al., 2011; Aaltonen, 2012; Nogueira et al., 2018; Schäfer et al., 2021). The first stage in lowering the calcium concentration of skim milk concentrates is to solubilize the calcium, which is commonly done by acidification, chilling, or adding calcium chelators (LE GRAËT and GAUCHERON, 1999; Broyard and Gaucheron, 2015; Marella et al., 2015). Then, microfiltration (with or without DF mode) removes the solubilized calcium from the retentate along with the permeate. Several DF steps are frequently used to remove as much soluble calcium as possible

(Singh and Bennett, 2002; Schäfer et al., 2018, 2019). Schafer et al., 2019 have evaluated quantitatively and qualitatively different process alternatives for calcium reduction in skim milk retentate.

1.9 Possible applications of MCC

1.9.1 Beverages

MCC could be used in making high protein and low carbohydrate beverages (e.g., sports drinks, meal replacement drinks) due to their high protein content and low lactose content. MCC is also stable for high temperatures without precipitating, so it can be utilized in beverages that need sterilization. Also, MCC has a bland flavor and can provide a good mouthfeel without fat.

1.9.2 Greek-style yogurt

It has been reported that the production and sales of Greek-style yogurt increased remarkably in recent years (Bong and Moraru, 2014). The MCC is a good source for protein for the fortification of the yogurt milk base (Bong andMoraru, 2014) due to MCC's nutritive value and functional properties (Nelson and Barbano, 2005; Affertsholt, 2009; Zhang et al., 2011; Sauer and Moraru, 2012). The protein fortification in yogurt led to a change in the chemical composition of the yogurt milk base, which affects yogurt's rheological and physical properties (Prentice, 1992; Skriver et al., 1999; Lucey, 2002; Peng et al., 2009). MCC at different concentrations of total protein (MCC-58 and MCC-88) have been utilized to fortify yogurt milk to 9.80% protein. The acidification rate was faster in the MCC-fortified Greek-style yogurt than the regular milk, regardless of

inoculation, attributed to the higher nonprotein nitrogen content in the MCC-fortified milk (Bong and Moraru, 2014).

1.9.3 Cheesemaking

MCC is utilized to fortify milk or as an alternative for milk for cheese making. The cheese yield increases when MF is applied to the skim used in cheese making (Papadatos et al., 2003) due to removing the whey. Papadatos et al. (2003) reported the economic benefits of using MF before cheese making, which resulted in the production of valuable co-products from the MF permeates, such as WPI. In addition, the MF permeate is ultrafiltered to utilize the UF permeate as a diafiltrant to increase the removal of SP from skim milk by maintaining the same concentrations of skim milk from soluble minerals, nonprotein nitrogen, and lactose (Nelson and Barbano, 2005). The gel firmness and coagulation time of milk fortified with 4-5% protein solution from MCC powder increased due to the higher calcium content complex with casein and retardation of rennet diffusion in higher protein cheese milk; respectively (Caron et al., 1997).

1.9.4 Low-fat cheese

The main components of low-fat cheese are protein, water, and minerals. The liquid MCC contains casein micelles, water, and minerals, similar to the fat-free portion of low-fat cheese composition. A study has been reported that 45% reduced-fat Cheddar cheese was made by using different protein concentrate powders to fortify milk with 3%, 4%, 5%, and 6% casein with 1.61 ratios of CN to fat (St-Gelais et al., 1998). The fortified

milk with diafiltered MF retentate was higher in the cheese yield than UF retentate and calcium caseinate, especially at 5% and 6% casein. It has been reported that curd made from fortified milk with calcium caseinate did not retain the fat well, increasing the fat content in the whey (St-Gelais et al., 1998). Therefore, the MCC is a valuable ingredient to produce low-fat cheddar cheese with a good structure (Amelia, 2012).

1.9.5 Process cheese

Process cheese has diverse applications and is frequently consumed as an ingredient with other food items. It is available in several forms, such as slices, blocks, shreds, and sauces (Biswas et al., 2008). Each application requires some unique functional properties. Process cheese is manufactured by blending dairy ingredients (various sources of protein, fat, carbohydrates, etc.) and non-dairy ingredients (salt, water, mold inhibitor, preservatives, emulsifying salts, color, flavor, additives, etc.), followed by continuous mixing and heating to extend shelf life (Guinee, 2007; Kapoor and Metzger, 2008; Kammerlehner, 2009). Processed cheese can be viewed as a complex gel with emulsified fat dispersed within a protein network (Bowland and Foegeding, 2001). The major principles for the manufacture of processed cheese involve calcium sequestration, pH displacement and stabilization, para-CN dispersion, water binding, and emulsification (Henning et al., 2006), followed by continuous mixing and heating to extend the shelf life primarily by stopping the ripening of cheeses (Guinee, 2007; Kapoor and Metzger, 2008; Kammerlehner, 2009) and finally cooling the product. The type and level of emulsifying salt, manufacturing parameters and natural cheese characteristics

influence the quality of process cheese (Zehren and Nusbaum, 2000; Kapoor and Metzger, 2008).

Chemically, PC manufacture involves the conversion of insoluble calcium phosphate para-caseinate (rennet cheese or RCN) or CN (isoelectric precipitation; acid casein, unripened direct acidified cheeses) into soluble or hydrated dispersible form (sodium caseinate or sodium calcium phosphate para caseinate) in the presence of emulsifying salts (buffering and calcium-binding salts), heat and shear action while mixing. This helps bind water and emulsify fat to make the cheese physicochemically stable (Guinee, 2011).

According to the CFR, four major categories of PC differ based on fat, moisture, and final pH and the quantity and the number of optional ingredients that can be used (21CFR133.169 to 133.180) (FDA 2006). The four major categories of PC are pasteurized process cheese, pasteurized process cheese food, pasteurized process cheese spread, pasteurized blended cheese, and processed cheese analogs (Henning et al., 2006; Lu et al., 2007; Guinee, 2007; Kapoor and Metzger, 2008; Chandan and Kapoor, 2011). There is another undefined category called pasteurized process cheese products (PCP), which has a composition similar to the various categories of PC; usually contain ingredients not permitted in the various pasteurized PC categories or do not meet the composition targets of the standard cheese categories (Lu et al., 2007; Kapoor and Metzger, 2008). PCP can further be categorized into substitute cheese or imitation cheese (Chandan and Kapoor, 2011). Ingredients such as milk protein concentrate that are not legally allowed under CFR are utilized in the formulation (Kapoor and Metzger, 2008) and are expected to provide similar textural and functional characteristics as found in

other process cheese categories. Since PCP can incorporate non-cheese dairy ingredients, they cost less (Lu et al., 2007; Kammerlehner, 2009) and can be tailored to specific functionality requirements (Lu et al., 2007; Kammerlehner, 2009). Major constituents that affect the quality of PC or PCP are intact CN and WP.

The PC characteristic is significantly affected by the type and amount of protein (Salunke, 2013). The addition of casein or caseinates in PC formulations ameliorates the consistency of PC. It has been reported that intact casein is the most important ingredient in PC formulations. It is selected depending on the type, flavor, maturity, consistency, texture, and pH of cheese (Zehren and Nusbaum, 2000). The PC properties are also affected by the amount of intact casein in natural cheese (Templeton and Sommer, 1930; Vakaleris et al., 1962; Berger et al., 1998; Zehren and Nusbaum, 2000; Piska and Štětina, 2004; Purna et al., 2006; Brickley et al., 2007; Kapoor et al., 2007; Kapoor and Metzger, 2008; Kammerlehner, 2009). Intact casein is the non hydrolyzed CN, which is high in fresh cheese and decreases during the ripening of cheese because of the proteolysis (Purna et al., 2006). Natural cheese and rennet casein are good sources of intact CN for PC Processors balance the ratio of young and aged cheese to have the optimum amount of intact casein in the final PC. Using aged natural cheese (less intact casein) in making PC results in decreasing the firmness and increasing the meltability of PC (Templeton and Sommer, 1930; Purna et al., 2006; Brickley et al., 2007; Kapoor and Metzger, 2008; Kammerlehner, 2009). It has been reported that the melting characteristic of cheese is affected by the interactions between CN molecules (Lucey et al., 2003). The amount of intact casein in cheese, pH, and calcium to CN ratio affect the extent of casein hydration

during PC manufacturing which influences the emulsification degree, CN aggregation degree, and elasticity of PC (Berger et al., 1998; Guinee, 2004).

The age of natural cheese is determined based on the characteristics of the final P.C. Young cheese (75% to 90% intact casein) is used to make block PC with good sliceability and elasticity (Fox et al., 1996), while PC spread is manufactured by using aged or mature cheese (60% to 75% intact casein). The addition of natural cheese in PC formulations increases the softness of the final PC product. Mild cheese is contributed with a high amount in block PC, while medium and aged cheeses are used by a high ratio in manufacturing spread PC (Tamime, 2011). The hydrolysis of αS1-CN in natural stages could be another reason for reducing the PC firmness (Vakaleris et al., 1962; Acharya and Mistry, 2005; Purna et al., 2006; Brickley et al., 2007; Tamime, 2011). Sliceable PC is thicker strands than spreadable PC. (Guinee, 2011) due to the difference in pH and temperature. The stand thickness and elasticity increase with decreasing the pH (Marchesseau et al., 1997) and increasing the holding time at high temperature before cooling (Kalab et al., 1987). This results in changes in the microstructure of PC due to a change in the proportion of protein interaction (Marchesseau and Cuq, 1995; Guinee, 2011). Protein-based interactions that occur during PC manufacturing produce a strong protein network with low flow characteristics (Purna et al., 2006). CN is used to form a gel network in many applications (Ann Augustin et al., 2011), and CN.provides unmelted firm texture and a stringy melted texture (Purna et al., 2006; Brickleyet al., 2007; Metzger, 2007; Kapoor and Metzger, 2008; Kammerlehner, 2009; Chandan and Kapoor, 2011). As a result, CN is more valuable in PC manufacturing (Metzger,2007). The fully ripened or too old natural cheese has a minimum amount of intact CN which results in the

loss of emulsifying characteristics due to the high amount of hydrolyzed protein (Chambre and Daurelles, 2000; Brickley et al., 2007).

1.10 Functional properties of process cheese

Depending on its end-use application, the desired functional properties of PC can be grouped into two major categories: unmelted texture and melted texture properties. In addition to individual functional properties, certain PC applications also require optimal interaction between melted and unmelted textural properties. Consequently, each processed cheese product's required functional properties are unique (Kapoor and Metzger, 2008). Various researchers have devised numerous empirical and instrumental techniques to evaluate and quantify the functional properties of processed cheese. Due to an array of options in ingredients, formulations, and processing conditions, manufacturers have numerous possibilities for producing processed cheese with different physicochemical properties (Kapoor and Metzger, 2008). Highly significant relationships between instrumental and sensory textural characteristics have been found in processed cheese over various compositions (Everard et al., 2007). Everard et al. (2007) varied the ratio between moisture and protein and found that the moisture-to-protein ratio influenced all instrumental and sensory parameters.

PC can be described as a viscoelastic material (Gunasekaran and Ak, 2003). The functional properties control its deformation and flow behavior when subjected to external forces. Texture profile analysis (TPA) is extensively used for measuring un-melted textural properties such as hardness, adhesiveness, springiness, cohesiveness, and gumminess. The constant cross-head speed leads to both forces–time and force-distance curves, and the work done to deform the cheese can be calculated (Breene, 1975). Breene

(1975) defines TPA hardness as a measure of the unmelted texture of a cheese that describes the firmness of the cheese. Drake et al. (1999) found a good correlation between G', G" and TPA hardness. Gupta and Reuter (1993) concluded that the lower penetration values of PCP with the increase in WPC solids are largely due to increased WP that become denatured upon subsequent heat processing.

1.10.1 Rapid visco analyzer viscosity

The Rapid Visco Analyzer (RVA) has been successfully used in small-scale manufacturing of PC (Metzger and Leman, 2001; Kapoor et al., 2004; Kapoor and Metzger, 2005; Prow and Metzger, 2005). The RVA is a computer-integrated instrument developed by Newport Scientific (Warriewood, Australia) to determine the viscous properties of foods. The RVA can measure apparent viscosity over variable shear and temperature conditions as defined by the operator. Kapoor and Metzger (2005) found a satisfactory correlation with the functional properties of PCF produced on the pilot scale and manufactured using RVA treatments. Additionally, the end apparent viscosity during RVA manufacture correlated with the functional properties of the PC. The RVA can be used as small-scale manufacturing and analysis tool to predict PC's functional properties and evaluate how various formulations and processing parameters affect these functional properties.

Moreover, adjustments in the RVA methodology can produce processed cheese with functionality similar to that produced in the BTS (Kapoor and Metzger, 2005). RVA hot viscosity and time at melting peak are measures of cheese meltability (Metzger and Leman, 2001). Prow and Metzger (2005) optimized the RVA to measure the melted

textural properties of PC. Using the R.V.A., Prow and Metzger (2005) developed a methodology to continuously measure the apparent viscosity of processed cheese during heating, holding, and cooling. The minimum apparent viscosity (hot apparent viscosity) of the PC was measured at the highest temperature, as well as the time required for the cheese to reach an apparent viscosity of 5000 cP (time at 5000 cP) during the cooling stage. According to Prow and Metzger (2005), the hot apparent viscosity measures how well the cheese flows when wholly melted; the time at 5000 cP is how quickly a melted cheese thickens during cooling. There was a good correlation between the hot apparent viscosity and time at 5000 cP with process cheese melt properties as determined by DSR, the Schreiber Melt Test, and the Tube Melt Test. Garimella Purna et al. (2006) used the RVA to study the effect of natural cheese age, trisodium citrate concentration and mixing speed on PCF functionality. The results demonstrate that natural cheese age, mixing speed during manufacture, and concentration of TSC have a significant impact on process cheese functionality (Garimella Purna et al., 2006). Changes in VAM were attributed to intact CN levels. Because young natural cheese has a larger amount of intact CN than mature natural cheese, processed cheese manufactured with young natural cheese should have more extensive protein-protein and protein–fat interactions and thus a higher viscosity at the end of manufacture. Berger et al. (1998) pointed out that processed cheese manufactured with young cheese will have longer protein strands and more protein-protein interactions than processed cheese manufactured with ripened cheese; this results in cheese that thickens faster during cooling. Similar observations were reported by Garimella Purna et al. (2006).

1.10.2 Meltability

Meltability is an essential functional characteristic for PC or IMC applications in pizza. Various methods, including the Schreiber Melt test, Tube Melt test, Dynamic stress rheology (DSR), or RVA melt test, have been used to study meltability of PC or IMC. The ingredients used in the formulation affect meltability. The DSR is a fundamental method for determining the rheological properties of viscoelastic materials. DSR measures the viscoelastic properties of processed cheese (Drake et al., 1999; Gunasekaran and Ak, 2003). The melted textural properties or meltability of cheese refers to the ease and extent to which the cheese will melt and spread/flow upon heating (Gunasekaran and Ak, 2003). DSR determines the storage modulus (G′), which measures the energy stored and subsequently released per cycle of deformation; the loss modulus (G″), which measures the energy dissipated per cycle of deformation; and the tan δ (which is G″/ G′) (Gunasekaran and Ak 2003). Sutheerawattananonda and Bastian (1998) developed a DSR-based method for processed cheese that heats the sample and utilizes DSR to measure the G', G", and the melting temperature. With the increase in tan δ, the material reacts to external stress relatively more viscous and less elastic. The modulus value at tan δ=1 (45° phase angle) is known as crossover modulus, where the material has equal solid and liquid-like characteristics. If tan δ is less than one, the material is more elastic; when tan δ is more than one, the material is more viscous (Gunasekaran and Ak, 2003). The loss modulus is generally more associated with fat and moisture content in cheese, whereas the storage modulus is related to the protein network matrix structure (Subramanian et al., 2006). Lucey et al. (2003) reported that the hydrolysis of both αs1- and β-CN during storage could increase the meltability of all cheeses by weakening the

number and the strength of the protein-protein interactions between CN molecules. The DSR melt temperature and RVA melt test measure the initial melt characteristics of processed cheese. A short melt time indicates easy meltability (Prow and Metzger, 2005). The RVA Melt viscosity (RVA hot viscosity) and time at 5000cp measure processed cheese flow properties. Cheese flowing easily after melting will have low RVA hot viscosity (Prow and Metzger, 2005). RVA Melt viscosity is a measure of the apparent viscosity of melted process cheese. Metzger (2007) reported significantly higher apparent viscosity after manufacture in PCP using CN-enriched protein concentrate (CEPC) compared to those produced from M.P.C. Garimella Purna et al. (2006) observed a decrease in the RVA Melt viscosity of PC manufactured with higher ripened cheeses and attributed it to the hydrolysis of the intact casein in the natural cheese during ripening, which in turn results in weaker protein-protein and protein–fat interactions and lower melted viscosity.

During heating of cheese, softening occurs. The viscous modulus may become greater than the elastic modulus when flow occurs (Lucey et al., 2003). A critical level of energy stored and dissipated within the cheese mass allows the CN network to melt and flow during heating (Lucey et al., 2003; Biswas et al., 2008). High protein in PC has been associated with high G' and G" (Joshi et al., 2004). The extent of protein-protein and protein–fat interactions in processed cheese is dependent on the characteristics of the protein (mainly CN) that forms the structure and framework (Guinee et al., 2004). The presence of intact (unhydrolyzed) CN results in extensive protein-protein and protein–fat interactions and a fibrous CN network, whereas the presence of hydrolyzed CN results in weaker protein-protein and protein–fat interactions and a nonfibrous CN network

(Taneya et al., 1980; Garimella Purna et al., 2006; Brickley et al., 2008). When cheese is heated, there is a dramatic decrease in the total number and/or strength of bonds in the cheese matrix, which is indicated by the steady reduction in the dynamic moduli and an increase in tan (Taneya et al., 1979; Horne et al., 1994; Guinee et al., 1999; Lucey et al., 2003).

This change in the dynamic viscoelastic parameters indicates that cheese changes into a more viscous-like material at elevated temperatures. Part of the initial softening of cheese at 40°C is related to the melting of fat, but the major overall effects relate to CN-CN interactions. This suggests that melting is primarily determined by the number and strength of the CN-CN interactions (Park et al., 1984). Increased protein concentrations have been shown to reduce PC meltability (Mleko and Foegeding, 2000; Everard et al., 2007). Lee et al. (2004) and Lee and Klostermeyer (2001) found that the storage modulus of PC spreads increased with an increase in pH. Increasing the concentration of CNs in the cheese matrix increases the intra- and inter-strand linkages. The matrix displays greater elasticity and is more difficult to deform (Fox et al., 2000). The increasing protein content increases the protein-protein interactions, resulting in a denser network. After cooling, the role of proteins in the texture of the final product dominates, resulting in products with increased viscoelastic properties and more solid-like behavior (Dimitreli and Thomareis, 2007). A simultaneous decrease in its elastic and viscous moduli facilitates the flow of the cheese mass (Taneya et al., 1979; Guinee et al., 1999). There is a critical level of energy stored and dissipated within cheese mass that allows the CN network to melt and flow during heating (Lucey et al., 2003; Biswas et al., 2008).

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1.12 Figures

Fig. 1.1 Schematic exchanges of minerals, water, and casein molecules as a function of different physico-chemical conditions (from Gaucheron 2004b)

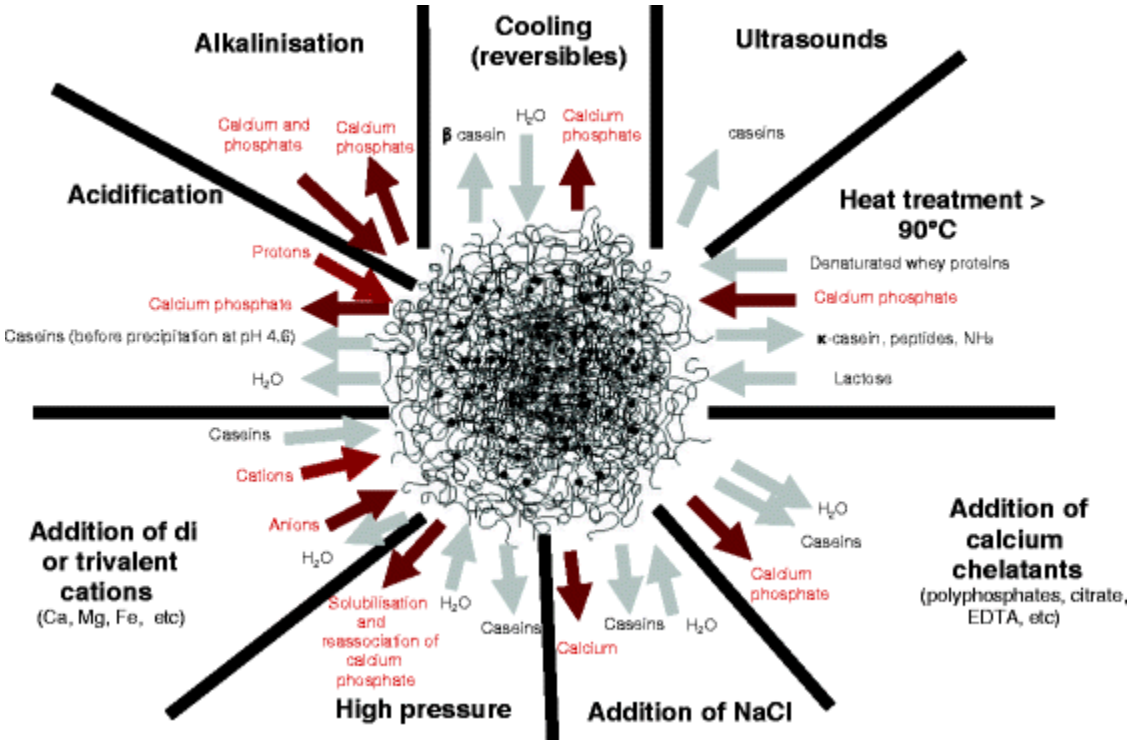


Fig. 1.2 Schematic relationships between structure and functionality of proteins (Broyard et al., 2015)

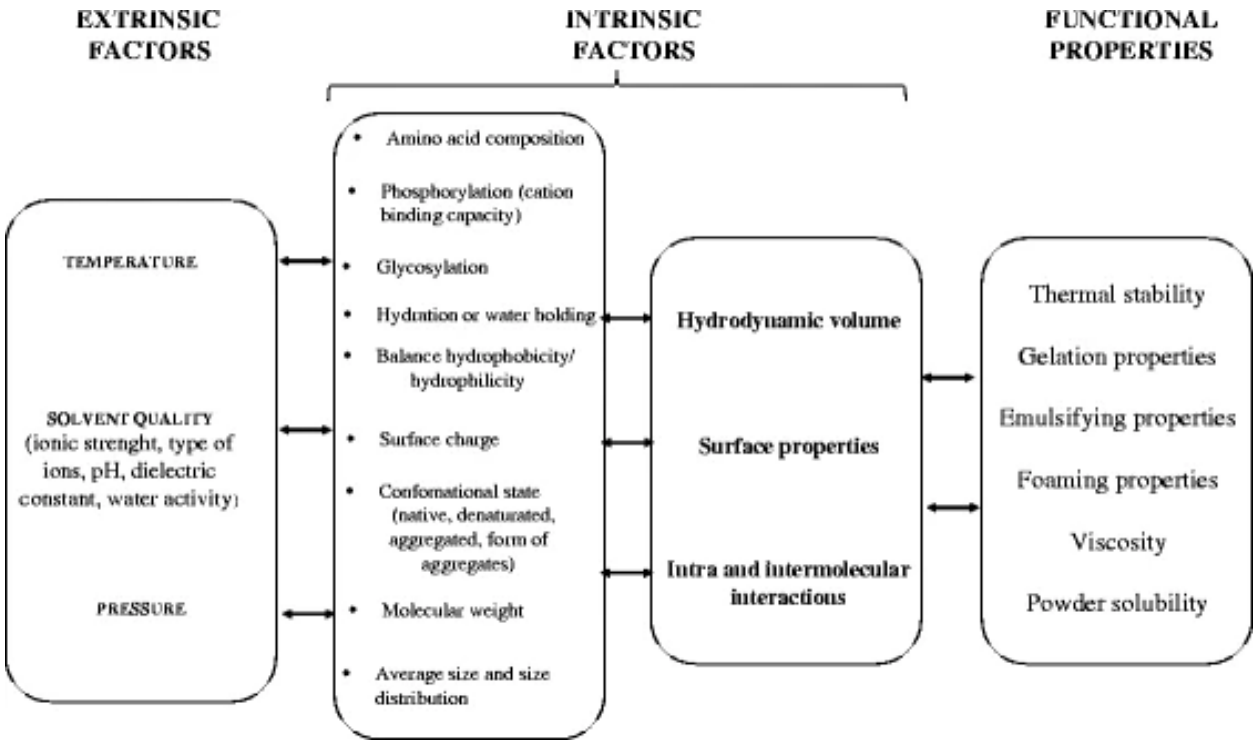
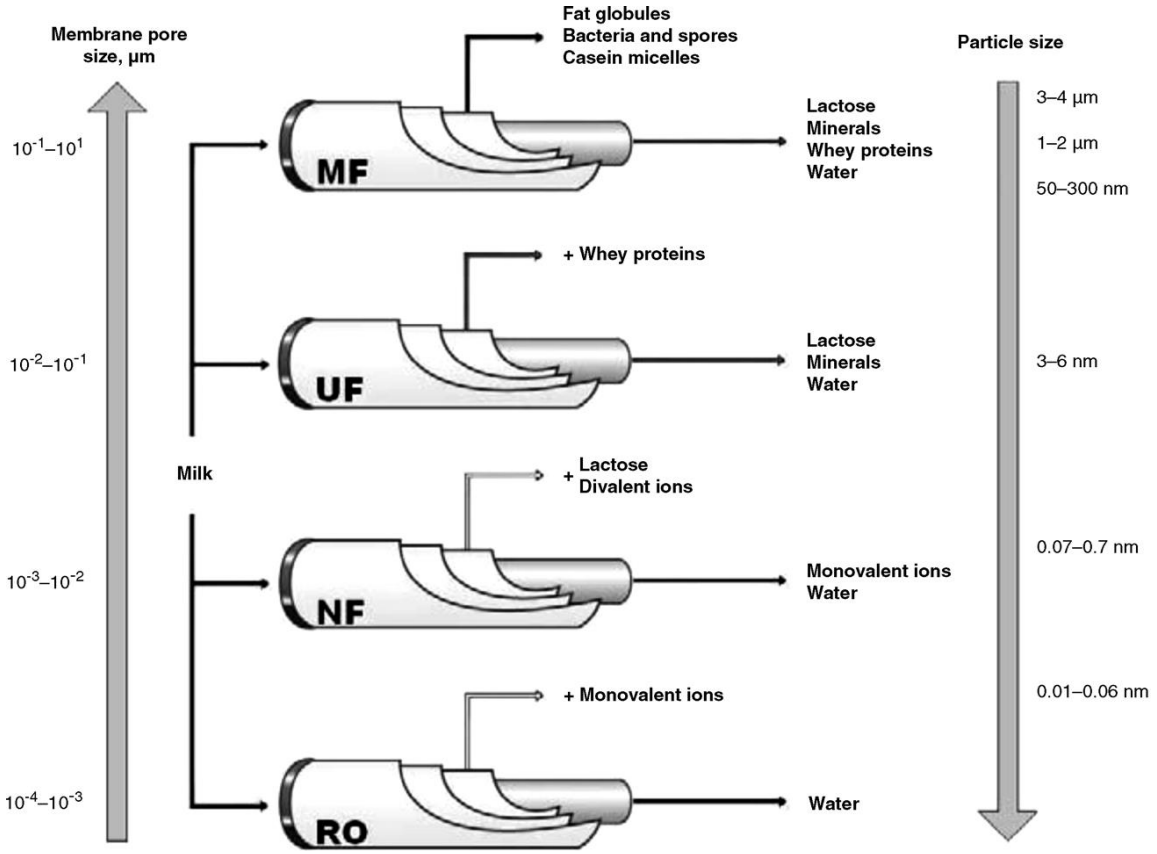


Figure 1.3 Milk processing with membrane technology (Adapted from Lipnizki, 2010).



1.13 Tables

Table 1.1 Quantity and molecular weight of protein fractions

Protein fractions	g/L	% of TP	MW (Daltons)
Total protein	33-34	100	
		79.5	
Total casein	26-27	- 80.5	
αS1-CN	10.0	30.6	23600
β-CN	9.3	28.4	23983
αS2-CN	2.6	8.0	25200
κ-CN	3.3	10.1	19550
γ1-CN	0.8	2.4	20500
γ2-CN			12300
γ3-CN			10300
Total whey protein	6.3 - 7.3	19.3 - 20.3	
α-LA	1.2	3.7	14176
β-LG	3.2	9.8	18283
BSA	0.4	1.2	66267
IgG1	0.7	2.1	150000
IgG2			150000
IgA			385000
IgM			900000
PP	0.8	2.4	4000
PP5			12176
PP			40000
Tf			76000
Lf			86000
Non protein nitrogen		5.1-6.0	<4000

Walstra and Jenness (1984)

CHAPTER II

PRODUCTION OF REDUCED CALCIUM MICELLAR CASEIN CONCENTRATE – MCC80 PROTEIN POWDERS

ABSTRACT

Typically, micellar casein concentrate (**MCC**) is manufactured by microfiltration (**MF**) of skim milk. Injecting CO_2 into skim milk during ultrafiltration (**UF**) has become a common practice to produce reduced calcium variants of milk protein concentrates (**MPC**) with better functional properties. However, the soluble portion of the caseins that are generated because of the reduction of pH during CO_2 injection, may permeate through MF, thus limiting the ability to produce reduced calcium micellar casein concentrate (**RC-MCC**) using the standard MCC process. The aim of this study is to evaluate the method of using a combination of MF, UF, and diafiltration (**DF**) along with CO_2 injection to control the loss of the soluble casein fractions and achieving the calcium reduction simultaneously. In addition, a control MCC powder (**C-MCC**), using only MF, UF-DF, and without injection CO_2 is also produced. Using this novel method, a significant calcium reduction (approximately 30%) was achieved in RC-MCC powders compared to C-MCC powders. The particle size reduction data indicated that by using the UF process, it is possible to produce reduce RC-MCCs without losing much of the serum phase casein fractions that are produced during pH reduction by injecting CO_2 to remove calcium in the final MCCs.

Keywords: micellar casein concentrate, microfiltration, ultrafiltration, CO_2 injection, reduced calcium micellar casein concentrate.

2.1 Introduction

Due to milk protein's unique functional properties such as water binding, viscosity, gelation, emulsifying, foaming, milk proteins are used in various food applications. Caseins make up around 80% of milk proteins and precipitate at its isoelectric pH of 4.6 (Walstra and Jenness, 1984). Caseins are by far the most essential and valuable component of milk from a product, technological, and industry standpoint (De Kruif et al., 2012). The textural, sensory, and nutritional qualities of liquid milk, cheese, and yogurt are derived from caseins. Because of their lack of complicated secondary and tertiary structure, these proteins exhibit good surfactant capabilities in emulsions and foams, gelling properties, and thermal resistance to denaturation (McSweeney and Fox, 1998). In addition, micelles of casein may survive mild heat and cold temperatures without aggregation or structural disturbance (Dalgleish and Corredig, 2012). Because of all these benefits, caseins are widely employed in various dietary applications due to their functional qualities, including stabilization, water binding, heat, and acid stability (Singh, 2004; Broyard and Gaucheron, 2015). Examples are whiteners and creamers for coffee, whipped toppings, processed meat products (for binding and emulsification), cultured products, and desserts. Caseinates (sodium, potassium, or calcium) are widely utilized in the food sector for their functional qualities. Caseinates can help improve the viscosity, structure, and texture of gels and certain foods' emulsifying and foaming capabilities (Fox and Mulvihill, 1983; Dickinson, 2006; Rollema and Muir, 2009).

Micellar casein has similar compositional and physical qualities to native casein micelles in milk (O’Regan and Mulvihill, 2011) which is commercially produced by MF

of skim by filtering out most of the soluble components, serum protein (**SP**) and non-protein nitrogen fractions, thereby increasing the ratio of casein to total protein (Marella et al., 2021). The MF and UF are two major membrane separation technologies used in the dairy industry. These two processes differ in membrane characteristics, pore size, and operating pressure. During UF, all the proteins are retained, whereas MF selectively retains casein (**CN**) and permeates SP. MF is used to manufacture both liquid, and dried MCC. The DF is a process where the retentates obtained from UF and MF are diluted with water to improve the separation efficiency and achieve higher purity fractions by permeating more impurities. In dairy ingredients processing DF is more commonly applied to enhance the permeation of lactose and minerals salts, thus allowing better control of the final composition of the product (Baldasso et al., 2022; Coşkun et al., 2022).

Some recent studies have detailed the chemical composition of MCC powders processed with MF (Schubert et al., 2018; Thienel et al., 2018; Schäfer et al., 2019) : 95-97 percent (w/w) dry matter, 70-74 percent (w/w) casein, 12-15 percent (w/w) lactose, 1.5 percent (w/w) fat, 0.2-0.4 percent (w/w) whey proteins, and 2.3-2.6 percent (w/w) calcium were recorded in MCC powders. Because most of the calcium in milk is micellar bound, the calcium content of MCC grows as the casein content rises. This rise in calcium is proportional to the increase in protein content, for example, from 1120 mg kg1 at 3.5 percent (w/w) protein content to 2850 mg kg1 at 8.5 percent (w/w) protein content (Adams et al., 2015; Jørgensen et al., 2015; Schäfer et al., 2018).

Colloidal calcium removal through acidification is one of several methods for modifying casein's functionality. The effect of acidifying milk to pH 6.0 and below,

where changes in mineral balance are apparent, is well documented in the literature. The pH value affects the structure and charge of casein molecules. Reduced ionization and alterations in intra- and intermolecular interactions are caused by lowering pH. The phosphoryl residues and carboxyl groups change their ionization state during the acidification of caseins because they have an affinity for protons. Their protonation relies on their pKa values. Casein molecules are negatively charged at neutral pH. Hence, Caseins bind to protons during Acidification. Casein molecules grow less and less negatively charged until they reach neutrality at their isoelectric pH. Casein molecules cluster at this pH, and their solubilities are negligible. Several hundred publications detail the various changes that occur as pH decreases (De Kruif, 1997; McMahon et al., 2009). Recently several attempts have been made to manufacture micellar casein with lower calcium content, and investigations on the effects of demineralization on the rehydration behavior of casein powders (Schokker et al., 2011; Aaltonen, 2012; Nogueira et al., 2018; Schäfer et al., 2021). The first stage in lowering the calcium concentration of skim milk concentrates is to solubilize the calcium, which is commonly done by acidification, chilling, or adding calcium chelators (LE GRAËT and GAUCHERON, 1999; Broyard and Gaucheron, 2015; Marella et al., 2015). Then, microfiltration (with or without DF mode) removes the solubilized calcium from the retentate along with the permeate. Several DF steps are frequently used to remove as much soluble calcium as possible (Singh and Bennett, 2002; Schäfer et al., 2018, 2019). Schafer et al., 2019 have evaluated quantitatively and qualitatively different process alternatives for calcium reduction in skim milk retentate. In that study, a method was developed for lowering the calcium content of a skim milk retentate (8.5 percent protein, w/w) by more than half (from 2850

mg kg1 to 1360 mg kg1). The following main process steps were advised to produce skim milk retentate with the lowest possible calcium content: (i) skim milk concentration through MF at pH 6.2, (ii) acidification of the corresponding Retentate to pH 5.6, and (iii) serum calcium elution via a MF-DF process.

Although a 50% reduction in calcium is achieved in this process, there might be a loss of soluble caseins through MF permeate when MF-DF is directly applied to acidified skim milk. Furthermore, the type of acidifier used plays a role in the ionic composition of the serum phase, which further affects the functionality of the casein micelle (Broyard and Gaucheron, 2015). (de la Fuente, 1998; Guillaume et al., 2002, 2004) reported that the Ca2+ concentration in the serum phase of the milk was a consequence of the acidifier type used. In addition, acidification of milk with any acid salts is nonreversible, whereas acidification of milk with CO_2 is reversible; hence an alternate strategy for improving casein functional properties via shifts in protein and mineral distributions is manufacturing modified milk protein ingredients by acidifying the milk through CO_2 injection giving a clean label functional MCC. The organic and inorganic phosphate, citrate, and carboxylic residues of caseins become increasingly protonated during milk acidification (or less ionized). At the same time, due to the dissociation of calcium phosphate, the aqueous phase becomes less saturated in this salt. As a result, as the calcium and inorganic phosphate concentrations in the aqueous phase rise, the micellar calcium phosphate dissolves (Visser et al., 1986; Dalgleish and Law, 1989; Mekmene et al., 2010). A dissociation of casein molecules transported in the aqueous phase occurs as a result. The pH level determines the degree to which minerals and casein dissociate. In the standard MF-DF MCC manufacturing process some portion of the dissociated soluble

caseins flows through MF, and this effect could be even higher when acidification of skim milk is utilized to proceed reduced calcium MCC.

Thus, this study aims to develop a novel multistage filtration process containing MF-DF and UF-DF to produce calcium-reduced MCC powder by acidifying the skim with CO_2 injection with an objective of retention of the dissociated soluble casein fractions in the resultant MCC powder. The method developed in this study to manufacture functional casein was based on the recent work published from our lab (Marella et al., 2015) that successfully manufactured 20% reduced calcium MPCs using the UF process with CO_2 injection during filtration.

2.2 Materials and Methods

2.2.1 Experimental design

Both control and treatment Micellar casein concentrates (MCC) were produced in triplicate from three different lots of milk. Final Micellar casein concentrates were made over two days, as shown in Figure 1 and Figure 2. One day -1, the pasteurized skim milk received from SDSU Dairy plant was processed into 9% protein liquid micellar casein concentrate (LMCC-9%) using ceramic microfiltration (MF) in the 1^{st} step. In the 2^{nd} step, LMCC-9% produced in step-1 was diluted with RO water (diafiltration water) to standardize the 2^{nd} MF feed total protein content to 3%. Then, this diluted 3% protein liquid casein concentrate was concentrated to a 6% protein liquid LMCC-6% using ceramic MF in the second step. Finally, LMCC-6 % obtained after the 2nd MF run was diluted with RO water to a 3% protein solution. After two MF runs at 3% protein, the final diluted liquid MCC was pasteurized and stored overnight at 4 °C as PLMCC-3%.

The PLMCC-3% was divided into two equal parts on the second day. As shown in Fig 2., one portion was processed into control liquid micellar casein concentrate (C-LMCC) using ultrafiltration (UF). Then, CO_2 was injected before and during the UF run into the other portion of PLMCC-3% to reduce and maintain the pH at 5.7 for the entire filtration time, to produce treatment liquid micellar casein (T-LMCC). The experiment was designed to have an 80.0% total protein to total solids ratio in the final UF retentates, C-LMCC and T-LMCC. Finally, these control C-LMCC and treatment T-LMCC were spray dried to produce control CMCC and reduce calcium RCMCC powders.

2.2.2 Membranes and filtration operation

Membranes: Membranes utilized during MF and UF processing in this experiment are described in Table 1. The membranes were subjected to a short caustic and sanitization clean before processing. After processing, a full clean-in-place protocol was used, including caustic, enzyme, acid, caustic, and soak steps with rinses between steps. Ceramic membranes were fully cleaned with caustic and acid washes only. Membranes were stored in a soak solution to prevent damage to the membranes.

Filtration operation

Micro-filtration (MF)

Ceramic MF (TIA Microfiltration Unit 7 P 1940 GL – UTP Mineral – 1.7m2 BP 12 Avenue jean Moulin 84501 Bollene Cedex, France)) was conducted at 120°F and 3.25× mass concentration factor (CF_m) in a continuous feed-and-bleed mode, which was controlled by opening and closing the retentate exit and monitoring the mass of permeate and Retentate exiting the system in a set period of time (e.g., 3.0× CF_m = 2 lb. of Permeate

and 1 lb. of Retentate in 60 seconds). Ceramic MF flux (L/m^2 per h, **LMH**) was held constant at 75 LMH during the run by using a flow-controlled automatic permeate exit valve. Pressures (Retentate inlet = 440 kPa, Retentate outlet = 170 kPa) and temperatures (120°F) during MF were maintained using valves and an inline heat exchanger, respectively. Skim milk (38°F) was heated to 120°F in a 300-gal tank using a heating jacket and external water heater before being pumped into the MF feed balance tank. Retentate and permeate from MF were continuously collected, transferred to large jacketed, temperature-controlled tanks for storage. As mentioned, above MF was conducted two times 1^{st} without diafiltration and 2^{nd} time with diafiltration as show on in Fig-1:

Ultra-filtration (UF)

The PLMCC-3% from Day-1 process was collected into a 600 gal jacketed tank after pasteurization and stored overnight under refrigeration at SDSU Davis Dairy Plant. On the following day, PLMCC-3% was split into two equal portions for control and treatment MCC manufacture as shown in Fig -2. The membranes used were listed in the table-1 below. UF was operated at a Base, boost, and inlet pressures of 30, 20, and 50 psi, respectively, and the temperature was maintained at ~70°F using an inline tube-in-shell heat exchanger located in the recirculation loop (Figure 3).

2.2.3 CO_2 Injection

A stainless-steel sparger cup (0.5 x 0.375 x 1 inch) with 10 µ porosity was used to inject CO_2 into skim milk. For injection of CO_2, 3% protein pasteurized liquid MCC (PLMCC-3%) from Day-1 was filled in the balance tank of the UF unit, membrane housings were isolated, and feed pump was used to recirculate the skim milk through the piping of the UF unit (Figure 1). Backpressure of 138 kPa (20 psi) was applied during the recirculation. The pressure of CO_2 was maintained at 527.1 kPa (75 psi) at the inlet to the sparger. The CO_2 flow rate was maintained at 14.1 L/ min, while the PLMCC-3% flow was at 15-18 gallons per minute. During sparging and for one hour equilibration time, the temperature of PLMCC-3% was maintained at 4-5 ^oC (37 - 40 F) utilizing the inline heat exchanger of the unit. The initial pH of PLMCC-3% was 6.90, and after 45 min, the pH dropped down to 5.70. At this point, CO_2 injection was stopped, and the milk was held at this temperature for 60 min. Before the UF, the PLMCC-3% was warmed up to 15 ^oC. During the UF, the pH of the PLMCC-3% was continuously recorded and was maintained at a pH of 5.70 with an additional injection of CO_2 (at a flow rate of 1.5 – 2 L/min).

2.2.4 Spray drying

The concentrated Micellular casein concentrate obtained from the UF process was spray-dried using a semi-industrial scale two-stage spray dryer. The spray dryer was equipped with a high-pressure nozzle. A high-pressure feed pump maintained a feed pressure of 2500 psi. The drying process used a feed temperature of 120 F, Air inlet

temperature of 380 F and air outlet temperature of 170 F. The dryer ran for about an hour and collected approximately 20lbs of MCC powder for each variable and stored at 4C.

2.2.5 Chemical analysis

The final dried MCC powders compositional analysis was done through the wet chemistry method. The total solids (**TS**), total fat, and ash of each sample were determined using standard wet chemistry procedures described by Hooi et al (2004). Total nitrogen (**TN**), non-protein nitrogen protein (**NPN**) and non-casein nitrogen (**NCN**) were determined using micro-Kjeldahl analysis as described by Hooi et al. (2004), except the modified NCN extraction method developed by Zhang and Metzger (2011) was used. The true protein, casein and serum protein was calculated by difference using the TN, NCN and NPN values as described by Hooi et al. (2004). The mineral analysis of the samples was done using ICP-OES (Inductive Coupled Plasma-Optical Emission Spectroscopy).

2.2.6 Particle size and Z-potential

Control and calcium reduced MCC were reconstituted in distilled water to obtain a final solution containing 8% (wt./wt.) protein. The solution was reconstitution at roughly 45°C and kept at 45°C under continual stirring for 30 minutes using a magnetic stirrer. When the pH of the reconstituted MCC was 7.0, it was corrected to 7.0 using 0.5 N NaOH. To achieve a stable emulsion, the pH-adjusted solutions were homogenized at 5,000 rpm for 30 s using a handheld homogenizer (Polytron PT 2500 E, Luzern, Switzerland).

Mean particle size and zeta potential of protein dispersions were measured using a dynamic light scattering analyzer (DelsaMax Assist, Beckman Coulter) using the method described by Anema and Li (2003a) For the measurement, samples were diluted to 1/100, and the diluted samples were injected into the flow cell using a syringe at 20 ± 1°C to obtain mean particle size and zeta potential.

2.2.7 Statistical analysis

The sample treatments and chemical analyses in the present study were run in duplicate. Values were means of replicate determinations, and the differences between the means of the treatments were compared by one-way ANOVA at a significance level of $P < 0.05$. The statistical analyses were conducted using Minitab (v.20, Minitab Inc., State College, PA). Differences between the treatments were tested using Tukey's honestly significant difference (HSD) intervals with $\alpha = 0.5$.

2.3 Results and Discussion

2.3.1 General powder composition

The general composition of C-MCC powders thus produced are summarized in Table 2. The total protein content of both the C-MCC and RC-MCC powders is close to 80%, making the products qualify as MCC80. There was no significant difference between the true protein content (and the casein) in C-MCC and RC-MCC, and the results were 77.81% (71.74%) and 78.99% (72.16%), respectively. Similarly, there were no significant differences in the rest of the composition between C-MCC and RC-MCC powders, i.e., total solids, whey protein, fat, and lactose.

2.3.2 Impact of CO_2 injection on ash and calcium contents of MPCs

Casein micelles are composed of submicelles consisting of casein proteins bound together by hydrophobic interactions and colloidal calcium phosphate (CCP) (Ranadheera et al., 2019). A mineral equilibrium exists between colloidal and serum phases in a stable milk system, which determines casein micelle integrity. However, when CO_2 is injected to the milk system, the reduction in pH disturbs the mineral equilibrium between the colloidal and serum phases by solubilization of CCP and colloidal calcium migrates into the serum phase. The solubilization of colloidal Ca phosphate (CCP) from the casein micelle upon the acidification of milk causes an increase in the content of soluble minerals such as various forms of pH-dependent phosphates and Ca2+ (LE GRAËT and GAUCHERON, 1999; Dalgleish and Corredig, 2012). The rate of solubilization increases at a pH of approximately 5.6 to 5.8 (Li and Corredig, 2014). The mineral composition of MCC powders manufactured in the study is given in Table 3. The total mineral and calcium content in RC-MCC powders (7.06%, 1727 mg/100g) were observed to be significantly ($P < 0.05$) lower than C-MCC (8.15%, 2503 mg/100g). A reduction of 15% in total minerals and 31% in calcium was achieved in RC-MCC compared to C-MCC by injecting CO_2. Additionally, we also observed a 23% reduction in the phosphorous levels of RC-MCC (1267 mg/100g) over C-MCC (1563 mg/100g). As discussed above, injection of CO_2 decreased pH of skim milk which led to solubilization of calcium phosphate nanoclusters associated with casein micelles. The soluble salts were washed out into permeate during the additional ultrafiltration-diafiltration process employed during the production of MCCs and caused the reduction of the mineral and calcium content in RC-MCC powders. However, there was a significant increase in the sodium

content of RC-MCC (700 mg/100g) compared to C-MCC powders (169 mg/100g) which could be attributed to the neutralization of liquid RC-MCC retentates using NaOH before spray drying.

2.3.3 Effect of CO_2 injection on ζ-potential and particle size of casein micelle

Data on ζ-potential of casein micelle in C-MCC and RC-MCC dispersions is presented in Table 4. The ζ-potential of casein micelles in RC-MCC powder dispersions (-36mv) was significantly ($P < 0.05$) higher than those in C-MCC dispersions (-23mV). The ζ-potential indicates the net negative charge casein micelle carries due to the presence of glycosylated residues of the κ-Casein. (Marella et al., 2015). Injection of CO_2 with consequent reduction in pH and solubilization of calcium phosphate resulted in a decrease in net negative charge (ζ-potential) on casein micelle. In the milk system, the cations present in the serum phase can interact with these negative charges shielding the charges on the micelle (Rabiller-Baudry et al., 2005; Huppertz and Fox, 2006; Ahmad et al., 2008). However, partial removal of soluble divalent calcium during the diafiltration step of MF and DF might have interfered with the shielding effect. It could also be attributed to an increase in net negative charge on the casein micelle.

The particle size of C-MCC and RC-MCC dispersions is presented in Table 4. There was a significant ($P < 0.05$) decrease in the diameter of casein micelles in RC-MCC dispersions (236 nm) was observed than C-MCC dispersions (135 nm). (Srilaorkul et al., 1991) reported a decrease in the size of casein micelle due to concentration and attributed this change to variation in the levels of calcium and phosphate. Additionally, the redistribution of mass within the casein micelles and the reorganization of internal structure that occurs during the acidification of PLMCC (liquid MCC) could also be

attributed to the decrease of the size in RC-MCC dispersions. The particle size distribution of C-MCC and RC-MCC dispersions are presented in Fig 4. Because of the CO_2 injection during the production process, the dissociation of casein micelles resulted in a bi model distribution in case of RC-MCC dispersion, whereas in case of C-MCC dispersions, the distribution is a mono model. This proves that using additional UF and DF step with the injection of CO_2 helps retain large position of dissociated casein fractions, otherwise would have permeated when only MF is used like in standard MCC production process.

2.4 Conclusion

In this study we have evaluated the novel process of producing calcium reduced micellar casein concentrate powders (RC-MCC) using MF, UF-DF and injecting the CO_2 into the liquid Micellar casein to reduce the pH to 5.7 and maintaining the same pH during UF-DF process. This novel production process resulted in a RC-MCC that is 30% less calcium than the C-MCC also retaining higher soluble casein fractions; otherwise, would have permeated through the MF when only MF is used to produce MCC powders. The retention of serum casein in the resultant RC-MCCs was established quantitively by comparing the ζ-potential and particle size distribution values of RC-MCC and C-MCC powders. The lower ζ-potential of RC-MCC powders indicates the retention of more serum caseins during the UF process that are produced because of CO_2 injection. This is further substantiated by a bi model particle size distribution (PSD) in the case of RC-MCC powders, whereas C-MCC powders showed a much tighter mono model PSD. Overall, we conclude that an additional UF-DF and CO_2 injection step to the current standard only MF

process could produce reduced calcium MCCs without losing a lot of serum casein fractions generating because of pH adjustment to solubilize calcium.

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2.6 Figures

Figure 2.1. Schematic of day-1 production process of MCC powders

Figure 2.2. Schematic of day-2 production process of MCC powders

Figure 2.3. Schematic of the pilot ultrafiltration unit utilized for MCC production

Figure 2.4. Particle size distribution of C-MCC and RC-MCC dispersions

Figure 2.1

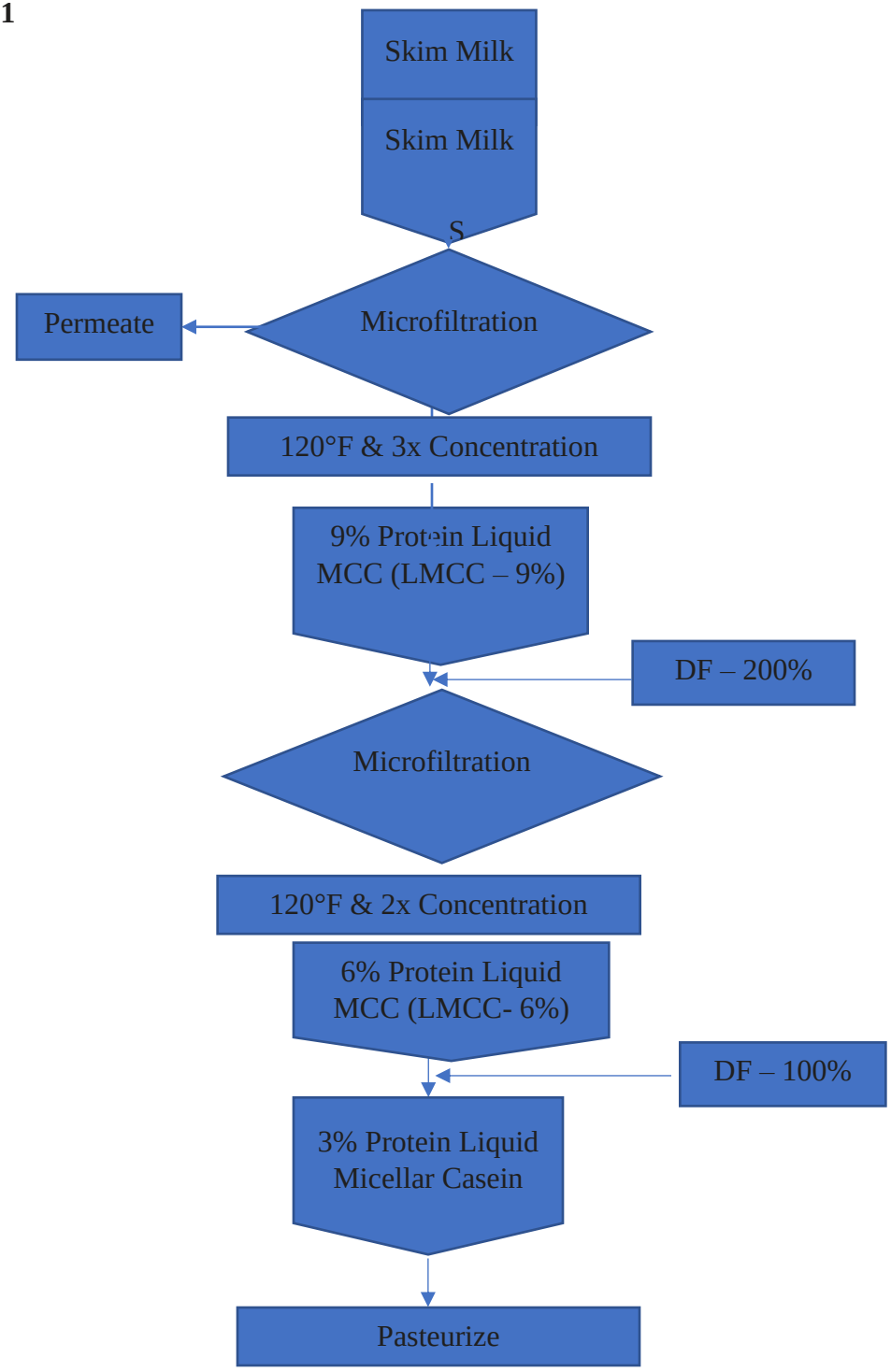

Skim Milk
Skim Milk
S
Permeate
Microfiltration
120°F & 3x Concentration
9% Protein Liquid
MCC (LMCC – 9%)
DF – 200%
Microfiltration
120°F & 2x Concentration
6% Protein Liquid
MCC (LMCC- 6%)
DF – 100%
3% Protein Liquid
Micellar Casein
Pasteurize

Figure 2.2

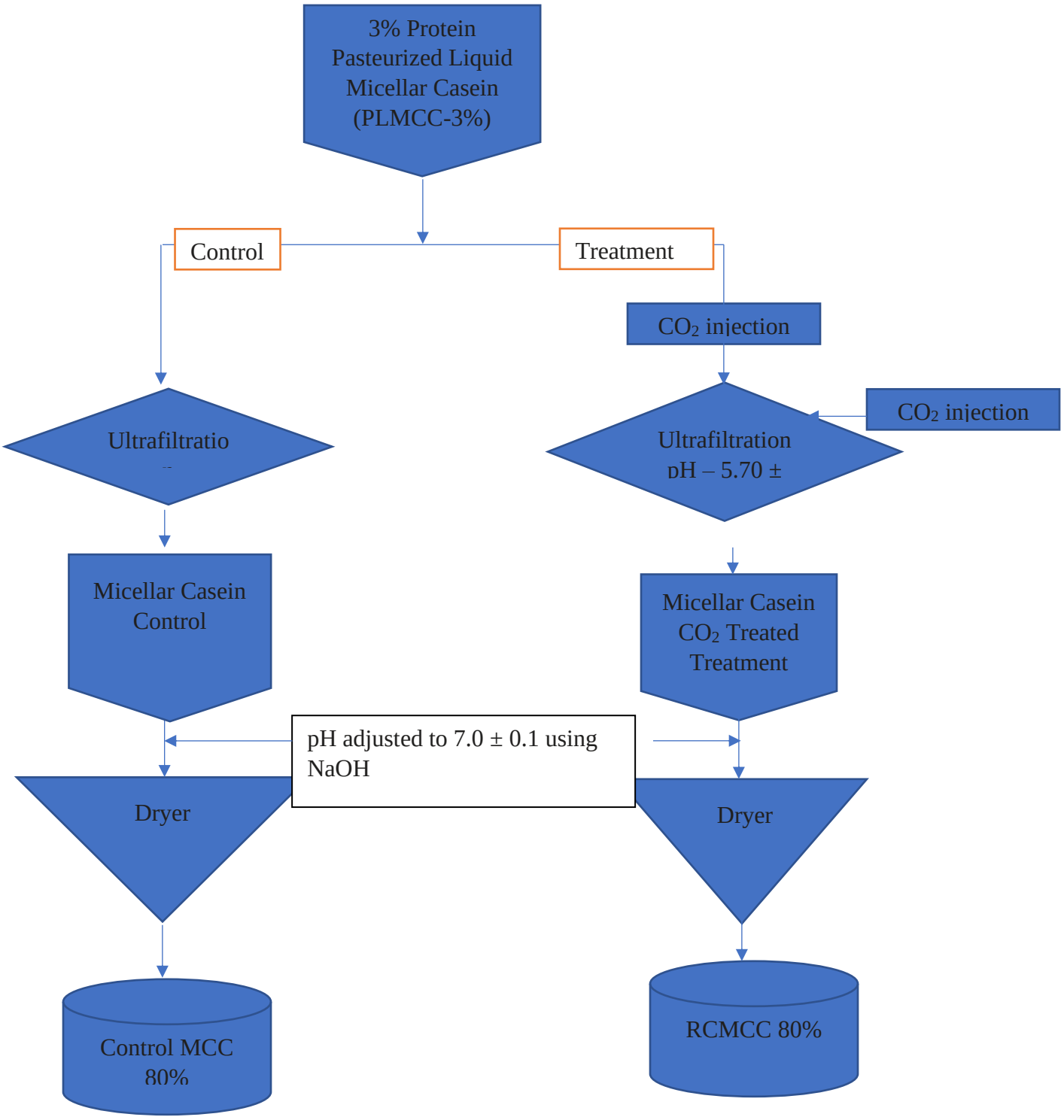

3% Protein Pasteurized Liquid Micellar Casein (PLMCC-3%)
Control
Treatment
CO_2 injection
CO_2 injection
Ultrafiltratio
Ultrafiltration pH – 5.70 ±
Micellar Casein Control
Micellar Casein CO_2 Treated Treatment
pH adjusted to 7.0 ± 0.1 using NaOH
Dryer
Dryer
Control MCC 80%
RCMCC 80%

Figure2. 3

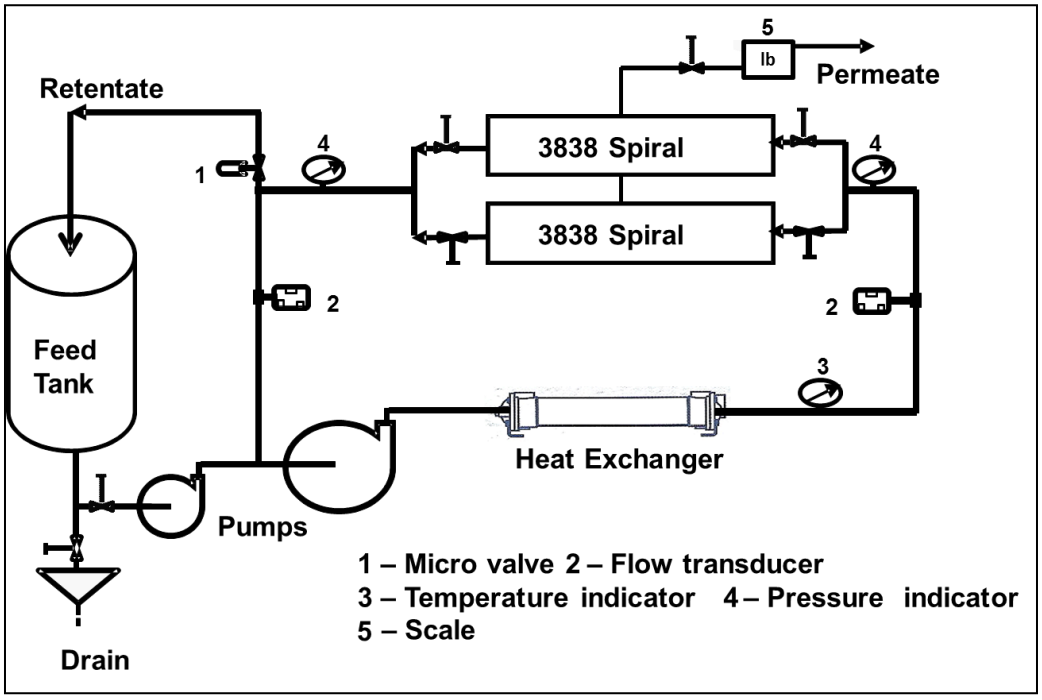
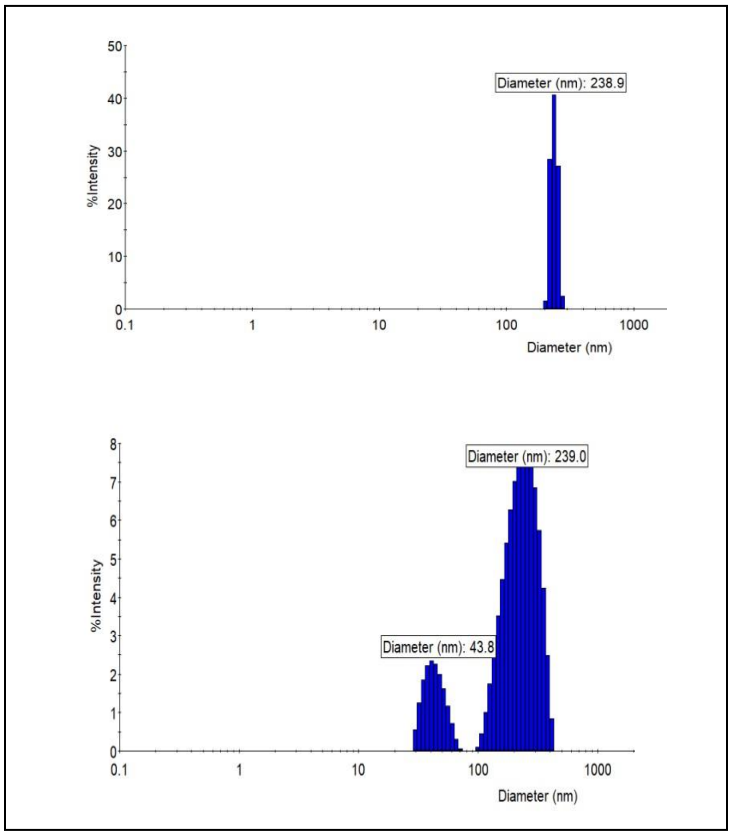

5
lb
Permeate
Retentate
4
1
3838 Spiral
4
3838 Spiral
2
2
Feed
Tank
3
Heat Exchanger
Pumps
1 – Micro valve 2 – Flow transducer
3 – Temperature indicator 4 – Pressure indicator
5 – Scale
Drain

Figure 2.4


Diameter (nm): 238.9
%Intensity
Diameter (nm)
Diameter (nm): 239.0
Diameter (nm): 43.8
%Intensity
Diameter (nm)

2.7 Tables

Table 2.1. Membrane type, description, molecular weight cutoff (MWCO) or pore size, feed spacer thickness or channel diameter, and surface areas used for this experiment.

Type	Description	MWCO (Da) or Pore Size	Spacer Thickness (mil)	Surface Area (m^2)
MF	Pall EP3730 Ceramic GP	0.1 µm	3 mm channel diameter	2.45
UF	Microdyn UP020-3838 PES	20000 Da	47 mil	5.7
	Microdyn UP020-3838 PES	20000 Da	47 mil	5.7

Table 2.2. Mean (n=3) chemical composition of the micellar casein concentrate powders (% w/w)

Powder[1]	Composition							
	Total Solids	Moisture	Total protein	True protein	Casein	Whey protein	Fat	Lactose
C-MCC	95.51 ± 0.11[a]	4.49 ± 0.11[a]	79.25 ± 0.82[a]	77.81 ± 0.88[a]	71.74 ± 1.13[a]	6.07 ± 0.54[a]	2.98 ± 0.41[a]	5.13 ± 0.94[a]
RC-MCC	95.34 ± 0.27[a]	4.62 ± 0.27[a]	80.33 ± 0.72[a]	80.00 ± 0.51[a]	72.16 ± 0.40[a]	6.83 ± 0.30[a]	2.91 ± 0.61[a]	5.08 ± 0.62[a]

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, calcium reduced micellar casein concentrate powder.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 2.3. Mean (n=3) mineral composition of the micellar casein concentrate

Powder[1]	Composition				
	Ash, %	Na, mg/100g	P, mg/100g	Ca, mg/100g	Ca reduction, %
C-MCC	8.15 ± 0.21[a]	169 ± 0.21[a]	1563 ± 0.06[a]	2503 ± 0.09[a]	-
RC-MCC	7.06 ± 0.42[b]	700 ± 0.42[b]	1267 ± 0.05[b]	1727 ± 0.10[b]	31.01 ± 3.39

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, calcium reduced micellar casein concentrate powder, Na, sodium; P, phosphorus; Ca, Calcium.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 2.4. Mean (n=3) ζ-potential and particle size of the micellar casein concentrate dispersions

Powder[1]	Parameter			
	Z-potential (mV)	Diameter (nm)	Particle size, [D,10]	Particle size, [D,50]
C-MCC(d)	-23.23 ± 0.12[a]	236.48 ± 0.76[a]	108.00 ± 1.60[a]	119.40 ± 1.10[a]
RC-MCC(d)	-35.82 ± 1.66[b]	134.80 ± 1.78[b]	27.24 ± 2.25[b]	93.61 ± 3.54[b]

[1] Abbreviations are: C-MCC(d), control micellar casein concentrate powder dispersion; RC-MCC(d), calcium reduced micellar casein concentrate dispersion.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

CHAPTER III

EFFECT OF 30% CALCIUM REDUCTION ON THE FUNCTIONAL PROPERTIES OF MICELLAR CASEIN CONCENTRATE – MCC80 PROTEIN POWDERS

ABSTRACT

The aim of this study was to examine the physicochemical properties of 30% calcium (**Ca**) reduced micellar casein 80% protein powders (**RC-MCC**) and functional properties of its resultant dispersions. The calcium reduction in the micellar casein (**MCC**) powder was achieved by subjecting the liquid micellular casein obtained from the microfiltration of pasteurized skim milk to carob dioxide (**CO_2**) treatment before and during ultrafiltration. The CO_2 injection was controlled to obtain 0 and 30% reduction in calcium in the C-MCC (control) and RC-MCC powders, respectively. The MCC powders were tested for physicochemical properties like composition, particle size distribution, and bulk density. Dispersions from these MCC powders in deionized water were tested for functional properties, i.e., solubility, viscosity, heat stability, emulsifying capacity, emulsion stability, foam capacity, and foam stability. The CO_2 injection did not result in any significant differences in the composition except mineral contents, particularly calcium. The particle size and bulk density of RC-MCC powders were significantly ($P < 0.05$) lower than control powders whereas solubility was higher. The RC-MCC powder dispersions showed increased heat stability as compared to control, whereas no significant changes in viscosity and emulsification capacity was observed between the two dispersions. However, the emulsion stability and foam stability of RC-MCC dispersions were significantly lower than C-MCC dispersions. This study shows that by utilizing a novel Microfiltration-CO_2 injection-Ultrafiltration process, 30% calcium reduced MCC powders is commercially feasible. his study also provides a detailed

understanding of the effect of calcium reduction on the functional properties of resultant MCC dispersions. It shows that calcium reduction could improve the overall functional properties of MCC dispersions.

Keywords: MCC, reduce calcium MCC, Micellar Casein, Functional properties

3.1 Introduction

Micellar casein concentrate (**MCC)** has received a lot of attention in recent years because of its unique functional properties and applications in various foods (Hammam et al., 2021). Micellar casein concentrate is manufactured by microfiltration (**MF**) of skim milk (Vadi and Rizvi, 2001; Metzger et al., 2012), which partitions serum proteins, non-protein nitrogen, lactose, and mineral into the permeate. The resulting retentate is casein enriched (higher casein to total protein ratio and casein to true protein) and is referred to as micellar casein concentrate. 80% (wt./wt.) protein MCC powders are produced from liquid micellar casein concentrate obtained by MF followed by d spray drying. The general physical and compositional properties of the casein in MCC are similar to that of the native casein micelles in milk (O'Regan and Mulvihill, 2011). Micellar casein concentrate may be used to produce cheese, processed cheese (as a rennet casein substitute), nutritional meal replacements, whipped toppings, RTD protein drinks, and coffee whiteners, among other applications (Nelson and Barbano, 2005; Zulewska et al., 2009). Additionally, they are an excellent raw material to produce bifunctional peptides (Korhonen and Pihlanto, 2006; Holder et al., 2014; Atamer et al., 2017) and for a variety of non-food applications such as coating agents (Audic et al., 2003) and glues (Strube et al., 2015). As a result, interest in casein products and fractions has increased steadily (Thienel et al., 2018).

Because MCC is a relatively new product, few detailed studies are available on its functionality. However, since MCC contains a higher proportion of casein and a lower proportion of whey proteins (**WP)**, specific properties such as heat stability are assumed. Nonetheless, the general properties of casein and casein micelles are thoroughly described (Walstra and Jenness, 1984; Swaisgood, 1992). Lower solubility of MCC powders that deteriorates over time has been reported and is ascribed to higher-order structural changes, such as cross-linking between casein micelles, which may involve the formation of intermolecular sheets (Schokker et al., 2011). The loss of reconstitute-ability is almost certainly a result of higher-order structural changes, such as cross-linking between casein micelles, which may involve the formation of intermolecular -sheets (Schokker et al., 2011). Biochemical and physical modifications to the concentrates and powders increased the solubility index to up to 92.6 percent (Schuck et al., 1994).

Several recent studies have detailed the chemical composition of MCC powders processed with MF (Schubert et al., 2018; Thienel et al., 2018; Schäfer et al., 2019). MCC generally contains more than 90% casein (**CN**) on total protein, whey protein (**WP**), and soluble constituents such as lactose and soluble minerals. Minerals, lactose, and non-protein nitrogen **(NPN**) all have a significant effect on the heat and alcohol stability of MCC. Due to its high CN content, MCC is expected to have excellent heat and alcohol stability. Previously, it was reported that CN is a heat-stable compound (Fox and Mulvihill, 1982). Recent studies, however, indicate that heat stability may not be as high as previously believed (Beliciu et al., 2012; Sauer and Moraru, 2012). Certain pH and temperature conditions, on the other hand, can cause the CN micelles to lose their integrity, resulting in flocculation, gelation, or protein separation (Singh and Fox, 1985,

1987), and can result in changes to CN micelle upon heating (Beliciu et al., 2012; Sauer and Moraru, 2012). The pH, calcium content, protein concentration, urea (NPN), lactose, and SP concentration significantly affect solubility and heat stability. Changes in solubility and heat stability occur due to the constituents and ratios of milk components changing during MF or ultrafiltration (**UF**) operations, with diafiltration (**DF**) further altering the composition. Sauer and Moraru, 2012, concluded that MCC is unstable at sterilization temperature. This instability increases with treatment temperature due to changes in mineral equilibrium and partial disintegration of the CN micelle, which results in aggregation and even coagulation. Minor differences in the composition and processing of the MCC can result in significant differences in their sterilization stability. Beliciu et al., 2012, reported that drying and reconstitution of MCC decreased its resistance to UHT treatment compared to the undried concentrates.

Most of the calcium in milk is contained in casein micelles. Micellar calcium is primarily present as colloidal calcium phosphate (**CCP**) nanoclusters. The amount of micellar calcium can be altered by altering environmental conditions such as temperature, pH, or the addition of chelators (Broyard and Gaucheron, 2015; Koutina and Skibsted, 2015). The concentration of salts, particularly calcium, significantly affects the sensory and functional properties of fermented dairy products (Lucey and Fox, 1993; Mistry and Maubois, 2004, 2017) and dairy powders (Baldwin, 2010; Sikand et al., 2013). For instance, it was reported that cheddar cheese (and processed cheeses made thereof) prepared from ultrafiltration (UF) milk retentate had a decreased melting ability due to its high calcium content (Mistry and Maubois, 2017).

Numerous researchers have found a close link between the solubility of MPC80 and its Ca content (Bhaskar et al., 2007; Sikand et al., 2011; Ye and Harte, 2013; Sunkesula et al., 2021), leading to the hypothesis that Ca present in MPC may promote protein-protein interactions during processing and storage. Several studies have been undertaken in recent years to determine the effect of demineralization on the rehydration behavior of casein powders (Nogueira et al., 2020; McSweeney et al., 2021) or calcium content (Schokker et al., 2011; Aaltonen, 2012; Schäfer et al., 2021). Mao et al., 2012 and Sikand et al., 2013, developed a novel method for mineral-modified MPC80 production by utilizing DF with varying concentrations of monovalent salts added to the DF water. Mineral modified MPC80 produced via this process exhibited enhanced functionality and solubility compared to conventionally produced MPC80. In addition, (Bhaskar et al., 2007; Dybing et al., 2007; Xu et al., 2016) used a cation-exchange method to replace divalent ions, particularly calcium, and reported improved functional properties for the calcium-depleted MPC that resulted. Based on these previous studies demonstrating enhanced functionality of MPC powders with reduced calcium content, we hypothesize that micellar casein concentrates with reduced calcium content will exhibit similar functional benefits.

Previous research has established that injecting CO_2 into cheese milk before rennet coagulation can be used to decrease the pH of the milk and solubilize micellar calcium phosphate, altering the mineral profile of cheese made from concentrated milk (Nelson et al., 2004; Kelly et al., 2008). Similarly, CO_2 injection could lower the milk pH and solubilize micellar calcium and phosphate before and during UF, resulting in decreased calcium and mineral content MPC (Marella et al., 2015). In addition, when

CO_2 is used as an acidulant, residual CO_2 can be easily removed via heating or vacuum, whereas other acidulants such as organic acids cannot.

The current study produced 30% calcium-reduced MCC powders by modifying the Marella et al. 2015 process developed initially to manufacture 30% calcium-reduced MPC's. Marella et al., 2015, reported improved functional properties in 30% reduced calcium MPC powders, and was attributed to the formation of more soluble caseins when colloidal calcium was removed. As a result, the study's goal was to determine the effect of a 30% calcium reduction in MCC produced through a novel multistage MF-UF filtration process that utilizes carbon dioxide to acidify milk before and during the UF stage.

3.2 Materials and Methods

3.2.1 Manufacturing of MCC80 powders

Micellar Casein Concentrate (MCC80) powders with two levels of calcium, 0% reduction (control, C-MCC), and 30% reduction (treatment, RC-MCC), were manufactured in triplicates using the novel MF-UF method. A schematic C-MCC and RC-MCC production process is shown in Fig. 1. Briefly, pasteurized skim milk was micro filtered to 3X concentration at 120 °F and a transmembrane pressure (**TMP**) of 0.1 MPa using a GP MF (TIA) cross flow membrane filtration unit with ceramic membranes (GP Memralox® modules) of 0.1 µm mean pore size, 1.68 m^2 total surface area, and 1.02 m long. The retentate from this MF step, with 9% (wt./wt.) protein was diluted back to 3% (wt./wt.) protein using deionized water, making it to a 200% DF rate. The liquid MCC thus obtained is again micro filtered (DF-MF) to 2X concentration to permeate more serum phase components and improve the casein fraction in the retentate. The

liquid MCC obtained from this DF-MF step, having 6% (wt./wt.) protein was diluted back to 3% (wt./wt.) protein and pasteurized (76 °C/16 s) and stored at 4 °C for further ultrafiltration processing. The pasteurized liquid MCC, 3% (wt./wt.) was split into two equal parts. One part was utilized to produce C-MCC powder, by ultrafiltering at a temperature of 70°F, and a base, boost, and inlet pressure of 30,20, and 50 PSI, respectively using spiral wound membranes (Microdyn® modules) of 20 kDa molecular weight cut off, a total surface area of 5.7 m^2. The second portion was utilized to produce treatment (RC-MCC) powder following the UF process used during the control manufacturing process, except that the CO_2 was injected into the liquid MCC before and during UF processing step to reach and maintain a pH of 5.7 ± 0.1 before and during UF. The pH of CO_2 treated UF retentates and the control UF retentates were adjusted to 7.0 ± 0.1, using 1.25N NaOH and subsequently spray dried using pilot scale spray dryer (NIRO) with an inlet and outlet air temperature of 193 °C and 76 °C, respectively. The powder manufacturing was performed in triplicates from three different lots of skim milk.

3.2.2 Chemical composition

The C-MCC and RC-MCCs were analyzed for Total solids (**TS**), total fat, ash using standard wet chemistry procedures (Hooi et al., 2004). The lactose content was determined using an HPLC method (Amamcharla and Metzger, 2011). Total nitrogen (**TN**), non-protein nitrogen protein (NPN) and non-casein nitrogen (**NCN**) were determined using micro-Kjeldahl analysis as described by Hooi et. al., 2004, except the modified NCN extraction method developed by Zhang and Metzger, 2011 was used. The true protein, casein and serum protein were calculated by difference using the TN, NCN and NPN values as described by Hooi et al., 2004. Mineral analysis of the samples was

conducted using ICP-OES (Inductive Coupled Plasma-Optical Emission Spectroscopy). The proximate composition of each powder sample was determined at least in duplicates.

3.2.3 Powder characterization

Particle size distribution analysis

The particle size of the MCC powders was measured using a Malvern Mastersizer (Mastersizer3000; Malvern Instruments Ltd, Malvern, Worcestershire, UK) equipped with an Aero S dry dispersion unit. The refractive index of the sample and air were set at 1.45 and 1.00, respectively. The air pressure was set at 2 bar for all samples, and the federate was adjusted (from 25–100%), to accommodate the inherent variability of the cohesiveness of the powders at a 3mm hopper gap. Size measurements were recorded as the median diameter (D50) and cumulative diameters (D90), where 50 and 90 refers to the percentile of the sample volume, with particle size less than the number indicated.

Bulk density of MCC 80 powders

Bulk density of MCC powder was measured for both loose and tapped conditions according to the IDF Standard 134A:1995. For the loose density, MCC powder was poured in a dry pre- weighed 100 mL calibrated glass cylinder up to the mark of 100 mL without any shaking and then weighed. After weighing, the same cylinder was tapped 100 times using UNILAB -009 Bulk Density Apparatus (Ambala Cantt, Haryana, India), and then volume after tapping was measured. Loose bulk density was calculated by dividing the weight of the powder by the volume of the powder before tapping, and the tapped bulk density was calculated by dividing the weight of the powder by the volume of the powder after tapping.

3.2.4 Functional properties

The MCC dispersions (both control and Ca reduced) were prepared as per the method outlined in the solubility test. Dispersions after overnight storage were adjusted to a pH of 7.0.

Powder solubility

The solubility of the MCC powders in room temperature water was determined gravimetrically. The 5% protein solutions (wt./wt.) were made by dissolving measured MCC powders into the water at 22 ± 1 °C. The powders were stirred for 30 min. into deionized water using a magnetic stirrer and a stir plate (Fisher Scientific) at a speed of 300 rpm. Post dissolution, the dispersions were hydrated overnight at 4 °C. Following over-night hydration, the protein solutions were allowed to equilibrate, and the pH was adjusted to 6.9 ± 1.0 using 2.0 and 0.2N NaOH. The solubility method described by (Havea, 2006) was used with some modifications. Aliquots of each reconstituted MCC (50 mL) were centrifuged (CR4-12, Jouan Inc., FL, USA) at 700 x g for 10 min. The samples of the 5% (wt./wt.) protein solution (before centrifugation) and the supernatant collected after centrifugation were analyzed for TS. The TS was determined using a forced draft oven (Fisher Scientific) by drying at 100 °C for 4 h. The solubility of powder was calculated as the TS of supernatant, expressed as percentage of the TS of 5% protein solution prior to centrifugation. The changes in the solubility of the MCC powders during storage at 38 °C were further tested for four weeks. Each MCC sample was tested for solubility in duplicates.

Apparent viscosity

The apparent viscosity of the dispersions adjusted to pH 7.0 after overnight storage was determined at 20 °C using a rheometer (MCR 92, Anton Paar GmbH, Germany). The dispersion was filled up to the mark into a concentric cylinder geometry consisting of a cup and bob. An equilibrium time of 25.0 s and a pre shear rate of 10/sec for 20.0 s was applied. The solution's viscosity was measured at a shear rate of 100 per sec. The viscosity data obtained are reported in centipoise (cP). The viscosity measurements on each sample were tested in duplicates.

Heat stability

Heat stability was determined on the MCC powder from each treatment using reconstituted 5% (wt./wt.) protein solutions. The dispersions prepared for viscosity measurements were used for heat stability testing. An aliquot of 3 ml was transferred to a capped glass vial (61 mm height x 17 mm dia.) and immersed in a clear mineral oil (99.9% mineral oil, USP, Vi-Jon, TN) maintained at 140^{o}C in a bath (Akash-Deep Scientific Industries, New Delhi, India) with constant agitation. The heat coagulation time (HCT) was determined as time in min, elapsed between immersing the samples in the oil bath and onset of visual clots (Singh, 2004).

Emulsifying capacity and Emulsion stability

To evaluate the emulsion capacity and emulsion stability of MCC powders, an emulsion was prepared by mixing soyabean oil with freshly reconstituted MCC samples (1% wt./wt.) at the ratio of 3:7 (wt./wt.). Dispersion of MCC 80 (1%, wt./wt.) was prepared by adding MCC80 powder in deionized water and then stirred using a magnetic stirrer (700rpm) for 60 min at 22˚C. The pH of the dispersions was adjusted in the range

of 6.8 to 7.0 using NaOH. A 7g of reconstituted MCC dispersion was taken in a 50mL centrifuge tube, and then 3g soybean oil was added to it. The MCC solution and oil mixture was heated to 55˚C and homogenized for 60 s at 10,000 rpm using a benchtop homogenizer (Polytron, PT 2500E). Approximately 8 g of the emulsion was transferred to another 15 mL centrifuge tube followed by centrifugation at 1100xg for 5 min. The height of the emulsified layer and that of the total contents in the tube were recorded. The emulsifying capacity was calculated as % of the volume of emulsified liquid to the total volume of the liquid homogenized using the below formula.

Emulsion Capacity, *EA* (%) = [H_E / H_T] x 100

Where:

H_E is the height of the emulsified layer in the tube

H_T is the height of the total content in the tube

To determine the emulsion capacity, the emulsion prepared was heated to 80 °C for 30 min. in a water bath, then brought down to room temperature (22◦C) and recentrifuged at 1100xg for 5 min. The emulsion stability was calculated as % of the volume of emulsified liquid after heating to the total volume of the liquid homogenized using the below formula.

Emulsion stability, *ES* (%) = [H_H / H_E] x 100

Where:

H_H is the height of the emulsified layer after heating, cooling, and re centrifugation

Foam capacity and Foam stability

Foaming capacity was determined using the method described by (Shilpashree et al., 2015). A 3g MCC80 powder was blended with 100 mL phosphate buffer (0.05 $molL^{-1}$, pH-7.0) in a mixer (auto-mix Osterizer blender, Model: 6630) and whipped for 6 minutes at 11,000 rpm. The developed foam was immediately transferred into a 250 mL measuring cylinder quantitatively, and the total volume was recorded. The foaming capacity was calculated using the below equation.

Foam capacity, *FC* (%) = [$(V_0 - V_{L)} / V_{L]}$ x 100

Where:

V_L is the volume of liquid before whipping (mL)

V_0 is the total volume (foam plus liquid) obtained immediately after whipping (mL)

To determine the foam stability, the cylinder containing foam was kept undisturbed for 30 min at 22˚C. The volume after the holding time was recorded again. The foam stability was determined as the volume of foam that remained after 30 min (at 22 ± 1° C) expressed as a percentage of the initial foam volume using the equation below.

Foam stability, *FS* (%) = [$(V_T - V_{L)} / (V_0 - V_L)$] x 100

Where:

V_T is the foam volume after 30 min of whipping

3.2.5 Statistical analysis

The treatments were run in triplicates, and the chemical analyses were run at least in duplicate. The values of the replicates were presented as mean ± standard deviation (SD). Using Minitab® (v.20.4, Minitab Inc., State College, PA), To assess for significant differences between the group means, a one-way analysis of variance (ANOVA) was used, followed by the Tukey post hoc comparison test. Statistical significance was defined as a p-value of less than 0.05.

3.3 Results and Discussion

3.3.1 MCC powder characterization

The principal goal of this study was to produce an MCC powder with 30% calcium reduction (RC-MCC) by injecting CO_2 and using membrane processing at a pilot scale (Fig.1). Along with RC-MCC, an MCC powder without any CO_2 injection and calcium reduction was also produced to serve as a control sample and for comparison with RC-MCC. The general composition and the mineral concentration of C-MCC powders thus produced are summarized in Table 1 and Table 2, respectively. The total protein content of both the C-MCC and RC-MCC powders ~80% (wt./wt.), qualifying the product as MCC80. There was no significant difference between the true protein content (and the casein) in C-MCC and RC-MCC, and the results were 77.81% (71.74%) and 78.99% (72.16%), respectively. Similarly, there were no significant differences in the rest of the general chemical composition i.e., total solids, whey protein, fat, and lactose. However, by using a novel MF-UF process and injecting CO_2 in the treatment, the calcium content was significantly reduced in RC-MCC powder (31.01%) compared to control MCC powders. The ash, calcium, and phosphorus contents were significantly

lower in RC-MCC, 7.06 %, 1727 mg/100g, and 1267 mg/100g respectively, compared to MCC, 8.15%, 2503 mg/100g, and 1563 mg/100g respectively. The equilibrium between colloidal and soluble Ca is dependent on pH of the milk system (Law and Leaver, 1998; LE GRAËT and GAUCHERON, 1999). During the acidification process of milk, the lowering of pH causes the serum phase to become less saturated in calcium phosphate due to the dissociation of this salt; consequently, the micellar calcium phosphate is progressively dissolved with an increase of the amounts of calcium and inorganic phosphate concentrations in the aqueous phase (Visser et al., 1986; Dalgleish and Law, 1988, 1989; Mekmene et al., 2010). During the UF process employed in the manufacturing of RC-MCC, the soluble minerals portioned into the permeate, resulting in a significant reduction of calcium (31%) and phosphorus (19%) in the retentate.

The physical characteristics of the MCC powders i.e., particle size and bulk density, are summarized in Table 3. The particle size distribution, [D,90] values of RC-MCC (65.77%) were significantly lower than that of C-MCC (73.97%). Colloidal calcium phosphate (CCP) plays an important role in the stability and the structural integrity of casein micelles, and it exists in equilibrium with the Ca present in the serum phase (Holt, 2004). Therefore, when the skim milk system is acidified, the CCP from the micelles moves into the serum phase. With progressive removal of CCP during acidification of skim milk, the casein micelles become smaller and more homogeneous (Moitzi et al., 2011) as shown in Fig. 2. A similar effect on the micellar size was observed when calcium is depleted by adding chelating agents, and an increasing micellar size was reported when calcium is added (Xu et al., 2016; Wu et al., 2020; Zhao et al., 2021). The effect of reduced particle size in RC-MCCs has a significant effect on both

tapped and loose bulk density parameters of the same (Table 3). This is because the smaller particles were much lighter and had much higher specific surface areas and thus trapped air much more efficiently in a bed of powder, which reduced the bulk density (Han et al., 2021).

3.3.2 Functional properties

The results of different functional properties i.e., Solubility, Viscosity, HCT, Emulsification capacity, Emulsion stability, Foam capacity, and Foam stability of MCC and RC-MCC are summarized in Table 4.

Solubility

One of the most important functional properties of any dried protein powders is the solubility. Protein powders tend to lose solubility because of protein-protein interactions during manufacturing and over storage. The proteins need to be rehydrated to ensure optimal functionality (Crowley et al., 2015), and the solubility during the rehydration of MCC powders has also been a limiting factor in its use in foods and beverages (Burgain et al., 2016; Zhang et al., 2018). The solubility of MCC and RC-MCC were compared using the suspensions of fresh powders and the powders stored at elevated temperatures (38 °C) for four weeks to understand and compare the impact of calcium depletion on the solubility of MCC powders during storage. The solubility of the MCC and RC-MCC suspensions are presented in Table 4. The strong influence of mineral content on the protein powders' solubility was studied in some of the previous research studies (Bhaskar et al., 2007; Sikand et al., 2011, 2013; Ye, 2011). In particular, the calcium content plays an important role in the solubility of micellar casein. Removal of calcium for the purpose of enhancing the solubility of MPCs was performed using

acidification of milk, addition of calcium chelators and replacement of calcium with monovalent salts had gained researchers interest (Khalesi and FitzGerald, 2021a). Therefore, changing the calcium equilibrium towards a higher proportion of serum calcium has become a promising approach to minimize protein aggregation and enhance solubility (Eshpari et al., 2014; Ramchandran et al., 2017). The solubility of RC-MCC dispersions (99.36%) was observed to be significantly ($P < 0.05$) higher compared to that of C-MCC (88.82%). A similar effect of reducing the pH of skim milk during ultrafiltration to manufacture reduced calcium MPC powders on the solubility of resulting MPC dispersions were reported by (Liu et al., 2019) and Marella et. al., 2015. Schafer et. al., 2021 reported a significant improvement in the solubility of reduced calcium MCC powder dispersions. However, the method and expression of the solubility that we have used in this experiment were different from the one used by Schafer et. al., 20021. They have used ADPI solubility method, and the solubility was expressed as the amount of insoluble material. In contrast, we have used the gravimetric solubility method as described in the material and methods section. Another reason for the difference in the solubility numbers between our test and that of Shafer et. al., 2021 was that they dried the calcium reduced MCCs at an unadjusted and notably lower pH (5.8). In contrast, in our processing, the pH of the RC-MCC liquids was adjusted to ~7.0 before drying, to avoid any unwanted buildup of viscosity and clogging the lines and nozzles that was observed during initial trials of the manufacturing. This improved solubility of RC-MCC powders could be explained by increased nonmicellar casein fraction in RC-MCC powders which was not susceptible to the development of insolubility during drying (Gazi and Huppertz, 2015). Also, the increase of non-micellar casein fractions and the net zeta potential of the

casein micelles (data not shown) might have prevented the aggregation of micellar casein in RC-MCC dispersions. In summary, the smaller particle size distribution, high surface negative electrostatic repulsions, and low ionic calcium activity (Wu et al., 2020; Sunkesula et al., 2021) of the neutralized RC-MCC dispersions were the principal reasons for the improved solubility in the case of calcium reduced micellar casein powders compared to those of control MCC powders.

It was theorized that during the storage of MCC powders, the progression of protein-protein interactions and cross linkage resulting in increased aggregation causes a decrease in solubility (Havea, 2006; Nasser et al., 2017). Several other studies (Davenel et al., 2002; Schuck et al., 2002; Hussain et al., 2011; Schokker et al., 2011; Mao et al., 2012; Sikand et al., 2013; Eshpari et al., 2014; Sun et al., 2017; Nogueira et al., 2020; Khalesi and FitzGerald, 2021a; b; McSweeney et al., 2021) have reported improved initial solubility of calcium reduced high casein containing powders (MPCs and MCCs); however there was no information available on the impact of calcium reduction on the storage solubility of RC-MCC powders.

The change in the solubility of MCC and RC-MCC dispersions is presented in Fig. 3. The RC-MCC powders were observed to retain their solubility for the entire duration of the storage period, and there was no significant drop in the solubility up to 30 days of storage at 38 °C. The control MCC dispersions showed a significant loss (approximately 30%) of solubility in the first week of storage itself. Towards the end of the storage period (30days) about 65% of the solubility was lost in the case of C-MCC, whereas an insignificant loss of 2% was observed with RC-MCC. As the storage time of MCC progresses, the hydrophobic protein-protein interactions between results in forming

a network of casein micelles via non-covalent bonding at the powder particle surface causing the deterioration of the solubility (Anema et al., 2006). In the case of control MCC, the initial solubility of 89% was similar to that of the non-calcium adjusted powders, dried after adjusting the retentate pH to 6.7 prior to drying as reported by Liu et al., (2019). Likewise, the loss of solubility after three weeks of storage at 38 °C in the case of control MCC (26 %, Solubility) was comparable to 19% solubility after a storage period of 84 d at 40 °C. Loss of storage stability can be reduced when skim milk is acidified to deplete the calcium prior to membrane filtration and spray drying (Liu et al., 2019; Schäfer et al., 2021). Whereas the calcium depleted RC-MCC powder showed 99% initial solubility and 98% solubility towards the end of the storage period of 3 weeks at 38 °C, retaining almost all the initial solubility. These findings are consistent with those of a previous study, wherein the skim milk, acidification was done using citric acid (from 6.8 to 5.9) before spray drying (Bhaskaracharya and Shah, 2001).

Viscosity

The viscosity of the reconstituted 5% (wt./wt.) solutions of MCC and RC-MCC powders at 20 °C, 2.37 cP, and 2.46 cP, respectively, were found to be non-significant ($P > 0.05$). Nevertheless, the RC-MCC dispersions had higher apparent viscosities, and this could be attributed to increased voluminosity of casein micelles due to a decrease in the colloidal calcium phosphate. Schafer et. al., 2019 reported no significant difference in the viscosity between control MCC and 50% calcium reduced MCC powder dispersion.

Heat stability

The casein micelles in milk are remarkably stable systems that can withstand the rigorous conditions applied during the commercial sterilization conditions (Fox and

Mulvihill, 1982). However, the heat stability of micellular casein is significantly influenced by the mineral equilibrium (Sauer and Moraru, 2012). The heat stability of RC-MCC powder dispersions were observed to be significantly higher ($P < 0.05$), 26.47 min., compared to 10.62 min for control MCC dispersion (Table 4). Although, this is a noticeable difference, approximately 2.5 times higher heat stability of MPCC dispersions could be achieved by reducing 30% calcium. Sunkesula et al., (2021) has studied the effect of calcium reduction in MPCs on the heat stability of reduced calcium MPC dispersions at different adjusted pH values. They reported a 30% reduction of calcium resulted in a significant improvement in the Heat Coagulation Time (HCT) of the dispersions at a pH of 6.9. They have summarized that the heat stability of reduced calcium casein dispersions is an interplay of colloidal reactions affected by Calcium ion activity, ionic composition, and dissociation of caseins, which affects the aggregation behavior of caseins during heating.

Emulsification capacity and Emulsion stability

Emulsion is generally described as a mixture of two immiscible liquids (for example, water and oil), wherein one of these liquids is dispersed as droplets in the other (McClements, 2004). Most of the food systems commonly contain particulate material that accumulates at oil-water and air-water interfaces and contributes to the colloidal stabilization of emulsions and foams (Dickinson, 2006). The emulsifying agents get adsorbed at the oil-water interface and reduce surface tension, thus stabilizing the emulsion (Lazzaro et al., 2017). Caseins can get adsorbed at the interface, either individual or aggregated form (Dickinson and Casanova, 1999), hence functioning as an emulsifying agent. Among the functional properties, the casein micelle's ability to

emulsify and stabilize oil in water emulsions is of great interest for the food industry, particularly the dairy industry.

The C-MCC and RC-MCC powders were assessed for their ability to facilitate the blending of the phases of the emulsion (emulsification capacity) and their ability to stabilize the emulsion (emulsion stability). The results are presented in Table 4. The emulsification capacity of the RC-MCC (66%) powders was observed to be higher than that of the control MCC (63%). The differences in the emulsification capacity can generally be attributed to the surface activity and/or the size of the emulsifying agent; the higher the surface activity and/or the smaller the size, the greater the emulsification capacity. The higher zeta potential (data not shown) and lower particle size distribution of RC-MCC compared to those of the control MCC powders could be affecting the emulsification capacity of RC-MCC powders positively. Similar results were reported by Lazzaro et al., (2017), where the emulsifying capacity and stability of casein aggregates were characterized after sequential calcium and simultaneous inorganic phosphorus depletion using Trisodium citrate (TSC). However, the emulsification stability of the RC-MCC (81%) powders was observed to be significantly ($P < 0.05$) lower than the control MCC (87%). Lazzaro et. al., 2017 has proposed that the destabilization of emulsions can result from three phenomena i.e., creaming, flocculation and coalescence and they observed that the emulsions stabilized by calcium depleted casein were stable against coalescence, but not so stable against creaming and flocculation phenomena. The creaming and flocculation are two concomitant phenomena and influence each other. Creaming happens in the emulsions due to the difference in the density between the oil and the aqueous suspension phases and is enhanced as the oil droplet combination

progresses. The creaming, on the other hand, facilitated flocculation by moving the droplets forward and encouraging contact, which is a critical step in the ultimate instability of emulsion (Dauphas et al., 2008). The presence of unabsorbed particles causes depletion-flocculation, an instability process that happens in emulsions. It occurs when two neighboring droplets are close enough to exclude any unabsorbed particles from the gap separating them. As a result, an osmotic pressure differential is created, which causes the emulsion droplets to attract each other (Dickinson and Golding, 1997; Radford and Dickinson, 2004). Ye et. al., has reported the effect of depletion-flocculation on decreasing the emulsion stability in calcium depleted MPC stabilized emulsions. This could explain the lower emulsification stability of the RC-MCC powders compared to that of control C-MCC powders.

Foaming capacity and Foaming stability

The foaming properties of proteins are related to the adsorption (surface activity) of proteins to the air/aqueous surface i.e., the rate at which the surface tension of the air-water interface decreases. The foaming performances are influenced by several factor like the type and concentration of protein, temperature, pH, ionic environment, ionic strength, and conformation of these proteins (Dickinson, 2006; Foegeding et al., 2006). The addition of calcium chelating agents like Ethylenediaminetetraacetic acid (**EDTA**) causes dissociation of casein micelles and the higher availability of β-casein would be preferentially adsorbed onto the foam, thus improving the foamability of protein dispersions (Zhang and Goff, 2004). Another study (Silva et al., 2013) demonstrated that different degrees of calcium demineralization by acidification using HCl and ultrafiltration of milk was possible and reported that in 9.5% wt./wt. milk protein

dispersions thus obtained have shown an increased β-casein and non sedimental casein fraction with the increase of acidification. As shown in Table 4, the 3% wt./wt. RC-MCC dispersions have shown a significantly high foaming capacity ($P < 0.05$) compared to the C-MCC dispersions. When CO2 is injected into liquid MCC before and during ultrafiltration to adjust the pH to ~5.7, the dissociation of casein micelles and increased non micellar casein (β-casein, in particular) could have improved foaming capacity RC-MCC dispersions. These results align with the finding of Silva et al., 2013. However, the foam stability (Table 4) of RC-MCC dispersions was observed to be significantly lower than that of C-MCC dispersions. One of the important physical properties of the dispersions that helps retain the stable foam is viscosity. Higher viscosities promote the formation and smaller size air bubbles and reduce the coalescence of air bubbles, thereby enhancing the stability of the resulting foam (Huppertz, 2010). Given that there is no significant difference between the viscosities of C-MCC and RC-MCC dispersions and more foam incorporated in the RC-MCC dispersions, the RC-MCC dispersions showed lesser foam stability than C-MCC dispersions.

3.4 Conclusion

This study evaluated the pilot-scale production of calcium-reduced MCC 80 powders using a novel Microfiltration-CO_2 injection-Ultrafiltration process and the effect of the calcium reduction on the physicochemical and functional properties of the RC-MCC powders and dispersions, respectively. Control micellular casein powders (C-MCC) without CO_2 injection were also produced to compare with RC-MCC. From our investigation, we conclude the following:

i. A 30 % calcium reduction in MCC powders is feasible at a commercial scale using CO_2 injection. Using CO_2 is a cleaner process than using other methods of using acidulants to lower the pH of milk/micro filtered milk to reduce the calcium content.

ii. Reducing the calcium content of MCC powders decreased the particle size and bulk density. This could be attributed to the dissociation of casein micelles during CO_2 injection. This has significantly improved the instant solubility, and the lower calcium levels retained the solubility of the RC-MCC powders. The reduction of calcium also improved the heat stability of the dispersions.

iii. Reduction of calcium was observed to improve foam capacity; however, the emulsions stability and foam stability were lower than control powder dispersions. This could be attributed to smaller particle size and not enough viscosity to retard the coalescence of smaller oil droplets or foam bubbles.

This research findings should add to the current understanding of the functionality of reduced calcium micellar casein powders and help users apply MCC power ingredients more effectively.

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3.6 Figures

Figure 3.1. Schematic of the production of MCC 80 powders with and without calcium reduction

Figure 3.2. Change in the micellar casein during acidification, adopted from (Moitzi et al., 2011)

Figure 3.3. The solubility of MCC and RC-MCC dispersions, stored at 38 °C for 30 days (four weeks)

Figure 3.1

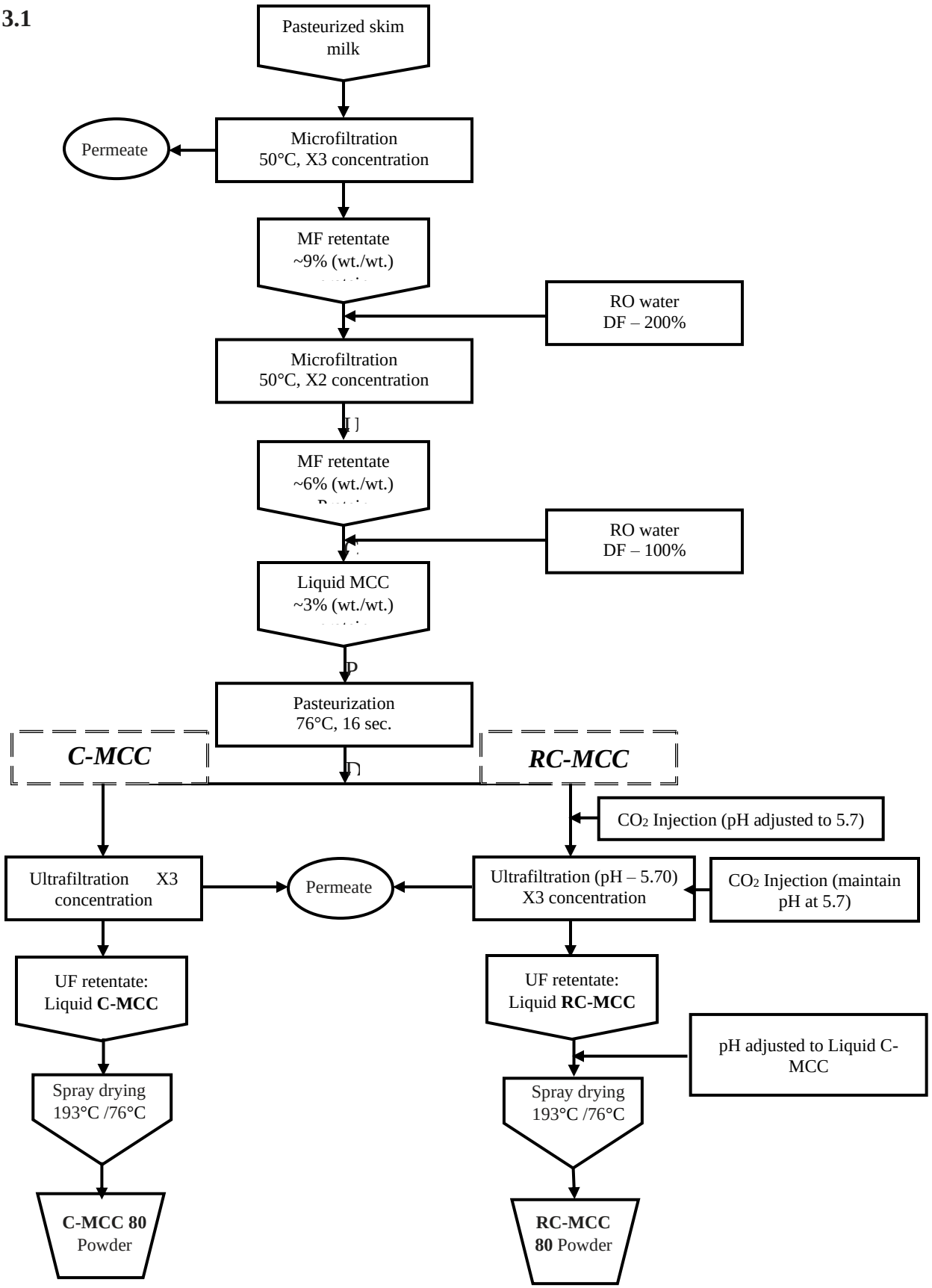

Pasteurized skim milk
Microfiltration 50°C, X3 concentration
Permeate
MF retentate ~9% (wt./wt.)
RO water DF – 200%
Microfiltration 50°C, X2 concentration
MF retentate ~6% (wt./wt.)
RO water DF – 100%
Liquid MCC ~3% (wt./wt.)
Pasteurization 76°C, 16 sec.
C-MCC
RC-MCC
CO2 Injection (pH adjusted to 5.7)
Ultrafiltration X3 concentration
Permeate
Ultrafiltration (pH – 5.70) X3 concentration
CO2 Injection (maintain pH at 5.7)
UF retentate: Liquid C-MCC
UF retentate: Liquid RC-MCC
pH adjusted to Liquid C-MCC
Spray drying 193°C /76°C
Spray drying 193°C /76°C
C-MCC 80 Powder
RC-MCC 80 Powder

Figure 3.2

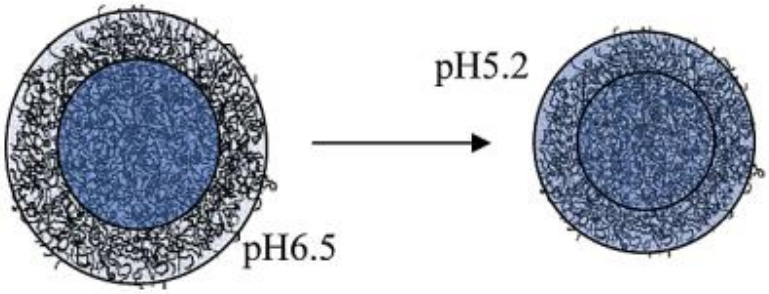
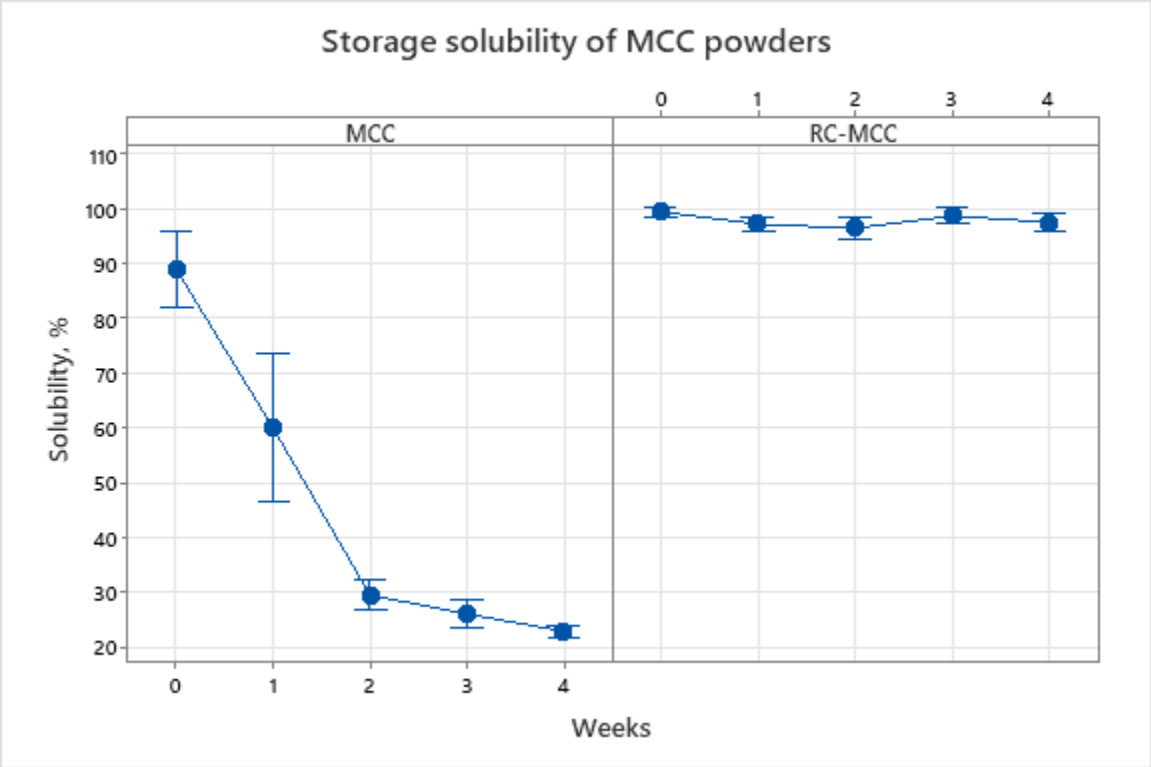


Figure 3.3


Storage solubility of MCC powders
MCC
RC-MCC
Solubility, %
Weeks
110
100
90
80
70
60
50
40
30
20
0
1
2
3
4

3.7 Tables

Table 3.1. Mean (n=3) chemical composition of the micellar casein concentrate powders (% wt./wt.)

Powder[1]	Composition							
	Total Solids	Moisture	Total protein	True protein	Casein	Whey protein	Fat	Lactose
C-MCC	95.51 ± 0.11[a]	4.49 ± 0.11[a]	79.25 ± 0.82[a]	77.81 ± 0.88[a]	71.74 ± 1.13[a]	6.07 ± 0.54[a]	2.98 ± 0.41[a]	5.13 ± 0.94[a]
RC-MCC	95.34 ± 0.27[a]	4.62 ± 0.27[a]	80.33 ± 0.72[a]	80.00 ± 0.51[a]	72.16 ± 0.40[a]	6.83 ± 0.30[a]	2.91 ± 0.61[a]	5.08 ± 0.62[a]

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, calcium reduced micellar casein concentrate powder.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 3.2. Mean (n=3) mineral composition of the micellar casein concentrate

Powder[1]	Composition				
	Ash, %	Na, mg/100g	P, mg/100g	Ca, mg/100g	Ca reduction, %
C-MCC	8.15 ± 0.21[a]	169 ± 0.21[a]	1563 ± 0.06[a]	2503 ± 0.09[a]	-
RC-MCC	7.06 ± 0.42[b]	700 ± 0.42[b]	1267 ± 0.05[b]	1727 ± 0.10[b]	31.01 ± 3.39

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, calcium reduced micellar casein concentrate powder, Na, sodium; P, phosphorus; Ca, Calcium.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 3.3. Physical characteristics of the micellar casein concentrate (n=3)

Powder[1]	Physical property			
	Particle size, [D,50]	Particle size, [D,90]	BD - untapped, Kg/m^3	BD - tapped, Kg/m^3
C-MCC	33.02 ± 2.88[a]	73.97 ± 3.24[a]	181.34 ± 12.17[a]	304.14 ± 13.81[a]
RC-MCC	33.72 ± 1.22[a]	65.77 ± 2.68[b]	160.27 ± 5.45[a]	271.31 ± 7.93[b]

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, BD, bulk density.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$)

Table 3.4. Functional characteristics of the micellar casein concentrate (n=3)

Powder	Functional property						
	Solubility, %	Viscosity, cP	HCT, min.	Emulsification capacity, %	Emulsion stability, %	Foam capacity, %	Foam stability, %
C-MCC	88.82 ± 2.77[a]	2.37 ± 0.10[a]	10.62 ± 0.47[a]	63.33 ± 0.87[a]	86.48 ± 1.79[a]	102.67 ± 6.11[a]	94.54 ± 2.08[a]
RC-MCC	99.36 ± 0.35[b]	2.46 ± 0.10[a]	26.47 ±0.60[b]	65.97 ± 0.94[a]	80.97 ± 1.07[b]	126.50 ± 3.62[b]	92.51± 1.65[b]

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, HCT, heat coagulation time.

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

CHAPTER IV

APPLICATION OF REDUCED CALCIUM MICELLAR CASEIN IN PROCESSED CHEESE – EVALUATION THE POSSIBLE REDUCTION OF EMULSIFYING SALT LEVELS AND THE EFFECT ON THE FUCTIONAL PROPERTIES

ABSTRACT

Process cheese (PC) and process cheese products (PCP) are common foods in the American diet. MCC is an ingredient that has been evaluated as a protein source to replace rennet casein in process cheese product (PCP) manufacture. However, MCC provides inferior functionality relative to rennet casein due to higher colloidal calcium content in MCC. A potential method to modify the functional properties of casein is by partial removal of colloidal calcium by acidifying the skim milk before and during the manufacture of MCC. Emulsifying salts (ES) are critical for the functional characteristics of PC. The ES is added to the processed cheese to create an emulsion during PCP manufacturing. The emulsion created by the ES is due to their unique function to chelate calcium in a colloidal phase in the casein and then transfer the chelated minerals into a serum phase during PCP manufacture. A 30% reduced calcium MCC (RCMCC) is manufactured by lowering the pH of skim milk. Acidification of skim milk allows the transfer of solubilized calcium from a colloidal phase to a serum phase. It then gets removed during filtration, making soluble caseins readily available for emulsification, possibly needing lesser levels of ES in PCP. The objective of the current study was to evaluate the effect of reduced calcium micellar casein (RCMCC) on lowering the usage of ES in PCP and its impact on the functional properties of the PCP.

Three different PCP formulations were prepared with CMCC-2%(control), treatment T1 with 25% reduced ES RCMCC-1.5%, and treatment T2 with 50% reduced ES RCMCC-1%. All 3 PCP formulations were formulated to have the same composition except for the differences in calcium and ES, and each treatment was manufactured in

triplicate. The PCP was analyzed for RVA-Viscosity, Texture profile analysis (TPA), Dynamic stress rheology (DSR), and melting temperature. The TPA-Hardness and DSR-melt temperature of PCP made from the RCMCC treatments was significantly ($p<0.05$) lower than the control. However, the RVA viscosity of T1 RCMCC-1.5% is significantly ($p<0.05$) higher than both control CMCC-2% and T2 RCMCC-1%. These results confirm that T2 RCMCC-1.5% had shown improved meltability, reduced hardness, and stronger emulsification. Furthermore, this study demonstrated that a 25% reduction in emulsifying salts is possible with improved functional properties when 30% reduced calcium RCMCC is used as an ingredient in PCP.

Keywords: Process Cheese, Micellar casein concentrate, emulsifying salt reduction, process cheese product, reduced calcium mcc.

4.1 Introduction

Process cheese (PC) and process cheese products (PCP) are dairy foods that are made by combining dairy ingredients such as natural cheese, protein concentrates, butter, NFDM, whey powder, and permeate with nondairy ingredients such as sodium chloride, water, emulsifying salts, color, mold inhibitors, and flavors and then heating the mixture with continuous agitation to create a homogeneous product with a long shelf life. (Meyer, 1973; Thomas, 1973; Guinee et al., 2004; Kapoor et al., 2007; Kammerlehner, 2009). The PC and PCP are categorized based on the composition and permitted ingredients utilized to make these types of cheese (Code of Federal Regulations, 2004). Thus, PCP contains not approved ingredients or does not meet the typical composition of the standard cheese listed in the Code of Federal Regulations (CFR) (Lu et al., 2007). Since the late nineteenth and early twentieth century, PC has been made to extend the shelf-life of natural cheeses. Approximately one-third of all-natural cheese produced in the United States is used in making process cheese. PC is one of the leading varieties of cheese globally and has several applications as an ingredient (Sorensen, 1997; Kapoor and Metzger, 2008).

The principle of making P. and PCP is calcium sequestration using emulsifying salts such as sodium citrate and disodium phosphate. Emulsifying salts are required for PC due to their role in improving the emulsification characteristics of casein by chelating the calcium phosphate complexes from the insoluble calcium-paracaseinate-phosphate network in natural cheese or aggregated casein network in casein containing ingredients. As a result, the primary molecular forces that cross-link the various monomers of casein in the network are disrupted by the calcium chelating emulsifying salts. This disruption leads to hydration and dispersion of the protein. The partially dispersed monomers of casein have hydrophilic and hydrophobic portions that have emulsification properties. This casein, in turn, links the hydrophilic aqueous phase with the hydrophobic fat phase

(Guinee et al., 2004), which prevents oil separation in PC and PCP in the presence of mixing and heating.

A critical parameter in all PCP formulations is the intact casein provided by cheese and other dairy ingredients. *Intact casein* is defined as a casein that has not undergone any hydrolysis. It forms the structural network of process cheese and plays a critical role in all PCP functional properties. Intact casein provides PCP with a firm unmelted texture and a stringy, elastic melted texture (Kapoor and Metzger, 2008). From a functionality perspective, rennet casein is the preferred ingredient to provide intact casein in the formulation. Compared to other dairy-based ingredients, the protein portion of rennet. Compared to other dairy-based ingredients, the protein portion of rennet casein is 100% intact and provides the maximum amount of viscosity per gram of protein.

Micellar casein concentrate (MCC) is a dairy ingredient manufactured through membrane filtration. When skim milk is microfiltered through the MF membrane (0.1 μm), the membrane retains caseins and casein-bound minerals while SP, lactose, and unbound minerals pass through the permeate. The typical composition of liquid MCC using a GP MF (3-stage, and 3× CF with DF) is >9% true protein (TP) and >13% total solids (TS) (Zulewska et al., 2009). This MCC can be further concentrated to increase the TP and TS to 18% and 22%, respectively, by using 2.2× CF UF followed by 3-stage 3× CF with DF, and finally UF for more concentration (Amelia and Barbano, 2013). In addition, the MCC can be dried to produce MCC powder with a long shelf-life (Amelia, 2012). The dried MCC can contain up to 84 % total protein and 96 % TS (Nasser et al., 2018).

MCC is a high protein dairy ingredient used as a partial or complete replacement of rennet casein in various food applications (Carter et al., 2021). (Salunke, 2013) processed-cheese products and mozzarella cheese analogs with MCC treated with or without transglutaminase, finding that MCC can effectively replace rennet casein in those applications. There is a difference between the intact casein found in cheeses or rennet

casein and native casein obtained in the form of MCC through membrane filtration (e.g., MF) as the former lacks glyco macro-peptide (GMP) at the macro level and a molecular level the charge profile, as well as steric stability of κ-casein, is quite different. The casein in MCC is different from that present in natural cheese as GMP is still attached to the κ-casein. Rennet casein action creates insoluble para-casein, whereas membrane filtration techniques give soluble protein where micelle structure integrity is intact.

The total calcium content of a processed cheese plays a role during its manufacture and influences its final functional properties. A high total calcium level in a process cheese formula affects the manufacturing process of the associated process cheese, since more calcium must be chelated from the natural cheese caseins by the emulsifying salts added during the process cheese manufacturing process.(Cavalier-Salou and Cheftel, 1991; Zehren and Nusbaum, 2000). The primary ingredient contributing to the variations in the total calcium content in a process cheese product (PCP) formula is natural cheese and MCC.

A reduced calcium MCC (RCMCC) manufactured through partial acidification of skim milk has shown improved functional properties (Salunke, 2013; Metzger, 2018; Schäfer et al., 2018) due to increased soluble casein fractions in RCMCC when compared to traditional MCC. The emulsifying salts are added to the processed cheese to create an emulsion during cheese manufacturing. The emulsion created by the emulsifying salts is due to a unique function of the emulsifying salts to chelate minerals (e.g., calcium) in a colloidal phase in the MCC and then transfer the chelated minerals into a serum phase of the MCC. The RCMCC is manufactured by lowering the pH of the MCC, which allows for a transfer of solubilized (i.e., chelated) minerals (e.g., calcium) from a colloidal phase to a serum phase and then get removed during filtration hence making soluble caseins readily available for emulsification. This is essentially the same function as the emulsifying salts necessary for cheese manufacturing. In addition, MCC reduction in calcium may promote

emulsification through the rearrangement of the micelle structure. As a result, less emulsifying salts may be required to make the processed cheese with the RCMCC. Hence, the objective of the current study is to determine if PCP could be produced with less emulsifying salts if RCMCC is utilized in the formulation and its impact on the functional properties of PCP

4.2 Materials and Methods

4.2.1 Preliminary PCP formulations

Initially, control PCP formulas were made, selecting disodium phosphate (DSP) as emulsifying salt (ES) with ES content of 1% to and in increments of 0.5% to determine the lowest emulsifying salt level needed to form an optimum emulsion in CMCC formulations. However, during the manufacture of control PCPs, the addition of DSP at 1% was insufficient to enable the formation of a stable PCP. as reflected by the presence of large quantities of free water and oil in the formulation after the normal cooking period in Rapid Visco Analyzer (RVA) for 4 min at 90 °C. These studies determined that the minimum ES needed for CMCC PC formulations is 1.5%. However, the RVA end apparent viscosity at 1.5% ES level was lower than 500 cp indicating very soft texture; hence 2% ES CMCC formula was selected as the control PCP formula.

4.2.2 Experimental design

Three replicates of process cheese product (PCP) were manufactured using two powders, CMCC and RCMCC, each manufactured in three different batches. CMCC-2% PCP is manufactured as control with 2% ES and a 25% reduced ES RCMCC - 1.5% and a 50% reduced RCMCC-1% were manufactured as treatments in RVA. The control PCP was coded as CMCC – 2%, treatment PCPs were coded as RCMCC-1.5%, and RCMCC – 1%.

4.2.3 Process cheese product (PCP) formulations

The ingredients used in each formulation are shown in Table 1. The ingredients used in making PCP were aged Cheddar (Great Value, Extra Sharp Cheddar Cheese, Bentonville, AR), MCC, water, unsalted butter (Land O Lakes Half Stick Unsalted Butter, INC., Arden Hills, MN), deproteinized whey (Bondgrads' Creameries, Perham, MN), dibasic sodium phosphate (Fisher Scientific, Fair Lawn, New Jersey), sodium chloride salt (Cargill, Minneapolis, MN), and trisodium citrate (KIC Chemical Inc., New Paltz, NY). Techwizard, an Excel-based-formulation software program, develops the PC formulations (Metzger et al.) provided by Owl Software (2301 Wood Street, Lancaster, PA). Each formulation was balanced for moisture, fat, protein, and salt at 48, 20, 19, and 1.5%, respectively. In addition, the protein content was balanced between cheddar cheese and MCC to get a ratio of 1:2, respectively. Disodium Phosphate and deproteinized whey powder were standardized in each formula depending on each MCC treatment to have the same composition in all formulations.

4.2.4 Process cheese manufacture

Pre-blend preparation

All ingredients (Table 2) were weighed and blended in a KitchenAid at room temperature for approximately 30-40 min to get a homogenous paste. Then, 300 g of each formula was prepared to make the PCP.

Cooking in the rapid visco analyzer (RVA)

A 25g sample of the paste was weighed in a canister when thoroughly mixed the blend. The canisters were tempered at 38°C/15-20 min in a water bath before being manufactured in the RVA (Perten RVA 4500, Macquarie Park NSW 2113, Australia). The canisters were then cooked in the RVA for 4 min at 90°C. The stirring speed was 1000 rpm for the first 2 min and 160 rpm for the last 2 min. The cooked PCP was poured in copper cylinders (20 mm diameter × 30 mm height) for texture profile analysis (TPA) and

plastic molds (28.3 mm diameter × 25 mm height) for dynamic rheological analysis (DSR.). Then the cylinders and molds were sealed with aluminum foil and kept at four °C for the next day for further analysis. Three replicates of PCP from each HC-MC treatment were manufactured.

4.2.5 Chemical analysis

MCC powders were analyzed for Ash (AOAC, 2000; method 945.46; 33.2.10), TS (AOAC, 2000; method 990.20; 33.2.44), total nitrogen TN (AOAC, 2000; method 991.20; 33.2.11), and fat (Mojonnier method: Atherton and Newlander, 1977) before being utilized in PCP formulations. Also, the PCP samples were analyzed for fat using the Mojonnier method (Atherton and Newlander, 1977), total solids content using a forced draft oven (model OV-490A-2; Blue M, Blue Island, IL), salt content using a Nelson Jameson Corning 926 chloride analyzer (Nelson Jameson Inc, Marshfield, WI), pH using a Corning pH/ion meter model 450 (Corning Glass Works, Medfield, MA) fitted with a Thermo Orion combination pH probe (Thermo Electron Corporation, Louisville, CO),

4.2.6 Analysis of PCP functional properties

RVA Cooked apparent viscosity

The cooked apparent viscosity of the PCP was measured at 90°C at the end of cooking time in the RVA by calculating the mean of the last five values of viscosity (Figure 1). This test was repeated six times for each replicate.

Texture profile analysis (TPA)

Texture profile analysis (TPA) was used to determine the hardness of the PCP samples. The PCP samples were removed from the copper molds and cut into cylinders (20 mm high) using a wire cutter. The TPA was performed using a T.A.XT-Plus Texture Analyzer (TA.XT-Plus, 6 Patton Drive, South Hamilton, MA). The following conditions were applied: Uniaxial 10% double bite compression, 50-mm diameter cylindrical flat probe (TA-25), and one mm/s crosshead speed. The maximum force during the first

compression was referred to as the hardness of PCP (Figure 2). TPA was performed on six samples of each replicate.

Dynamic rheological analysis (DSR)

A dynamic rheological analysis was performed using a rheometer (MSR 92, Anton Paar, Graz, Austria) to analyze PCP meltability using 25-mm parallel plate geometry. The DSR test was done using the modified method described by (Sutheerawattananonda and Bastian, 1998). The sample was prepared by removing the PCP partially from the plastic molds and then cutting it into slices (2 mm thick) using a wire cutter. All cheese samples were tempered at room temperature for 10 minutes before the test. Initially, a stress sweep test for PCP was performed at a frequency of 1.5 Hz and a range of 1 to 1000 Pa stress at 20°C using the rheometer (MSR 92, Anton Paar, Graz, Austria) with parallel plate geometry. The stress sweep experiment determined that the maximum stress limit for the linear viscoelastic region was 50 Pa. The DSR properties of the PCP were then analyzed using a dynamic temperature ramp test. The ramp test was performed using the same rheometer at a temperature ranging from 20 to 90°C with a ramp rate of 1°C/min using a frequency of 1.5 Hz and the constant stress of 50 Pa (linear viscoelastic region). Elastic modulus (G′), viscous modulus (G″), tangent angle (tan δ), and melt temperature were determined. The temperature at which tan δ=1 (G″/ G′) was referred to as the cheese melt temperature (Figure 3). DSR test was performed in duplicates.

4.2.7 Statistical analysis

The treatments were run in triplicates, and the chemical analyses were run at least in duplicate. The values of the replicates were presented as mean ± standard deviation (SD). Minitab® (v.20.4, Minitab Inc., State College, PA) was used to assess significant differences between the group means. A one-way analysis of variance (ANOVA) was used, followed by the Tukey post hoc comparison test. *Statistical significance* was defined as a p-value of less than 0.05.

4.3 Results and Discussion

4.3.1 PCP composition and chemical properties

The principal goal of this study was to study the impact of lower calcium MCC (RCMCC) on the lowering of the ES needed and compare its functional properties with corresponding control (CMCC), which is determined by comparing the results of CMCC-2% Vs. RCMCC-1.5%, i.e., at 25% E.S. reduction and CMCC-2% Vs. RCMCC-1 %, i.e., at 50% reduction in E.S.

The general composition and the mineral concentration of MCC powders thus produced are summarized in Table 2. The total protein content of the C-MCC and RC-MCC powders is ~80% (wt./wt.), qualifying the product as MCC80. There was no significant difference between the true protein content (and the casein) in C-MCC and RC-MCC, and the results were 77.81% (71.74%) and 78.99% (72.16%), respectively. Similarly, there were no significant differences in the rest of the general chemical composition, i.e., total solids, whey protein, fat, and lactose. However, using a novel MF-UF process and injecting CO2 in the treatment, the calcium content was significantly reduced in RC-MCC powder (31.01%) compared to control MCC powders. In addition, the ash and calcium contents were significantly lower in RC-MCC, 7.06 % and 1727 mg/100g, respectively, compared to MCC, 8.15% and 2503 mg/100g, respectively.

The mean composition values for total solids, protein, fat, pH, and ash are shown in Table 3 for the control (CMCC-2%) and 2 PCP treatments (RCMCC-1.5% AND RCMCC-1%). The chemical analysis results of the PCP formulas showed no significant difference ($P > 0.05$) for total solids, protein, and fat content among the 3 PCP treatments. No significant ($P > 0.05$) differences in total solids were observed among the three PCP samples, even with different ES levels. Therefore, total solids were adjusted to be the same for all formulations using deproteinized whey powder. pH results showed no significant ($P > 0.05$) differences among the three PCP samples even with different Ash and ES levels.

The pH of the samples was adjusted by adding Lactic acid in the 25f pre-blend weighed into the RVA canister before the PCP cooking. As expected, significant differences ($P < 0.05$) were observed in ash content among the 3 PCP samples due to differences in ES content and lower ash content in RCMCC powder samples. The lower calcium content in treatment formulations (RCMCC-1.5% and RCMCC-1%) as shown in the formulations Table 1 might influence the functional properties as the total calcium content of a processed cheese not only plays a role during its manufacture but also influences its final functional properties (Kapoor and Metzger, 2008).

4.3.2 Functional properties

Hardness

TPA-hardness is an important parameter to PCP and measure of its unmelted characteristics.). Texture in food is affected by strong (covalent) and weak (hydrophobic, hydrogen, and electrostatic) bonds and their optimum balance in the matrix (Ercili-Cura et al., 2010). The mean values of the hardness of PCPs measured by TPA are shown in Table 4. The hardness of control (CMCC-2%) and treatments RCMCC-1.5% and RCMCC-1% PCPs is 127.67, 105.14 and 80.18 g, respectively. The control C-MCC hardness was significantly ($P < 0.05$) higher than both 25% reduced ES RCMCC-1.5% and 50% reduced ES RCMCC-1% PCPs. This significantly higher hardness of the control PCP formulation might be due to higher calcium content in this formulation as explained by Kapoor and Metzger 2008 that the total calcium content of a processed cheese plays a role during its manufacture also influences its final functional properties. In a study performed by (Cavalier-Salou and Cheftel, 1991) on cheese analogs using sodium caseinate, they found that their firmness increased as the calcium content of the cheese analogs increased. Hardness is significantly ($P < 0.05$) different between two treatments formulations RCMCC-1.5% and RCMCC-1%. The hardness of the 1.5% ES RCMCC-1.5% PCP is higher than 1% ES RCMCC-1% PCP. This difference in hardness between the two

treatment PCP samples was due to their ES level in the formulas. These results agree with previous studies by (El-Bakry et al., 2010) who reported higher hardness with a higher emulsifying salt level in imitation process cheese studies.

DSR Melt temperature

The DSR melt test measures the initial melt characteristics of PCP and indicates molecular interactions. The DSR melt temperature has been used to quantify the melting characteristic of process cheese. DSR was used to measure the melt temperature where tan δ (G''/G') = 1 is a convenient measure of the melting point of PCP because this is the lowest temperature where a material changes from primarily elastic to primarily viscous (Sutheerawattananonda and Bastian, 1998; Prow and Metzger, 2005). The mean values of melt temperature (°C) of PCP are shown in Table 4. The DSR melt temperature of control (CMCC-2%) and treatments RCMCC-1.5% and RCMCC-1% PCPs is 86.73, 65.80, and 59.63 °C respectively.

The melt temperature of CMCC-2% is significantly ($P < 0.05$) higher than the treatment PCPs RCMCC-1.5% and RCMCC-1%. The high melt temperature of CMCC-2% may be related to the high insoluble calcium in this formulation. These results agree with the previous studies that concluded that higher insoluble calcium leads to the formation of heat-induced irreversible gel and produces a PCP with higher restricted melt characteristics (Purna et al., 2006; Kapoor and Metzger, 2008; Kammerlehner, 2009). Like viscosity and hardness results above, the melt temperature of RCMCC-1.5% is significantly ($P < 0.05$) higher than the RCMCC-1%, which might be due to differences in ES content of these formulas as explained above. This lower melt temperature in lower ES PCPs results agrees with the Guinee and Kennedy, 2012 who reported that, as added DSP was increased, increase in the water solubility of the protein, which would favor increases in the degree of fat emulsification and viscosity of the aqueous phase. In contrast, a

reduced degree of solubilization of the protein at the lower DSP levels would result in a more liquid aqueous phase with less meltability of the PCP

RVA Cooked apparent viscosity

The mean values of cooked viscosity (cP) of PCP determined by the RVA are shown in Table 4. The viscosity of control (CMCC-2%) and treatment RCMCC-1.5% and RCMCC-1% PCPs is 627.30, 889.0, and 317.33 cP, respectively. The 25% reduced ES RCMCC-1.5% had significantly ($P < 0.05$) higher viscosity that control CMCC-2%, However the 50% reduced ES treatment RCMCC-1% has significantly ($P < 0.05$) lower viscosity than control CMCC-2% and treament-1 RCMCC-1.5%.

The higher viscosity in RCMCC-1.5% compared to C-MCC-2% might be due to the higher emulsifying capacity of the soluble caseins in RCMCC. The RCMCC powder used in this formula has 30% less calcium than the CMCC powder used in the control PCP formula. This reduced calcium RCMCC powder should have higher soluble caseins in this formulation that help form a stronger emulsion. During PCP manufacture, when soluble proteins like MCC from membrane filtrations are used, due to high negative charge on the surface, the emulsifying action is poor, causing a protein network in PCP to be weaker (Kapoor and Metzger, 2008; Guinee and O'Kennedy, 2012; Salunke and Metzger, 2022). However, modifying casein by reducing calcium makes MCC more soluble and readily available for emulsification as there is less calcium to be chelated by emulsifying salt in its PCP manufacture. When casein is hydrolyzed into small peptides that are easily hydrated and dispersed during process cheese manufacture, and under normal process cheese manufacturing conditions, extensive protein-based interactions occur, leading to a strong protein network with restricted flow properties (Meyer, 1973; Purna et al., 2006). The significantly lower ($P < 0.05$) viscosity of RCMCC-1% when compared to RCMCC-1.5% might be due to lower emulsifying salt at the same level of calcium in both formulations as increased Disodium phosphate levels are likely to be associated with the increase in the

water solubility of the protein, which would favor increases in the degree of fat emulsification and viscosity of the aqueous phase (Guinee and O'Kennedy, 2012)

4.4 Conclusion

Compositional analysis showed no significant difference between treatments except for the significantly lower ash content among three PCP samples tested. PCP formula made with reduced calcium MCC at 25% less emulsifying salt than control PCP had improved the functional characteristics. Using reduced calcium MCC, PCP manufactured with 25% less emulsifying salts showed a significant decrease in hardness, improved meltability, and optimal viscosity, confirming improved emulsification in the process of cheese products. Overall, compositional, and functional analyses indicated that PCP formula with reduced calcium MCC at 25% less emulsifying salt had improved functional properties. However, PCP formula with reduced calcium MCC at 50% less emulsifying salt results in suboptimal functional characteristics. Consequently, a 30% reduced calcium MCC powder could be used to partially replace emulsifying salt up to 25% in PCP manufacture.

4.5 References

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4.6 Figures

Figure 4.1. Measuring the apparent cooked viscosity of process cheese products (P.C.P.) by using the R.V.A.

Figure 4.2. Measuring the hardness of process cheese products (P.C.P.) by using the T.P.A.

Figure 4.3. Measuring the melting point of process cheese products (P.C.P.) by using the DSR

Figure 4.1

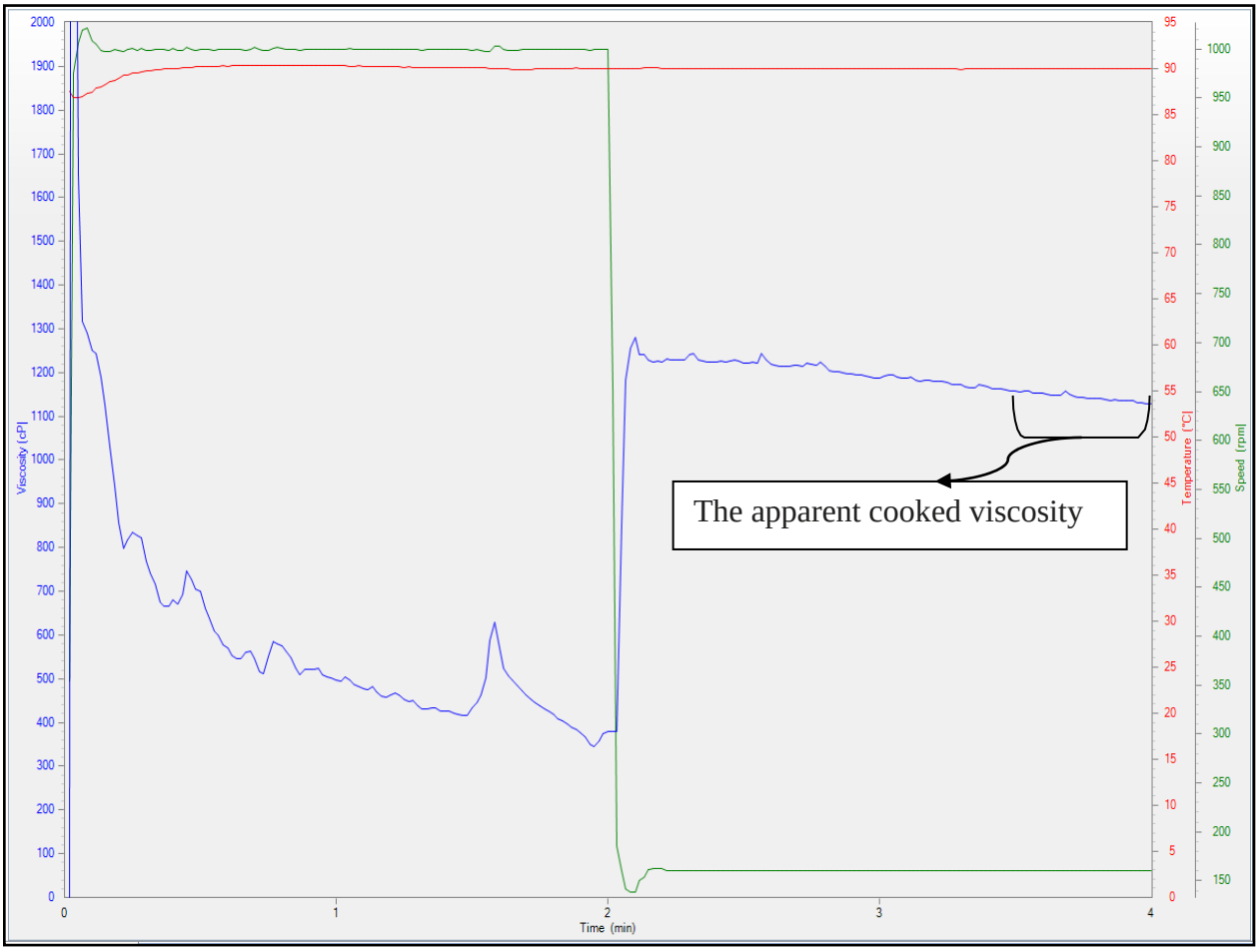

Viscosity [cP]
Temperature [°C]
Speed [rpm]
Time (min)
The apparent cooked viscosity

Figure 4.2

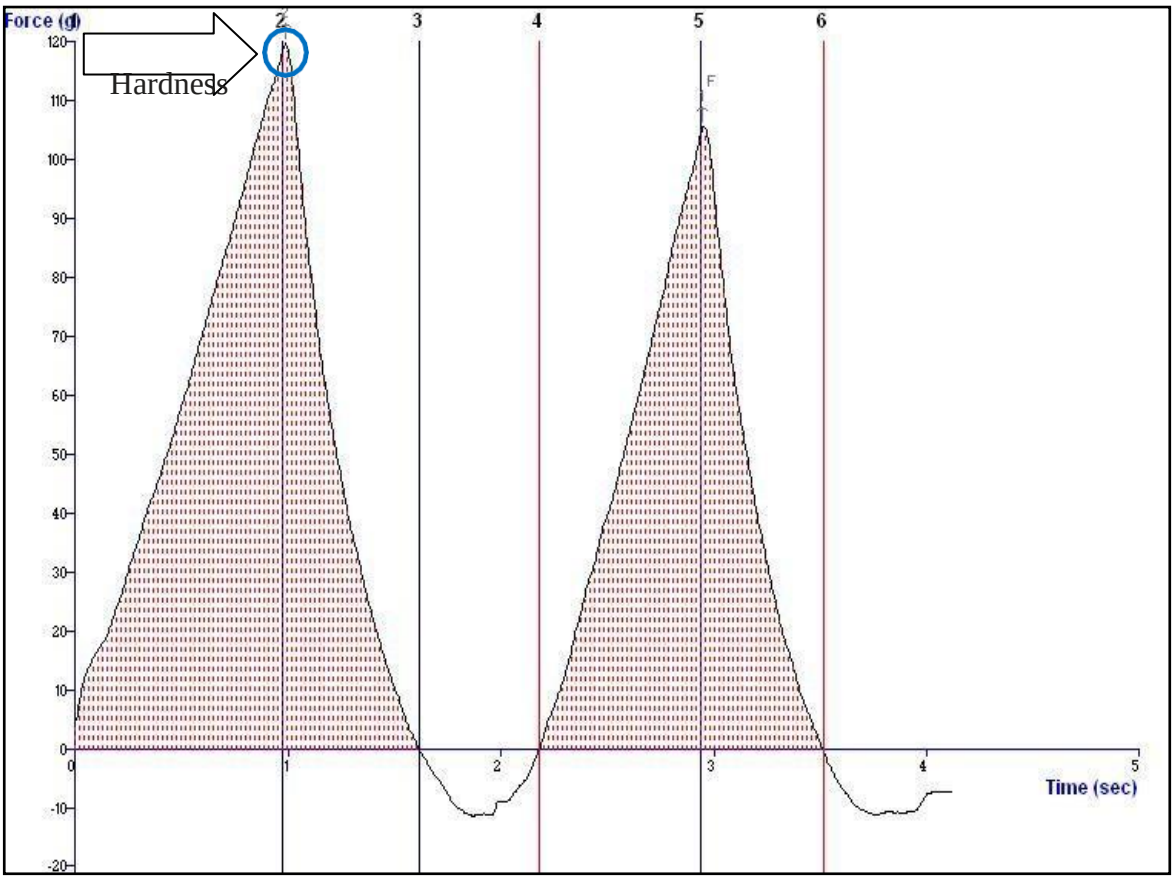
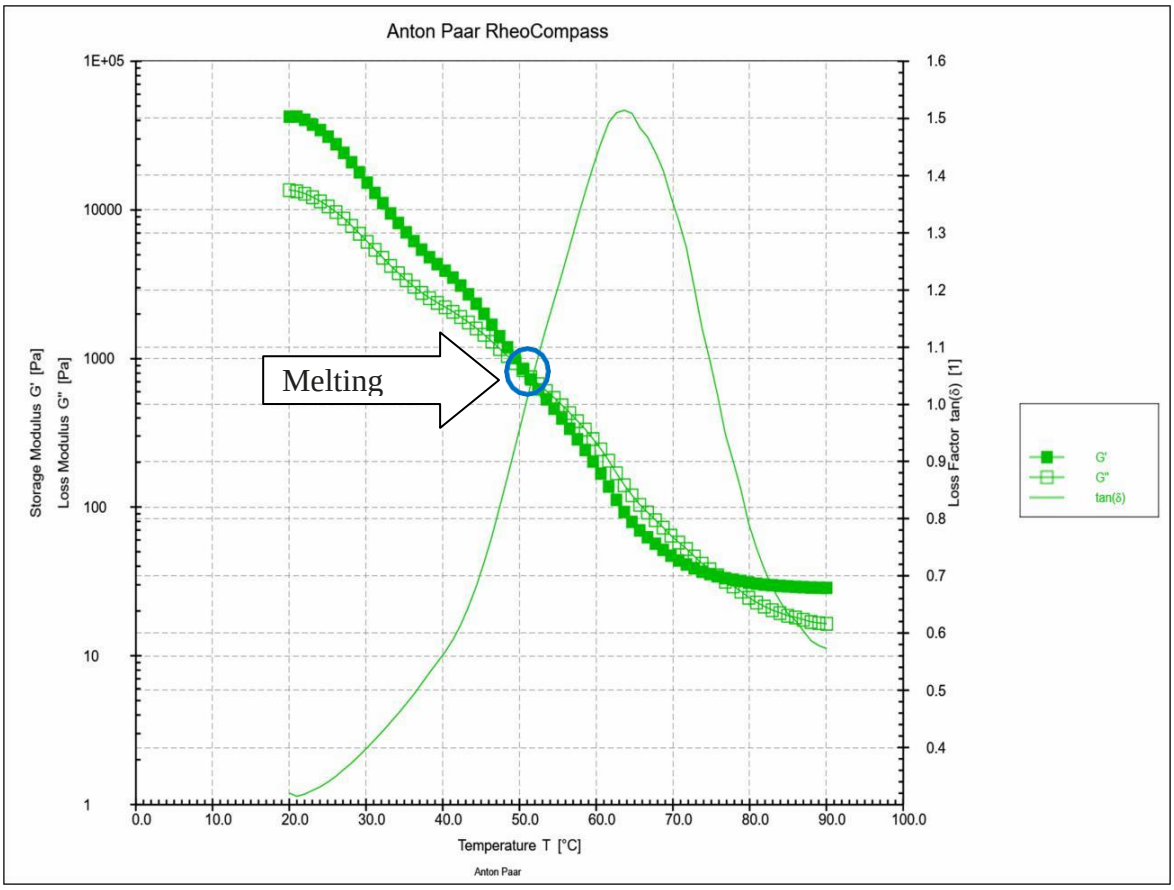


Figure 4.3


Anton Paar RheoCompass
Melting
Storage Modulus G' [Pa]
Loss Modulus G'' [Pa]
Loss Factor tan(δ) [1]
Temperature T [°C]
G'
G''
tan(δ)
Anton Paar

4.7 Tables

Table 4.1. Mean (n=3) chemical composition of the micellar casein concentrate powders (% wt./wt.)

Powder[1]	Total Solids	Fat	True protein	Casein	Ash, %	Ca, mg/100g	Ca reduction, %
C-MCC	95.51 ± 0.11[a]	2.98 ± 0.41[a]	77.81 ± 0.88[a]	71.74 ± 1.13[a]	8.15 ± 0.21[a]	2503 ± 0.09[a]	-
RC-MCC	95.34 ± 0.27[a]	2.91 ± 0.61[a]	80.00 ± 0.51[a]	72.16 ± 0.40[a]	7.06 ± 0.42[b]	1727 ± 0.10[b]	31.01 ± 3.39

[1] Abbreviations are: C-MCC, control micellar casein concentrate powder; RC-MCC, calcium reduced micellar casein concentrate powder. Ca, Calcium

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 4.2. Mean (n=3) of Process cheese products (PCP) formulations

Ingredients	Process cheese Products formulas		
	Contr	T1	T2
	CMC	RCMCC	RCMC
Aged Cheddar cheese	27.00	27.00	27.00
Unsalted Butter	13.01	13.04	13.04
Water	35.00	35.64	35.62
Salt	1.50	1.50	1.50
Deproteinized whey	6.58	6.59	7.15
C-MCC Powder	14.91	-	-
RC-MCC Powder	-	14.70	14.70
Disodium Phosphate	2.00	1.50	1.00
		COMPOSITION	
Calcium, mg/100g	607	475	478

C-MCC, control micellar casein concentrate powder;

RC-MCC, 30% calcium reduced micellar casein concentrate powder

Table 4.3. Mean (n=3) mineral composition of the process cheese products.

Process cheese product composition			
	Control	Treatments	
	CMCC-2%	RCMCC-1.5%	RCMCC-1%
Total Solids, % w/w	53.56 ± 0.30[a]	53.35 ± 0.53[a]	53.36 ± 0.49[a]
Protein, % w/w	19.91 ± 0.12[a]	20.46 ± 0.74[a]	20.17 ± 0.25[a]
Fat, %w/w	20.99 ± 0.15[a]	21.45 ± 1.22[a]	21.19 ± 0.45[a]
Ash, % w/w	5.46 ± 0.18[a]	5.03 ± 0.13[b]	4.71 ± 0.20[C]
pH	6.01 ± 0.05[a]	6.03 ± 0.06[a]	6.00 ± 0.05[a]

[1] Abbreviations are: CMCC-2%, control micellar casein concentrate 2% ES PCP; RCMCC-1.5%, 30% reduced calcium micellar casein concentrate 1.5% ES PCP; RCMCC-1%, 30% reduced calcium micellar casein concentrate 1% ES PCP,

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

Table 4.4. Mean (n=3) functional properties of the process cheese products.

Process cheese functional properties			
	Control	Treatments	
	CMCC-2%	RCMCC-1.5%	RCMCC-1%
TPA Hardness, g	127.67 ± 5.24[a]	105.14 ± 10.93[b]	80.18 ± 14.13[c]
DSR Melt temperature, °C	86.73 ± 0.29[a]	65.80 ± 3.15[b]	59.63 ± 2.54[c]
RVA Viscosity, cP	627.30 ± 32.3[b]	889.00 ± 34.70[a]	317.33 ± 11.59[c]

[1] Abbreviations are: CMCC-2%, control micellar casein concentrate 2% ES PCP; RCMCC-1.5%, 30% reduced calcium micellar casein concentrate 1.5% ES PCP; RCMCC-1%, 30% reduced calcium micellar casein concentrate 1% ES PCP,

[a,b] Mean ± SD values not sharing a common superscript within the same column are significantly different ($P < 0.05$).

FUTURE STUDIES

Future studies might help achieve higher calcium reduction in micellar casein than the 30% reduction achieved in this study. Higher calcium reduction may drive further improvement in MCC functional properties. Also, a higher reduction in micellar calcium may help the complete replacement of emulsifying salts in process cheese products vs. partial reduction in ES achieved in this study. It would also be helpful to study the impact of sodium hydroxide vs. potassium hydroxide as a pH adjuster in reduced calcium MCC. Further studies on soluble casein fractions and micelle rearrangement mechanisms in reduced calcium MCC might provide insights into casein chemistry.