Stability of Proteins and Organic Acids in Production of Milk Protein Concentrate (MPC) and Milk Protein Isolate (MPI) and Effect of Temperature and Time on Functional Properties of MPC and MPI

A Thesis

Presented in Partial Fulfillment of the Requirements for the
Degree of Master of Science
with a
Major in Animal Science
in the
College of Graduate Studies
University of Idaho
by

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August 2021

Authorization to Submit Thesis

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Abstract

Milk is a complex food containing many constituents in a variety of forms, like fat in an emulsion, proteins in colloidal dispersion, and lactose and minerals in a soluble phase. Each milk component has its own biological, nutritional, and technological significance. Milk is one of the most complete foods nutritionally being rich in protein, fat, carbohydrates, vitamins and minerals essential for sustaining life and maintaining good health. Milk proteins contribute to functional properties such as solubility, emulsion formation, and foaming ability and stability for products in food systems utilizing dairy ingredients. Processing such as heating and drying during production of milk protein concentrates (MPC) can affect milk proteins. MPCs have become common dairy ingredients because of their nutritional quality and functionality. A variety of organic acids (e.g., lactic, citric, and orotic) are also present in milk. Organic acids are important because they contribute to the flavor and aroma of dairy products. Organic acids are also used as additives to stabilize or increase the palatability of dairy products. Many organic acids in dairy products originate from the metabolism of larger organic compounds such as lipids, proteins, and carbohydrates. Stability of proteins and organic acids during production of MPC was evaluated. The quantity of various proteins and organic acids in raw milk, nanofiltration product (NF) and MPC-80 and milk protein isolate (MPI)-85 powder was analyzed by reverse phase-high performance liquid chromatography. MPC-80 is a specific dairy ingredient category containing 80% protein on “as is” basis while MPI-85 is another category containing 85% protein on a “dry matter” basis. Concentrations of the major caseins (α-CN and β-CN) were increased in NF compared to raw milk and further increased in MPC-80 and MPI-85. The concentration of κ-CN was greater in NF compared to raw

milk but not different in MPC-80 or MPI-85. Most whey proteins (α-lactalbumin, β-lactoglobulin, IgM) were similar in concentration between raw milk and MPC-80 as well as between raw milk and MPI-85. The major caseins (α-CN and β-CN) were greater in concentration in MPI-85 than MPC-80.

The effects of storage temperature and time on the solubility of MPC-80 and MPI-85 were investigated using solubility tests, emulsification, foaming ability and stability, viscosity and gelation time on powder stored at 25 ℃ and 55 ℃ for 49 d. Over time, storage at 55 ℃ compared to 25 ℃ reduced solubility of MPC-80 and MPI-85 but no difference was detected in solubility between MPC-80 and MPI-85. Emulsions made from either MPC-80 or MPI-85 stored at 25 °C had no visibly detectable separation or instability but emulsions stored at 55 ℃ had clear separation of cream and water with sedimentation of powder. Viscosity increased across all days of storage in MPC-80 and MPI-85 stored at 55 ℃ compared to that stored at 25 ℃. Foaming ability and stability of MPC-80 and MPI-85 were reduced during storage at 55 ℃, presumably due to the reduction in powder solubility. Gelation time increased in MPC-80 and MPI-85 stored at 55 ℃ compared to powder stored at 25 ℃. Overall, greater storage temperature adversely affected functionality of MPC-80 and MPI-85 with little difference between the milk powders. Preventing reductions in functionality of milk protein concentrates during storage at higher temperatures would likely aid in the economic value of these dairy ingredients.

Key words: milk, milk protein concentrate, protein, organic acids, functionality

Acknowledgements

I would like to acknowledge the tireless efforts of Dr. Mark McGuire for his guidance and patience throughout the process as well as for the opportunity to pursue my Master of Science in Animal Science in the United States. Furthermore, I would like to thank my committee, Dr. Michelle McGuire, Dr. Brennan Smith, and Dr. Mireille Chahine, for their guidance and support. I would like to acknowledge Idaho Milk Products for helping with the product and especially Dr. Chenchaiah Marella and Dr. Venkat Sunkesula for their unconditional support and advice while conducting research. I would like to thank my lab members, especially, Haley Peterson, Dr. Janet Williams, and Dr. Brennan Smith for allowing discussion, listening to my problems, and helping me in scheduling, planning, and conducting my experiments. I would like to thank Dr. Mostafa Taghvaei for giving me firsthand experience with HPLC and helping me understand the basic concepts. I am grateful to Dr. William Price for help in understanding the statistical analysis of the data.

Dedication

This work is dedicated to my parents, who have been my force to work towards my dreams

and

My friends in Moscow, Idaho, USA who have always supported me and made me feel at home during my master's degree.

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Chapter 1: Literature Review

1.1 Introduction

Milk is a complex food that contains a variety of elements in different forms, including fat in an emulsion, proteins in colloidal dispersion, and lactose and minerals in a soluble phase. Each milk component has its own biological, nutritional, and technological significance. Quantitative analysis of organic acids and proteins is used to monitor bacterial growth and activity, and for assessment of nutritional value. Protein, being a source of amino acids, is a necessary nutrient in the human diet throughout the rest of one's life. Milk proteins are used in a wide range of foods, as they provide functional properties such as viscosity, gelation, and foaming. Individual casein (β-Casein, κ-Casein, α-Casein) and whey proteins (α-lactalbumin, β-lactoglobulin) are known to affect the nutritional and technological properties of milk as a result of qualitative and quantitative variation. Many organic acids (e.g., lactic, citric, and orotic) are also present in milk. Organic acids are important in dairy products because they add to the taste and flavor. A wide range of dairy beverages utilizes milk protein concentrate (MPC) to improve functional, nutritional, and sensory properties. Several variables, including raw milk composition, drying and storage conditions, and physicochemical and biochemical changes such as lactose crystallization, Maillard reaction, and oxidation, might negatively impact MPC's overall functional properties. Therefore, it is important to perform quantitative analysis of milk and MPC powder for proteins and organic acids to understand the impact of raw milk processing, the changes associated with storage, and the technological functionality when applied in food formulations. The following literature review, therefore, covers milk and the chemistry of

major components of milk, namely proteins and organic acids, potentially impacted by the manufacturing of MPC, and affecting the functionality of MPC.

1.2 Milk

Milk and milk products are an important part of the Western diet. Milk has many appealing properties as a dietary item serving as a major source of dietary energy, protein, and fat, and contributing on average 134 kcal of energy/capita per day, 8 g of protein/capita per day and 7.3 g of fat/capita per day (FAOSTAT, 2012). The FAO (2008) correctly predicted the demands for milk in developing countries would increase 25 percent from 2008 to 2025. While humans consume the milk of many species (e.g., goat, sheep, buffalo), bovine (*Bos taurus*) milk is the most economically important (Chandan, 1997). The nutritional value and technical qualities of milk and milk products are heavily influenced by milk composition. In addition, composition is an important part of the price farmers receive. The composition of milk, therefore, has a great deal of significance for the dairy industry.

Milk is considered a near-complete food in nature because it contains nearly all essential nutrients required for growth and development in adequate and assimilable forms (Chandan, 1997). Milk can be described in many ways. Chemically speaking, milk is a complex fluid containing many different chemical compounds (Chandan, 1997). Water, fat, lactose, proteins, fatty acids, amino acids, and minerals are the main components of milk with concentrations of each component varying across animal species. From a visual point of view, milk is an opaque, whitish fluid with multi-disperse phases in terms of physical chemistry. Lactose, some inorganic salts, vitamins, organic acids, and enzymes are present in the real solution, while caseins, calcium phosphate, and globular proteins are

present in colloidal form. Fat occurs in the form of an emulsion style of oil-in-water, with fat globules ranging in diameter from 0.1 to 22 μm. Milk is an excellent source of nutrients for the human consumer when eaten alone or included as an ingredient in other foods (Chandan, 1997).

Worldwide, bovine milk is by far more commercially available than any other mammal's milk. In the United States, the word "milk" legally refers to cow's milk (Division of the Federal Register, the National Archives, 1943) although plant-based drinks are challenging the definition. Milk from other species, such as sheep or goats, is labeled to indicate its specific kind. By definition, milk is the lacteal secretion from healthy cows, with collections starting three to five days after calving. From a legal point of view, colostrum, the secretion immediately after giving birth, is not considered milk. The U.S. Public Health Service's definition of Grade A milk refers to milk produced under adequately hygienic conditions and nearly colostrum-free lacteal secretion acquired during entire milking of one or more healthy cows, including at least 8.25% solids-not-fat (SNF) and at least 3.25% milkfat (Grade “A” PMO, 2017).

Regarding nutrition, milk is one of the most complete foods because it possesses nearly all of the recognized nutrients essential for maintaining a healthy lifestyle and body. Proteins specific to milk have all the major amino acids required for building tissues and the repair of damaged cells in our body. Finally, milk has a pleasant and appealing taste and mouthfeel for most consumers due to the presence of complex macromolecules, such as colloidal proteins and fat globules.

1.3 Milk Composition

Commercial bovine milk contains water, milk fat, and milk solid non-fat (SNF) components including protein, lactose, and minerals (Figure 1.1). A portion of the solids are also called skim-solids or serum solids, when the fat is removed. The term total solids refer to SNF plus milk fat.

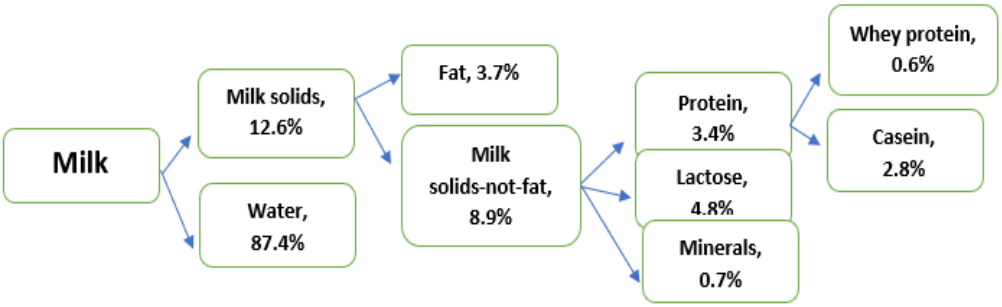


Figure 1.1. Typical composition of major constituents of raw bovine milk found in the commercial industry (Adapted from McSweeney and Fox, 1998).

The composition of milk varies widely in milk among cows due to health, stage of lactation, parity, feed components, and genetics (Fox and McSweeney, 1998; Table 1.1). Several other factors influence milk composition, such as breed, milking times, milking phases, different quarters of udder, lactation time, season, ambient temperature, health status, parity, nutrition, age, environment, estrus, pregnancy, and energy expenditure (Goff et al., 1993; Wong et al., 1998). In general, variation in milk composition significantly impacts properties of the resulting dairy products.

Table 1.1. Composition of bovine milk[1]		
Component	Average Content (Wt %)	Range (Wt %)
Water	87.3	85.5 - 88.7
Fat	3.9	2.4 - 5.5
Protein	3.25	2.3 - 4.4
Casein	2.6	1.7 - 3.5
Whey	0.65	0.40 - 0.65
Lactose	4.6	3.8 - 5.3
Minerals	0.7	0.53 - 0.80
Organic acids	0.18	0.13 - 0.22
Other	0.14	0.10 - 0.19
[1]Values are mean values of a range of different components of milk of dairy cows (adapted from Walstra and Jenness, 1984).		

Milk proteins can provide essential nutrients to the human consumer throughout life (Wedholm et al., 2006). The provision of nutrients can be direct through consumption of fluid milk or other dairy products. However, use of dairy products in various foods to provide viscosity, gelation, foaming ability, and stability can support other sources of consumption. Milk protein composition is especially important for the technological properties of milk. Cheese yield, for example, is directly related to the amount of casein in milk (Wedholm et al., 2006). Milk with greater casein content (i.e., a higher casein number) yields more cheese than milk with the same total protein content but less casein (Wedholm et al., 2006). Major milk proteins include caseins such as β-casein (β-CN), κ-casein (κ-CN), α-casein (α-CN), and whey proteins such as α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). Other minor milk proteins such as immunoglobulins (Ig) and bovine serum albumin (BSA) are categorized in the whey protein fraction. As a result of qualitative and quantitative differences, these proteins are known to impact the nutritional and

technical aspects of milk. Organic acids and a range of vitamins that do not fit conveniently into the four major categories (protein, fat, minerals, and carbohydrates) are also found in bovine milk. Information regarding the presence of organic acids is important in understanding the quality of milk products and potentially the metabolic status of the cow which produced the milk. Organic acids appear in milk as a result of fatty acid breakdown, natural bovine metabolic processes, bacterial development, or direct addition as acidulants during processing (Tormo and Izco, 2004). Little attention has been given to the presence of organic acids (e.g., citric, lactic, acetic) in milk (Mullin and Emmons, 1997) likely since price of raw milk does not consider these components and the impact on milk processing is limited. Quantitative determination of organic acids has value in monitoring bacterial growth and activity of milk and dairy products, but is limited for nutritional value. Contribution to flavor and aromatic characteristics of milk, most cheeses, and other dairy ingredients suggest more assessment of organic acids in milk could provide improved consumer acceptability (Fox, 1993; González de Llano et al., 1996).

1.4 Milk Proteins

Research into milk proteins has a long and honorable history. Early research on milk proteins can be traced back to 1814 when Berzelius published a paper on the subject. A critical assessment of milk proteins was the primary method of separation of caseins devised by Braconnot in 1830 (discussed by Wisniak et al., 2007). In the early investigations of milk, identified casein was recognized as the primary component of milk protein, with little attention paid to the comparatively weak solution of proteins in the supernatant (whey) after casein was precipitated from the solution. Rowland (1938) described the general complexity and heterogeneity of the milk protein system still used

for the most part today (Table 1.2). Milk proteins were understood to consist of caseins, α-lactalbumin, β-lactoglobulin, protease peptone, and non-protein nitrogen representing approximately 78%, 12%, 5%, 2%, and 3%, respectively, based on nitrogen concentration (Rowland, 1938). As one of the major components of milk (30-36 g/L), proteins play a vital role in the functional aspect of milk as a food and during processing of various dairy products including food ingredients (Fox and McSweeney, 1998; Swaisgood, 1992, Swaisgood, 1996; Walstra and Jenness, 1984). Milk proteins play significant physiological roles within the human body relating to the uptake of nutrients and vitamins and they are a source of biologically active peptides (Table 1.2).

Table 1.2. Major bovine milk proteins and some of their biological functions[1]

Proteins	Nomenclature	Concentration (g/L of milk)	Major biological functions
Casein		24 - 28	Mineral carrier and peptide source
	α_{s1}-casein	12 - 15	
	α_{s2}-casein	3 - 4	
	β-casein	9 - 11	
	κ-casein	3 - 4	
	γ-casein	1 - 2	
Whey Protein	β-lactoglobulin	2 - 4	Retinol carrier, antioxidant and vitamin−A−binding protein
	α-lactalbumin	1 - 1.5	Lactose synthesis in mammary gland
	Bovine serum albumin	0.1 - 0.4	Peptide source
	Immunoglobulins	0.6 - 1.0	Immune protection

[1]Adapted from Korhonen and Pihlanto (2007) and Robin et al. (1993).

Milk proteins are classified as either casein or whey proteins based upon response to addition of acid (reduced pH). The proteins (about 80% of the total) that precipitate out of suspension when milk is acidified to pH 4.6 (isoelectric point) are called caseins. Whey is the liquid that remains after casein precipitation from skimmed or whole milk. It is a dilute solution of proteins that make up around 0.7 percent of bovine milk (Table 1.2, O'Mahony and Fox, 2013). Caseins provide a complete protein source for the human consumer. Their presence in milk impacts physical properties as well as caseins cluster together with calcium and phosphate to form tiny particles called micelles. When light hits casein micelles, the light refracts and scatters resulting in milk appearing white (Walstra et al., 2006). Caseins are the most commonly used milk protein in the food industry today (Dairy Australia, 2013; World Casein & Caseinates Market, 2014; FAO, 2013).

In addition to being a complete protein, whey protein contains biologically active components that enhance human health. In particular, whey protein is rich in the amino acid cysteine. This amino acid may enhance concentrations of glutathione, which has been shown to have strong antioxidant properties combating various diseases in the human body (Bounous et al., 2000). Whey protein also contains a high concentration of branched chain amino acids important in the maintenance of tissue and prevention of catabolic actions during exercise (MacLean et al., 1994). In addition, whey protein positively affects immune function through some minor proteins (Ha and Zemel, 2003).

1.4.1 Milk Protein Chemistry

Functional properties of milk proteins occur due to intrinsic properties (amino acid composition and sequence, molecular weight, genetic variation, charge, hydrophobicity, and protein configuration) and extrinsic factors (temperature, pH, salts, and concentration) present (Henning et al., 2006; Kinsella et al., 1984). Milk protein plays a significant role in the structural and rheological characteristics of dairy products with common dairy processing techniques such as heating and drying affecting the functionality of milk proteins in foods (Henning et al., 2006; Kinsella et al., 1984). The typical quantity and molecular weight of the various protein fractions in milk are shown in Table 1.3.

Table 1.3. Quantity and molecular weight of protein fractions in bovine milk[1].			
Protein fractions	g/L	% of Total Protein	MW (Daltons)
Total protein	33 - 34	100	
Total casein	26 - 27	79.5 - 80.5	
α_{S1}-casein	10	30.6	23,600
β-casein	9.3	28.4	23,983
α_{S2}-casein	2.6	8	25,200
κ-casein	3.3	10.1	19,550
γ_1-casein	0.8	2.4	20,500
γ_2-casein			12,300
γ_3-casein			10,300
Total whey protein	6.3 - 7.3	19.3 - 20.3	
α-Lactalbumin	1.2	3.7	14,176
β-Lactoglobulin	3.2	9.8	18,283
Bovine serum albumin	0.4	1.2	66,267
Immunoglobulin G_1	0.7	2.1	150,000
Immunoglobulin G_2			150,000
Immunoglobulin A			385,000
Immunoglobulin M			900,000
Protein phosphatases 1	0.8	2.4	4,000
Protein phosphatases 5			12,176
Protein phosphatases 6			40,000
Transferrin			76,000
Lactoferrin			86,000
Non protein nitrogen		5.1 - 6.0	<4,000
[1]Adapted from Walstra and Jenness (1984).			

The relative ratio of total casein to whey protein in bovine milk is 80:20. The individual CN proteins, α_{S1}-CN, α_{S2}-CN β-CN, κ-CN, and γ-CN, are in the proportion of 4:1:4:1:0.4, respectively. The major individual whey proteins (α-lactalbumin and β-lactoglobulin) are in the ratio of 35:65 (Fox and McSweeney, 1998; Swaisgood, 1992; Walstra and Jenness, 1984). All CNs range in molecular weight (MW) from about 20 to 25 kDa, whereas most whey proteins have a molecular weight below 20 kDa (Fox and McSweeney, 1998;

Swaisgood, 1992; Walstra and Jenness, 1984). The heterogeneity in MW is used during membrane separation ((reverse osmosis (RO), ultrafiltration (UF), nanofiltration (NF)) of milk proteins, and for analytical techniques (electrophoresis, capillary gel electrophoresis (CGE), Rowland extraction, and Kjeldahl) identifying each milk protein.

As shown in Table 1.3, the main four CN fractions, α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, and two whey protein fractions α-LA and β-LG, make up approximately 90% of the total proteins and are of primary interest to processors and scientists. In addition to the major milk proteins, some minor milk proteins (e.g., enzymes, peptides, and hydrolyzed peptides) and non-protein nitrogen (NPN) compounds are present which represent 5 to 6% of the total milk nitrogen (Fox and McSweeney, 1998). Proteins are amphoteric, which means they may respond as both a base and an acid; a protein's net charge is determined by the pH of the fluid in which it is located. Table 1.4 shows the main physicochemical features of casein and whey proteins with key differences noted in Table 1.5. Beyond the stability in mildly acidic conditions, one significant difference between casein and whey proteins is sensitivity to heat, where whey proteins denature at high temperatures (Visser and Jeurnink, 1997). These properties are important from a technological and functional point of view and provide physicochemical and structural aspects that impact technological, nutritional, and functional characteristics in raw and processed dairy products.

Table 1.4. Principal physicochemical properties of major protein component in milk	
Caseins	Whey proteins
Contains strong hydrophobic bonds	Balance of hydrophobic and hydrophilic residues
Contains little cysteine	Contains cysteine and cystine
Random coil structure	Globular structure and helical structure
Heat stable	Easily heat denatured
Unstable in acidic conditions	Stable in mildly acidic conditions

Table 1.5. Differences between casein and whey protein		
Characteristics	Casein	Whey proteins
Solubility at pH 4.6	No	Yes
Rennet coagulation	Yes	No
Heat stability	High	Low

1.4.2 Caseins

As stated previously, caseins comprise approximately 80% of milk proteins and precipitate at the isoelectric pH point of 4.6 (Walstra and Jenness, 1984) with four individual proteins (α_{s1}−, α_{s2}−, β− and κ−casein) present in bovine milk. All four casein fractions are phosphorylated to variable degrees that are characteristic to each form (Holt, 1992; Walstra, 1979). Caseins are by far the most essential and valuable component of milk in terms of product, technology, and industry (De Kruif et al., 2012). Caseins provide the textural, sensory, and nutritional qualities to the key dairy products of liquid milk, cheese, and yogurt. Because of their lack of complicated secondary and tertiary structure,

caseins offer good surfactant capabilities in emulsions and foams, gelling characteristics, and heat resistance to denaturation (Fox and McSweeney, 1998). Casein micelles are resistant to mild heat and cold without aggregation or modification of the core structure (Dalgleish and Corredig, 2012). The specific casein proteins have some unique properties, which are described here.

a. **α −Caseins:** α−Caseins (α_{s1}−, α_{s2}−) stabilize milk proteins through both refolding properties and chaperone−like activity (Sakono et al., 2011). The solubility of α−caseins is marginally influenced by temperature but strongly influenced by pH (Post et al., 2012).

b. **β −Casein:** β−Casein has an unordered structure, which provides a high degree of segmental motion (Nasir and McGuire, 1998). The strong negatively charged N terminus and an uncharged hydrophobic tail (Krisdhasima et al., 1993) can stabilize calcium phosphate in solutions (Holt, 2004) in a temperature−dependent manner (Singh and Flanagan, 2006). The mechanism is likely through a temperature−dependent conformational change in which the content of poly−proline helix decreases as a function of temperature (Singh and Flanagan, 2006). Through a sequential process, β−casein can form micelle−like aggregates (O'Connell & Fox, 2003), primarily attributed to diffusive motion, long range concentration fluctuations (De Kruif and Grinberg, 2002) and hydrophobic interactions (Pierre and Brule, 1981).

c. **κ −Casein:** κ−Casein has a flexible structure that includes positively charged hydrophobic regions, negatively charged polar regions, and connecting domains (Fox and McSweeney, 1998). The high amount of β−sheet structure can form close

association with other phosphorylated caseins (Farrell et al., 2003). κ−casein is markedly different from the other caseins in terms of solubility characteristics. It has the ability to stabilize α-casein and β-casein by producing an external coating of κ-casein, ending casein aggregation and preventing additional casein protein adsorption even in the presence of calcium. This is consistent with the notion that κ-casein forms the outer layer of casein micelles, aiding in the stabilization of colloidal particles (Nagy et al., 2012).

1.4.2.1 Casein Properties

a. **Solubility:** The solubility of proteins in fluid is the most important property, since insoluble proteins cannot perform typical functions. In a calcium-free system, casein solubility changes with pH and temperature (Bingham, 1971). Caseins are insoluble at their isoelectric point (pH 4.6) with greater range of insolubility as temperature increases (Fox and McSweeney, 1998). Highly viscous solutions can be formed due to the open structure and relatively high-water binding capacity of caseins. No more than 20% casein protein, however, can be dissolved even at elevated temperatures (Fox and McSweeney, 1998).

b. **Heat stability:** Caseins have remarkable heat stability because of the structure and contribution of κ−casein. First, being amorphous unstructured proteins, the caseins do not denature and aggregate on heating (Fox and McSweeney, 1998). Second, the structure of casein micelles is such that the calcium-sensitive caseins are located inside the micelles and are protected from further aggregation at elevated temperature by the outer layer of κ−casein (Griffin et al., 1986).

c. **Emulsifying capacity:** The surface activity of caseins is also an important property that makes them good as foaming agents and emulsifiers (Fox and McSweeney, 1998). To be an effective emulsifying agent, a molecule should be relatively small and capable of adsorbing into an oil−water or air−water interface. In milk, α_{s1}− and β−casein have relatively high surface hydrophobicity and meet these requirements well (Dickinson, 1989).

d. **Gelation:** Caseins undergo gelation when the environment is changed in one of several ways. The most important of these are rennet-induced coagulation for cheese production and acidification to the isoelectric point. Organic solvents or extremely severe thermal conditions may also lead to gelation or coagulation of caseins (Fox et al., 1998).

1.4.3 Whey

Whey proteins are classified as globular proteins that do not precipitate at pH 4.6 but denature upon heating at high temperatures (>70 ℃) (Sawyer et al., 2002). Whey proteins are soluble in saturated NaCl and after rennet-induced coagulation of caseins. Whey protein (WP) constitutes approximately 20% of the total proteins in bovine milk (Eigel et al., 1984). The WP exists in solution as globular proteins and is stable in mildly acidic conditions; however, they are highly susceptible to heat denaturation (above 70 ℃). A critical characteristic of β-LG from a processing standpoint is the presence of a reactive-free sulfhydryl group in its primary structure (Wong et al., 1996). Above 70 ℃, WP (particularly β-LG) aggregate with themselves, other proteins, or κ-CN at the micelle

surface, significantly modifying the properties of milk (Oldfield and Singh, 2005). Various applications of WPs in dairy-based foods are presented in Table 1.6.

Table 1.6. Application of whey proteins in dairy-based foods.[1]

Product	Effect
Yogurt, cheese (Ricotta)	Yield, nutritional, consistency, urd cohesiveness
Cream cheese and cheese spreads, sliceable/squeezable cheeses, cheese filling and dips	Emulsifier, gelling, sensory properties
Soft drinks, fruit juices, powdered or frozen orange beverages	Nutritional
Milk-based flavored beverages	Viscosity, colloidal stability
Ice cream, frozen desserts coating, frozen juice bars	Skimmed milk solid replacement, whipping properties, emulsifying properties, body and texture

[1]Adapted from Mulvihill (1992).

Like other globular proteins, WP may be heat-denatured, resulting in the formation of complexes of gel-β-lactoglobulin in milk that are subject to intensive heat treatment.

Whey proteins have caught the interest of many scientists in recent years due to their biological value, which is the highest of any known protein (Hambraeus and Lonnerdal, 2003). Whey proteins are useful because they can be utilized successfully by humans and supply a considerable amount of needed amino acids for development (Hambraeus and Lonnerdal, 2003). Whey proteins are easily denatured during thermal processing, commonly applied to milk during industrial processing to ensure the microbial safety of dairy products while extending shelf life (McKinnon et al., 2009). As a result, WP undergo conformational changes due to the unfolding of the initially folded molecules. The denaturation of WP by heat is a significant processing problem in the dairy industry

(Al−Attabi et al., 2009) with formation of WP aggregates or complexes fouling heat exchangers, which in effect restricts the running times of industrial plants, decreases heat transfer, and increases a drop in pressure during separation (Delplace et al., 1997; Visser and Jeurnink, 1997). Denaturation and aggregation reactions tied to WP are of great importance to dairy scientists as their expertise is important for creating ways of altering chemical and nutritional properties of dairy products.

1.5 Organic acids

The organic acids (Table 1.7) were chosen as they were identified in the literature as commonly found in milk and dairy products (Ledford et al., 1969; Marsili et al., 1985, Masrsili et al., 1981; Zeppa et al., 2001). Organic acids are used as additives to stabilize or increase the palatability of dairy products, while others are required for nutritional and biochemical processes (Ashoor et al., 1984, Masrsili et al., 1981, Marsili et al., 1985, Mullin et al., 1997). Except for orotic acid, which contains an aromatic moiety, and uric acid, which contains a purine, most of the organic acids investigated are tiny, extremely polar carboxylic acids ranging in length from one to five carbon atoms. In the literature, these smaller, aliphatic organic acids are frequently referred to interchangeably as fatty acids, short-chain fatty acids, or volatile fatty acids.

Table 1.7. Quantity of common organic acids in milk serum.[1]

Organic acid	Average concentration (mg/kg)
Citrate	1600
Formate	40
Acetate	30
Lactate	20
Oxalate	20
Others	10

[1]Milk serum is milk minus fat globules and casein micelles (Adapted from Walstra, 1999).

The occurrence of most organic acids in dairy products is from the metabolism of larger organic compounds such as lipids, proteins, and carbohydrates (Pereira, 2015). However, some such as acetic and lactic acid may be added directly to products or arise from bacterial fermentation and act as preservatives (Butler, 2011). The addition of such acids lowers pH, hindering microbial growth (Pereira, 2015). Milk is considered a good source for many of the organic acids which are derived from both animal feed (silage, grain, and pasture) and bovine metabolism (Parodi, 2004). Knowledge about organic acids is important in understanding the quality of milk products. Lactose undergoes considerable reactions during the heating of milk. There is the formation of compounds such as formic acid, acetic acid, pyruvic acid, hydroxyl methyl furfural, and furfuryl alcohol. Formic acid is the primary compound responsible for the increased acidity of heated milk (Dursun et al., 2016).

Factors associated with animal (e.g., genetics, lactation stage, ruminal fermentation and mammary infections) and feed (e.g., types of grain, dietary protein intake, season, maturity at harvest) may account for variations in organic acids in raw milk (Garnsworthy et al., 2006; Islam et al., 2013; Walstra et al., 2006). As a result, raw milk concentrations, the technological process (i.e., severity and method of heat treatment, homogenization pressure, and temperature), and storage conditions affect the concentration of organic acids in processed milk (Claeys et al., 2013; Nishimura et al., 2015; Van Boekel et al., 1998). The mean and range of organic acids in ultra-high temperature (UHT) pasteurized milk are shown in Table 1.8. Compared to other components of milk, organic acids varied widely, with a coefficient of variation ranging from 10.1 to 59.7 %.

Table 1.8. Organic acids (mg/100 mL milk) in UHT milk samples.[1]				
Acid	Mean	SD	Minimum	Maximum
Oxalic	9.2	2.2	6.2	14.3
Orotic	7.5	4.2	2.2	17.1
Citric	133.0	30.7	94.7	203.3
Pyruvic	5.9	1.6	4.4	9.7
Uric	1.5	0.3	1.2	1.9
Succinic	44.7	15.6	23.0	71.7
Lactic	97.9	9.9	73.8	108.7
Formic	114.4	19.4	84.9	141.2
Acetic	8.7	1.3	4.8	9.5
Propionic	6.8	4.1	0.9	15.4
[1]Adapted from Ahmet et al. (2016).				

a. **Lactic acid:** Lactic acid (Figure 1.2) is the common name for 2-hydroxypropanoic acid, is an important fuel source for the body (Pereira, 2015), formed through anaerobic glycolysis from its precursor, pyruvic acid. Lactic acid is then catalyzed by the enzyme, lactate dehydrogenase.

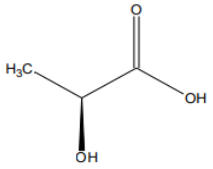


Figure 1.2. L-lactic acid

b. **Butyric acid:** There are two forms of butyric acid present in dairy products (Figure 1.3), iso-butyric (2- methylpropanoic acid) and n-butyric (butanoic acid). Both are volatile fatty acids that humans cannot directly produce but are products of carbohydrate fermentation by bacteria in the human colon and cow's rumen. Butyric acids, particularly iso-butyric acid, are an important source of energy for colonic epithelial cells, commonly known as colonocytes,

and play an important role in disease prevention and proper colon function (Cook, 1998; Lin, 2012; Rumsey, 1964). Butyric acid is sometimes used as a food additive (Hawthrone, 1991; Johnson, 1983) and to enhance flavor. n-Butyric acid has a logP value of 0.79, which means it has hydrophobic characteristics and has a pKa value of 4.82. Iso-butyric acid has a logP value of 1.02 which means it has hydrophobic characteristics and a pKa value of 4.60.

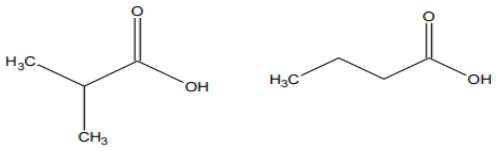


Figure 1.3. Forms of butyric acid. The left structure is iso-butyric acid, and the right structure is n-butyric acid.

c. **Propanoic/ Propionic acid:** Propanoic acid (Figure 1.4) is a product of bacterial fermentation in dairy products (Tormo, 2004). It is also a product of carbohydrate fermentation by lactic acid bacteria and prevents spoilage in dairy products. Propanoic acid may promote a healthy colon for humans. Propionic acid inhibits the growth of mold and some bacteria at levels between 0.1 and 1% by weight. As a result, some propionic acid produced by fermentation is used as a preservative for both animal feed and food for human consumption. Another major application is as a preservative in baked goods, which use sodium and calcium salts. Propanoic acid has a logP value of 1.21, which means it has hydrophobic characteristics and a pKa value of 4.87.

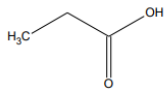


Figure 1.4. Propanoic acid

d. **Oxalic acid:** Oxalic acid (Figure 1.5) is the common name of ethanedioic acid and occurs in both insoluble and soluble form in foods. Research suggests that the water-soluble form can hinder the absorption of milk calcium in the body (Pingle, 1978). The binding of calcium ions to oxalate may also limit bacteriophage development in dairy products, and so addition can limit spoilage (Kadis, 1962). Oxalic acid has a logP value of -0.26 which means it has hydrophilic characteristics and two pKa values 1.25 and 3.67.

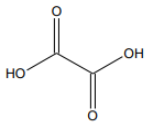


Figure 1.5. Oxalic acid

e. **Citric acid:** Citric acid (Figure 1.6) is present in milk in its ionized form, citrate, and is the most predominant organic acid found in milk (Pereira, 2015; Walstra, 1999). Citric acid degrades rapidly in storage, and many of the organic acids present are breakdown products of citrate hydrolysis; this also applies to lactose and lipids in dairy products (Pereira, 2015; Walstra, 1999). Citric acid has a logP value of -1.32 which means it has hydrophilic characteristics and three pKa values, 3.13, 4.76 and 6.40.

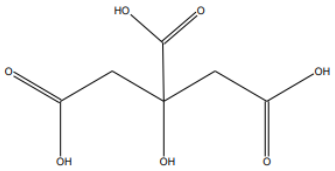


Figure 1.6. Citric acid

f. **Formic acid:** Formic acid (Figure 1.7) is the common name for methanoic acid and is present in dairy products as a degradation product of lactose. It contributes to the flavor of milk (Tormo, 2004). Formic acid has a logP value of -0.27 which means it has more hydrophilic characteristics and a pKa value of 3.74.

Figure 1.7. Formic acid

g. **Acetic acid:** Acetic acid (Figure 1.8) is the common name for ethanoic acid. It is also volatile and a degradation product of lactose. Acetic acid is used as an antibacterial addition, but it is also thought to enhance the flavor of dairy products (Izco, 2002; Pereira, 2015; Tormo, 2001). Acetic acid has a logP value of -0.22 and a pKa value of 4.76.

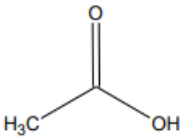


Figure 1.8. Acetic acid

h. **Succinic acid:** Succinic acid (Figure 1.9) is the common name for butanedioic acid and occurs in fermented dairy products mainly due to the metabolic activity of the starter cultures used. Concentrations of succinic acids in dairy products can vary significantly depending on the type of starter culture used (Ammor, 2006; Pereira, 2015). Succinic acid has a logP value of -0.40 and two pKa values, 4.21 and 5.72.

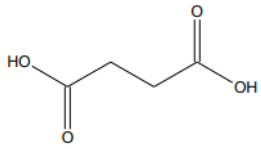


Figure 1.9. Succinic acid

i. **Orotic acid:** Orotic acid (Figure 1.10) is an important molecule involved in nucleotide synthesis. Its major end product is uridine monophosphate, (Voet, 2007). It occurs in milk as a result of normal bovine metabolic processes. Orotic acid is used as a marker for bacterial activity and flavor studies and has nutritional significance. The main source of orotic acid in the human diet is from milk (Robinson, 1980). Orotic acid has a logP value of - 1.23 and has three pKa values, 2.40, 9.50 and 13.00.

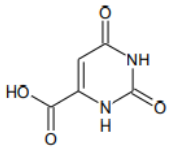


Figure 1.10. Orotic acid

j. **Uric acid:** Uric acid (Figure 1.11) is the common name for 7, 9-dihydro-1*H*-purine-2, 6, 8 (3*H*)-trione. It is a purine that has four ionizable hydrogens at

positions 1, 3, 5 and 7 and thus, four ionization constants. Uric acid is a purine degradation product that is insoluble in water and is present due to normal bovine metabolism. Like orotic acid, it is used in flavor studies and to monitor bacterial activity (Larsen et al., 2010). Uric acid has a logP value of -2.17 and has four pKa values, 3.89, 5.40, 5.80 and 11.30.

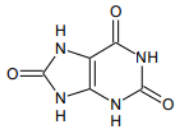


Figure 1.11. Uric acid

1.6 Milk Protein Concentrates (MPC)

After 18 months of research, the FDA Regulatory Guidance Division issued an opinion letter in March 1992, stating that a product "made by ultrafiltration to remove non-protein components such as lactose, water, and minerals from skim milk, thereby concentrating protein components to higher levels" could be called a milk protein concentrate. In the same statement, the FDA said that the MPC should contain protein from "all fractions of milk proteins in the same ratio as that observed to be naturally occurring in milk." The invention of milk powders, especially MPC powders, came from the necessity for a milk product that could overcome the economic constraints of fresh milk in terms of storage and transportation. MPC is a concentrated version of milk proteins that comprises whey protein and caseins in the same proportions as milk (Augustin et al., 2011). MPCs are often added to milk or cheese recipes in order to standardize the products or increase the protein content (Singh, 2007). Pasteurized skim milk is concentrated by ultrafiltration (UF) and occasionally coupled with diafiltration (DF) for creating high concentrate protein powders

(over 70% protein on a dry powder basis) in MPC manufacture (shown in Figures 1.12 and 1.13) (Augustin et al., 2011). After skim milk has gone through UF and DF, the retentate is concentrated by nanofiltration (NF) and evaporated to remove further water before spray drying. The manufacturing conditions, product content, and storage all have an impact on the properties of MPC powders.

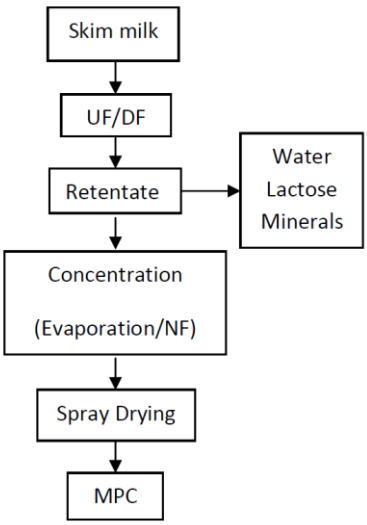


Figure 1.12. Schematic for the manufacture of milk protein concentrate (MPC) powders. UF: Ultrafiltration, DF: Diafiltration and NF: Nanofiltration.

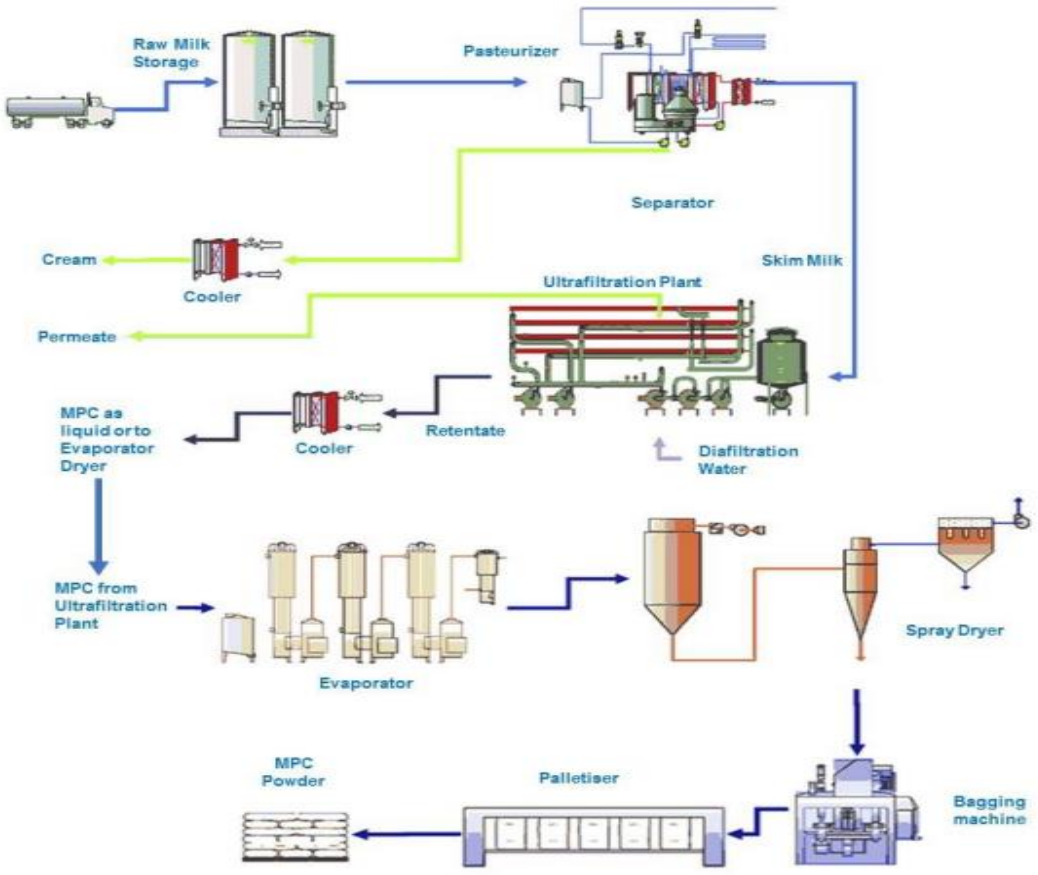


Figure 1.13. The processing flow for milk protein concentrate (MPC) and MPC powder production (From GEA Filtration, GEAF Process Engineering Inc., Hudson, WI, USA).

Different drying procedures, such as freeze-drying or spray drying, can remove up to 87 percent of the water in milk (Henning et al., 2006). To boost protein content in powders, it is preferable to remove lactose and minerals before drying, resulting in high protein content MPC powders (Le Graet and Brulé, 1982; McKenna, 2000). Membrane ultrafiltration (UF) is a popular technology in the dairy business for removing lactose and minerals, which have lower molecular sizes than proteins (TetraPak, 1995).

1.6.1. Ultrafiltration/ Diafiltration (UF/DF)

Filtration (UF and DF) can be performed at either 10 °C (50 °F) or 50 °C (122 °F). The concentration of retentate varies depending on the processing temperature and the kind of membrane or combination of membranes used. Cold UF may concentrate milk to 20-24% total solids with more mineral depleted from retentate, whereas hot UF can concentrate milk to 28-33% total solids (Schuck, 2009). Mineral removal during filtering appears to inhibit rennet coagulation of reconstituted MPC solutions (Williams et al. 2010). Adding monovalent salts to the retentate or partially replacing calcium with sodium ions in the UF process has been observed to improve the functional characteristics of MPC powders (Bhaskar et al., 2004; Singh et al., 2001). The solubility of MPC powder rises with protein interactions in the retentate changed by adding NaCl during DF (Tong et al., 2012). Lowering milk pH from 6.7 to 5.5 boosts the capability and stability of MPC but increases membrane fouling (Tong et al., 2012).

1.6.2 Concentration and Drying

Milk pH drops after evaporation due to mineral balance shifting to the colloidal phase (Augustin et al., 2011). The milk system is cleansed of both inter- and intra-micelle water (Dunstan et al., 2012). During the process, protein interactions (e.g., soluble protein contacts and their relationship with casein micelles) have been documented (Augustin et al., 2011). Some of the alterations are irreversible (Dunstan et al., 2012), which may have an impact on the functional qualities of the final products. However, the NF concentration procedure may result in MPC powders with poor solubility because to a reduction in surface hydrophobicity and protein interactions in the UF retentates. NF concentrated

MPCs offer better rehydration and storage stability than evaporative concentrated MPCs, and their soluble protein and mineral content are greater (Wang et al., 2016). Spray drying, fluid bed drying, and occasionally a combination of the two are the most often utilized methods in the manufacturing of milk protein powder in terms of economics and end product quality (Schuck, 2009). Spray drying dehydrates milk liquid by breaking it down into small droplets and exposing them to hot air (150-250 °C), with droplets at the drier output reaching 70-90 °C (Augustin et al., 2011; Schuck, 2009). The powder's quality is determined by processing circumstances such as air temperature, drying equipment type, and drying stage. Increasing the intake or outlet temperature of the dryer may reduce the solubility and rate of hydration of MPC powders, which is harmful to powder characteristics (Augustin et al., 2011; Chen et al., 2010; DeCastro et al., 2001). According to Fang et al. (2012), increasing inlet air temperature can produce alterations to the surface structure of MPC powder (i.e., wrinkling) with enhanced protein denaturation. Two-stage and multi-stage driers consume less energy and have less of an influence on the characteristics of milk powder than single stage driers (Schuck, 2009). To obtain high-quality MPC powders on a commercial scale, a tall dryer with external fluids for cooling is preferred (Getler et al., 1997).

Prior to drying, high shear treatment of retentate (homogenization and microfluidization) enhances MPC powder solubility (Augustin et al., 2012), presumably owing to milk protein breakdown and protein structural alterations (Augustin et al., 2012; Nguyen and Anema, 2010). It has been found that after production and during storage, homogenization at 350/100 psi and micro fluidization at 800 psi improve the nitrogen solubility of MPC (Augustin et al., 2012). Micro fluidization had a greater impact on nitrogen solubility,

increasing it by more than 17% before and after storage, whereas homogenization only raised it 4-7% (Augustin et al., 2012). Prior to spray drying, high pressure treatment (200 MPa, 40°C of MPC) might enhance the solubility of MPC powders by at least 19% after production and throughout storage, possibly due to an increase in serum protein at the droplet interface during pre-treatment (Udabage et al., 2012).

1.6.3 Composition of MPC powders

The FDA states that MPC should include all proteins found in milk in the same proportions (AOAC, 1998; Garde, 2008). MPCs are thus full-fledged milk proteins that include both caseins and whey proteins. The usual composition of skim milk and MPC powders is shown in Table 1.9. Mineral content can vary because to changes in skim milk as well as the temperatures employed in UF and DF (Augustin et al., 2011).

MPC powders contain tiny particle sizes and large specific surface areas, as well as decreased bulk density and compressibility as compared to MPC powders with low protein concentration (Crowley et al., 2014). However, increasing the protein amount of MPC powders may reduce their solubility (Crowley et al. 2015). MPC powders derived from acidified milk have a high solubility, which could be attributed to a decrease in calcium content in the retentate, which has little effect on powder size, density, and surface structures (Eshpari et al., 2014). Dispersions of MPC derived from the DF process have lower heat stability than UF products (Eshpari et al., 2014).

Table 1.9. Typical composition of powder from skim milk and milk protein concentrate (MPC)[1]		
Component	Skim milk powder	MPC-80
Protein (%)	36	80
Moisture (%)	4	5
Ash (%)	7.7	6.5
Fat (%)	1	2
Lactose (%)	5.3	6.5
Minerals		
Ca (ppm)	12,500	23,300
P (ppm)	10,000	14,500
Na (ppm)	4,800	1,100
K (ppm)	16,500	2,400
Cl (ppm)	11,000	500
[1]Adapted from Anon (1991), Augustin et al. (2011), and Zwijgers (1992).		

1.6.4 MPC Functionality

The elements of MPC impact functional characteristics of the powder. When employed in a particular formulation, MPC may contribute to enhanced whey protein levels, but its application is limited owing to whey protein denaturation at higher processing temperature. Whey protein may crosslink with itself and caseins at high temperatures, resulting in more faults and poorer functioning. Whey protein denaturation can cause precipitation and flocculation, affecting characteristics such as freezing temperature and viscosity. As a result, the usefulness of MPC's natural casein in the formulation may be underused. In some applications, MPC offers an advantage over nonfat dried milk (NFDM) since it requires less lactose and soluble minerals (Martin et al., 2007) given their reduction in concentration.

Most applications need prior dissolving of MPC in water, ideally at room temperature and as rapidly as feasible with low agitation to minimize operating expenses. A genuine solution or complete dispersion of colloidal particles is essential since an insoluble powder does not completely express its functional qualities (Martin et al., 2007). Commercial MPC powders have poor reconstitution characteristics, whether freshly made or stored (Baldwin and Truong, 2007; Mimouni et al., 2009; Singh, 2006). Dissolution of powder particles, rather than wetting or deagglomeration, is the rate-limiting phase in rehydration (Mimouni et al., 2009), with storage-induced effects further modifying the dissolution kinetics. These changes might be caused by some type of micelle association during storage (Anema et al., 2006; Havea, 2006; McKenna, 2000). Moisture content and storage temperatures control the stability of high protein powder (Schuck, 2009). The pH, temperature, ionic concentration of the solution, and type of the powder all influence MPC powder solubility; pH has an effect on the charge and electrostatic interactions of protein molecules. MPCs containing up to 70% protein are very soluble in water at pH 7, whereas MPCs containing more protein are poorly soluble in water at ambient temperature (Augustin et al., 2011). With increasing reconstitution temperature, solubility rises, with α and β-caseins representing the bulk of the insoluble component of MPC powders (Havea, 2006). As the protein level of MPC suspensions increases, their thermal stability diminishes (Crowley et al., 2014).

1.6.5 Storage

Increased interactions between and within casein micelles occur during powder storage, resulting in micelle compaction and the formation of a protein monolayer outside the powders. These changes inhibit the movement of water inside particles, ultimately

decreasing powder solubility (Anema et al., 2006; Fyfe et al., 2011; Mimouni et al., 2010; Singh, 2007). Furosine, free HMF (free hydroxymethylfurfural (HMF), and browning increase with storage in MPC powders, showing that the Maillard process occurs (Bhandari et al., 2011). Understanding the elements that determine MPC functional qualities during storage should lead to higher MPC quality for customers.

Objectives and hypotheses:

Objectives

- Develop and validate quantitative analysis of proteins and organic acids by RP-high performance liquid chromatography.
- Understand the stability and enrichment of proteins and organic acids in milk during processing to milk protein concentrate (MPC-80) and milk protein isolate (MPI-85).
- Evaluate the effects of storage temperature and time on functional properties of MPC-80 and MPI-85 with elevated temperature providing a model for accelerated storage effects.

Hypotheses

- The concentrations of caseins and whey proteins will be greater in MPC-80 and MPI-85 compared to raw milk.
- The concentration of organic acids will be reduced during production MPC as proteins are enriched.

- Storage of MPC-80 and MPI-85 at high temperature will reduce functionality of the powders.

Chapter2: Methods and Materials

2.1 Experimental design

Samples of raw milk, nanofiltration (NF) concentrate, and final powder from 5 lots of 2 different milk protein concentrate (MPC) and milk protein isolate (MPI) products (MPC-80 and MPI-85) were procured from Idaho Milk Products (Jerome, ID) to study the effect of processing on the proteins and organic acids present. The two milk protein products were manufactured from fresh pasteurized skim milk using a low-heat membrane filtration process. The target composition of MPC-80 was 80% protein (as-is basis), 1.15% fat, 6.75% lactose, 6.5% ash, and 5.1% moisture. The target composition (dry matter basis) of MPI-85 was 85 percent protein, 1.15 percent fat, 6.75 percent lactose, 6.4 percent ash, and 5.3 percent moisture (Resource Center, Idaho Milk Products, 2021). From each lot of MPC-80 and MPI-85, samples were collected at the different process points (raw milk, NF concentrate, and final powder). A sample was collected from the raw milk silos before processing through separators (GEA Westfalia, NJ, USA) (around 4500 rpm and 80 psi) to remove the fat. The skim milk was pasteurized (GEA) at 74 ± 2 ℃ for 15 s and moved further to ultrafiltration (UF) (UF Skids 0, 1, 2, 3 at 15-20 ℃ and about 20 psi) for the removal of non-protein components (lactose, water, and minerals) from skim milk to concentrate protein components. With the UF process, skim milk was also processed with 50-60% diafiltration (DF). After UF and DF processing, retentate was concentrated by NF (NF skids 1 and 2 at 15-21 ℃ with loop pressure around 450 psi). After this procedure, water was removed by spray drying through a high-pressure pump (GEA Niro Soavi, New Hampshire, USA) with the help of dryers at about 420 °F. Samples collected were shipped to the University of Idaho frozen on ice before storage at -80 °C. Raw milk, NF, and

powder samples of MPC-80 and MPI-85 were freeze dried (Labconco Freezone 18 freeze dry system) to determine dry matter.

2.2 Quantitative Analysis of Proteins and Organic Acids by High Performance Liquid Chromatography

Each sample was analyzed in duplicate for quantitative analysis of milk proteins and organic acids through reverse phase – high performance liquid chromatography (RP-HPLC).

2.2.1 Protein Analysis

For quantitative analysis of milk proteins, a RP-HPLC method was adapted from those of Bonfatti et al. (2008) and Bobe et al. (1998). Reagents included HPLC-grade acetonitrile (Fisher Chemical, IL, USA), deionized and distilled water, with all other chemicals of analytical grade. Bis-tris buffer, β-mercaptoethanol, guanidine hydrochloride (GdnHCL), sodium citrate, trifluoracetic acid and purified bovine proteins (κ-casein, β-lactoglobulin, β-lactoglobulin B, α-casein, β-casein, immunoglobulin M, and bovine serum albumin were purchased from Sigma (St. Louis, MO). Aliquots containing 800 µL of milk, NF, and powder (diluted 1:9 wt:vol with water) were made. Solution A containing 0.1 M bis tris buffer (pH 6.8), 6 M GdnHCl, 5.37 mM sodium citrate, and 39 mM β-mercaptoethanol (pH 6.5) was added directly to the aliquots (1:1, vol:vol) at room temperature. Each mixture was shaken for 10 - 15 s, incubated for 1 h at room temperature, and centrifuged for 5 min at 15,000 x g in a microcentrifuge. The fat layer was then removed with a spatula and the remaining solubilized sample was diluted 1:3 (vol:vol) with solution B containing 4.5 M GdnHCl and solvent A, which consisted of acetonitrile, water, and trifluoroacetic acid (TFA) in a ratio 100:900:1 by volume. The resulting solubilized

sample was passed through 0.45 µm polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, USA) before HPLC analysis.

The HPLC equipment consisted of an Agilent 1100 Series HPLC Systems (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump (Agilent 1100 Series, G1312A). Detection was via UV (Agilent 1100 Series) with a G1379A Degasser used. The equipment was controlled by the Agilent ChemStation (version A.06.54). Separation was performed on a reversed-phase analytical column C8 Zorbax 300SB-C8 RP, (Agilent Technologies) with a silica-based packing (3.5 µm, 300 Å, 150 mm × 4.6 mm I.D.). A guard cartridge system was used for pre-column protection. Gradient elution was carried out with a mixture of two solvents for HPLC. Solvent A consisted of 0.1 % TFA in water and solvent B was 0.1% TFA in acetonitrile. After injection (5 µl), separation was performed with the following program: linear gradient from 10 to 38 % Solvent B in 3 min followed by an isocratic elution at 38 % solvent B for 1 min, then linear gradient from 38 to 45 % solvent B in 16 min, from 45 to 50 % solvent B in 2 min followed by elution at 50 % for more 2 min. The column was re-equilibrated at the starting condition for 6 min before injection of a new sample. Total analysis time per sample was 30 min. Flow rate was 0.5 mL/min with the column temperature kept at 45 °C. Detection was made at a wavelength of 214 nm. Peaks on the chromatogram were determined through analysis of purified proteins (Figure 2.1).

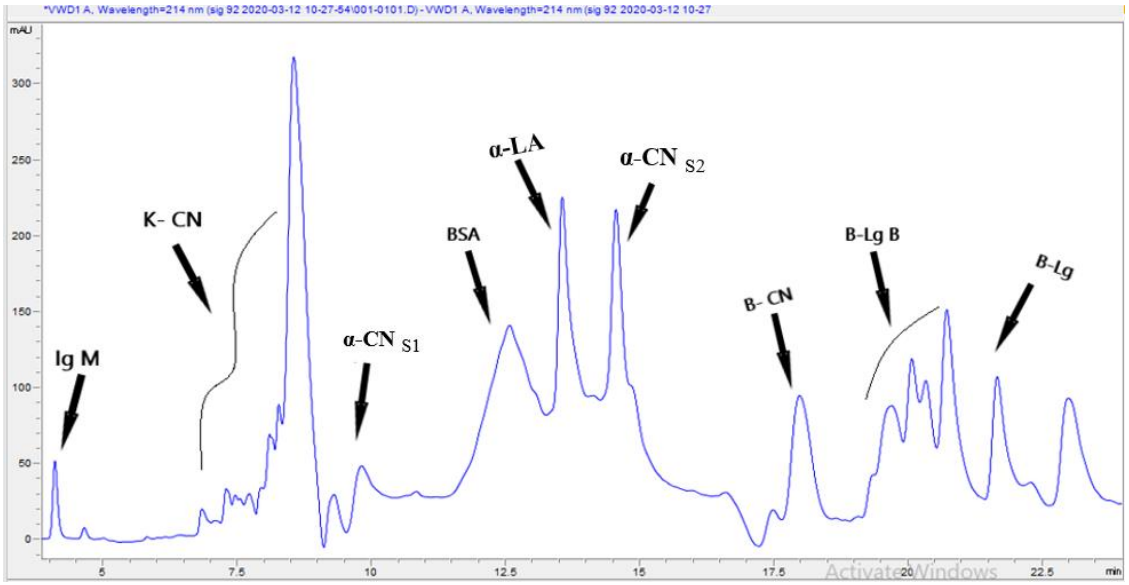


Figure 2.1. A chromatogram representative of the separation of milk protein with associated peaks identified with proteins including α- CN (α_{s1}−, α_{s2}−): α-casein; β- CN: β-casein; κ-CN: κ-casein; β-Lg: β-lactoglobulin; β- Lg B: β-lactoglobulin B; IgM: Immunoglobulin M; α-LA: α- lactalbumin and BSA: bovine serum albumin.

2.2.2 Analysis of Organic Acids

For quantitative analysis of organic acids, a RP-HPLC method (Tormo and Izco, 2004) was modified. Oxalic, citric, formic, succinic, orotic, uric, pyruvic, acetic, propionic, lactic, butyric, maleic and phosphoric acids, and sodium phosphate were purchased from Sigma. One gram of milk, NF concentrate, and powder were diluted to 10 mL with water containing 2 µg/mL of maleic acid as an internal standard. The preparation was vigorously shaken and blended with a vortex before centrifugation at 14,000 x g for 15 min. One mL of the supernatant was filtered through 0.45 µm PVDF membranes before HPLC analysis.

Separation was performed using an Atlantis dC18 column (Waters) (5 µm, 250 mm × 4.6 mm I.D.) in the Agilent HPLC system. Phosphate buffer (20 mM) adjusted to pH 2.10

with phosphoric acid was prepared fresh daily for the analysis. Solvent A was 1% acetonitrile in the 20 mM phosphate buffer and solvent B was acetonitrile. The flow rate for both solvents was 1.5 mL/min with solvents at room temperature. The gradient program started with 100% of solvent A. Seven min after sample injection (10 µl), solvent B was increased linearly to reach 7% in 5 min. From 12 to 19 min the rate was kept at 93% of Solvent A and 7% of Solvent B before returning to the starting conditions for 15 min of equilibration before injection of the next sample. Detection was made at a wavelength of 210 nm. Peaks on the chromatogram were determined through analysis of purified organic acids (Figure 2.2).

Pasteurized milk samples (n=12 and n=16 for protein and organic acids, respectively) were used to validate assays. The precision of the method was evaluated by estimating repeatability across the pasteurized milk samples by running 3 consecutive replications of the same sample and calculating the relative standard deviation (RSD) for peaks area and elution times (sample injection volume = 5 µl). The RSD for retention time was below 0.29% and 0.12% for proteins and organic acids, respectively, while the RSD for peak area was below 0.26% and 1.2% for protein and organic acids, respectively. In addition, the external standard method was used to calibrate the chromatographic system for proteins and organic acids. For this purpose, standard solutions consisting of purified milk proteins and organic acids were used. Calibration curves (5 points) were computed for each protein and organic acid by estimating parameters of the linear regression of the peak area.

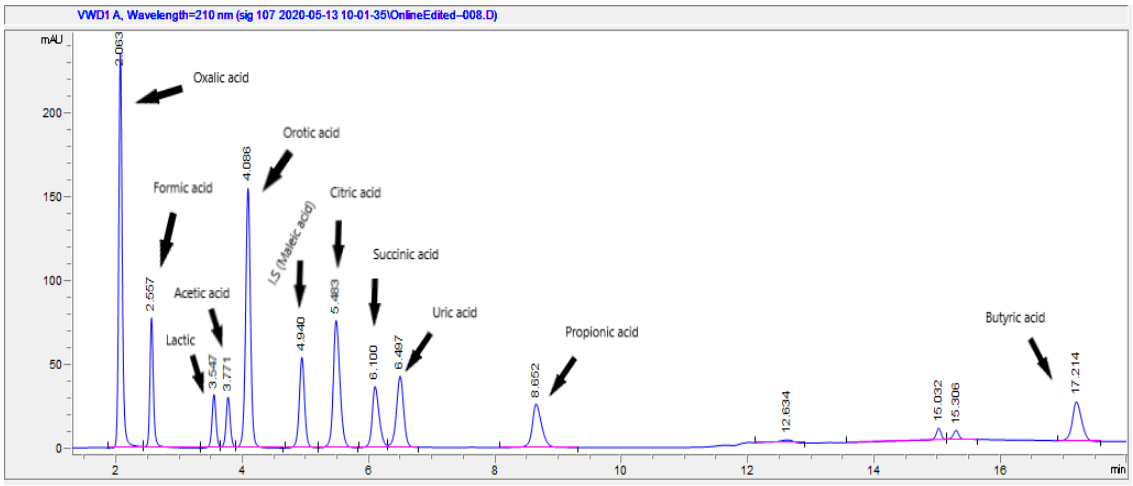


Figure 2.2. A representative chromatogram of organic acids in milk with peaks identified.

2.3 Storage Study

The effect of storage temperature and time on the functionality of MPC-80 and MPI-85 was tested through replicates stored individually in sealed airtight bags at two different temperatures (25 °C and 55 °C) from two lots of MPC-80 and MPI-85. Samples were evaluated at 0, 7, 14, 21, 28, 35, 42, and 49 d of storage. At each timepoint, powders were assessed by the investigator for color, odor and texture before direct analysis of solubility, emulsion, foaming ability and stability, gelation time, and viscosity.

Chemical analyses of powders

Moisture content of the powder was determined as described (American Dairy Products Institute, 1990) using a vacuum oven. Sample moisture content was determined by drying approximately 4 g at 102 ± 2 °C for 3 h in triplicate for each sample (IDF/ISO, 2004).

2.3.1 Functionality Tests

Solubility was determined using the method of Rupp et al. (2018) with slight modifications. Each powder replicate was dissolved in distilled water at 60 °C for 1 h to achieve 4.5%

(wt:wt) and 3.95% (wt:wt) for MPC-80 and MPI-85, respectively. The solution (100 mL) was stirred for 30 min at 20 °C on a stir plate. The stirrers used had the same configuration and were run at a controlled speed (300 rpm). After stirring, the protein solution was stored for overnight hydration at 4 °C. After overnight hydration, the protein solution was again stirred in identical conditions as mentioned previously. Aliquots of the solution (25 mL) were centrifuged at 2460 x g for 10 min at room temperature. Both the samples of protein solution (before centrifugation) and the supernatant (after centrifugation) were analyzed for total solids (TS) by oven drying at 102 ± 2 °C for 24 h. The solubility of powder was calculated as the TS of supernatant, expressed as percentage of the TS of solution prior to centrifugation.

For viscosity, samples were prepared using the solubility test method and left overnight at 4 °C to get complete rehydration and a protein profile similar to that of skim milk (Meena et al., 2017). The viscosity profile was evaluated with an Anton Paar MCR 302 rheometer (Anton Paar; Gratz, Austria) equipped with a cup and bob attachment. Shear-dependent behavior was evaluated by collecting the shear rate sweeps (from 0.01 to 100/s at 25 °C). Viscosity profile data were collected from the Rheocompass^{TM} software (version 1.26.22) and were fitted in a behavior model.

For rennet gelation, sample dispersion, as described in the solubility test method, was made in duplicate in 2% sodium azide solution at 60 °C for 5 min at 600 rpm to ensure complete solubilization (Eshpari et al., 2017; Martin et al., 2010). Samples were supplemented with 2 mM $CaCl_2$ after cooling to room temperature and reconstituted for 3 h at 300 rpm. The sample was kept overnight at 4 °C to ensure complete rehydration. The next day samples were equilibrated at 30 °C for 5 min at 300 rpm, and 0.0315 international milk clotting

(ICM) units of rennet (CHY-MAX® Extra, CHR Hansen, Inc., Milwaukee, WI, USA) per mL were added. Samples were vortexed for 10 s before transmitting to the Anton Paar MCR Rheometer in the cup and bob attachment. Gelation time was taken as the crossover point of storage and loss modulus, indicating a shift from viscoelastic solid behavior at 30 °C with an oscillatory strain and frequency of 0.5% and 1 rad/s, respectively. The initiation of gel formation was indicated by an increase in storage modulus (Eshpari et al., 2017).

For phase formation, samples of MPC-80 and MPI-85 were dissolved in distilled water at 60 °C for 1 h to achieve 4.5% (wt:wt) and 3.95% (wt:wt) solutions, respectively. These reconstituted samples were homogenized with 5 mL canola oil (Crisco, Pure Canola Oil) using a Brinkmann homogenizer at the highest setting for 1 min. Ten mL of each emulsion was transferred to graduated tubes, capped, and stored for 14 d at room temperature (25 ± 2 °C) for visual observation and characterization of stability of the concentrated emulsions. Phase formation or volume separation was evaluated after 7 and 14 d of quiescent storage with confirmation by photography (Anvari and Joyner, 2017).

Foaming ability and stability were calculated using the method of Augustin and Clarke (2008) and Phillips et al. (1987), respectively, with modifications. Milk powder samples were reconstituted at 4.2% and 3.3% (wt: vol) in deionized water for MPC-80 and MPI-85, respectively, at 20 °C for 1 h at 600 rpm. Ten mL of sample was homogenized at maximum speed for 5 min in a 50 mL graduated cylinder. Foam capacity (%) was measured as follows (Shilpashree et al. 2015).

Foam capacity (%) $= \frac{B\ A}{A} * 100$, where A equaled the volume of liquid before whipping (10 mL), and B equaled the total volume (foam plus liquid) obtained immediately after whipping (mL).

Foam stability (%) was measured as the foam volume which remained after 30 min at room temperature with respect to initial foam volume after homogenization at room temperature.

Statistical analysis

The quantitative analysis of proteins and organic acids by RP-HPLC was a 2 by 3 factorial treatment design. The factors were two different products MPC 80 and MPI 85 and three sample points. Values presented are means of replicate determinations, and the differences between the means of the treatments were compared by one-way ANOVA with significance declared at $P < 0.05$. Microsoft Excel and SAS (version 9.3) software were used to analyze the data. A 2x2 factorial treatment design was used to test the effect of storage temperature and time on functional properties of MPC and MPI. The factors were 2 different products MPC 80 and MPI 85 and two different temperatures. Mixed model analysis for repeated measures, regression analysis for change over time and descriptive multivariate analysis were performed to test for effect of temperature and time on functional properties of milk protein concentrates. The model included the main effects of temperature and time, and the interaction of temperature and time. An independent paired t-test was performed to test for differences in functionality between MPC-80 and MPI-85 at different time points.

Chapter 3: Results and Discussion

3.1 Validation of HPLC methods

Assessment of variability for retention times and peak areas in the analysis of repeatability for proteins and organic acids (OAs) indicated that the precision and repeatability of the methods were acceptable (Table 3.1). The RSD values for retention times for protein and OAs were below 0.2911% and 0.1291%, respectively, within analytical day (repeatability) and values of RSD for peak areas were below 0.2621% and 1.2502% respectively.

Table 3.1. Relative standard deviation of retention times and peak areas for milk protein fractions and organic acids (OAs) obtained in the analysis of repeatability.

Protein[1]	Repeatability			OAs[2]	Repeatability	
	Retention time RSD (%)	Area RSD (%)			Retention time RSD (%)	Area RSD (%)
α-casein $_{S1}$	0.1841	0.0124		Oxalic	0.0178	0.1603
α-casein $_{S2}$	0.0146	0.0913		Formic	0.0435	0.5115
β-casein	0.221	0.0051		Lactic	0.0673	1.2502
κ-casein	0.0804	0.026		Orotic	0.1291	0.4291
β-lactoglobulin	0.0625	0.0326		Citric	0.0902	0.3958
β-lactoglobulin B	0.2911	0.2621		Succinic	0.068	0.2022
Bovine serum albumin	0.1985	0.0378				

[1]Samples (n=12) of whole milk analyzed for repeatability of protein analysis.
[2]Samples (n=16) of whole milk analyzed for repeatability of OAs analysis.

Five levels of pure standards in triplicate were used to build calibration curves. The data points from calibration curves were subjected to a least square regression analysis for determination of a standard curve and limits of detection (LOD) for the measured proteins and organic acids. The r-square for every calibration curve was greater than 0.99. The LOD

values (µg) for α- CN, β- CN, κ-CN, β-lg, β-Lg B, α-LA, BSA and IgM were 0.28, 0.84, 0.82, 0.55, 0.84, 0.1, 0.79, and 0.05, respectively. The LOD values (mM) for oxalic, formic, lactic, orotic, citric, and succinic acids were 0.007, 0.28, 0.27, 0.06, 0.3, and 0.18, respectively.

3.2 Effect of different MPC/ MPI processing on proteins and organic acids

The concentration of various proteins in raw milk, NF and powder samples demonstrate notable differences among the three process points (Table 3.2). The concentration of proteins in raw bovine milk were similar to those previously published (Korhonen et al., 2007; Walstra et al., 1984) with similar concentrations of alpha and beta caseins. The concentration of CN fraction increased in NF compared to raw milk due to concentration during filtration processes which removed water. Caseins in MPC-80 and MPI-85 further increased in concentration due to drying of powder (Table 3.2).

Table 3.2. Concentration (g/kg) (mean ± SD) of proteins ([2]DMB) in raw milk, nanofiltration (NF) concentrate, and powder during processing of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85.

Protein	Raw	NF	MPC-80	MPI-85
α- CN	$134.90^{a} \pm 9.8$	$161.67^{b} \pm 24.9$	$210.85^{c} \pm 10.6$	$221.42^{c} \pm 16.6$
β- CN	$129.12^{a} \pm 33.5$	$299.46^{b} \pm 54.6$	$326.72^{c} \pm 30.5$	$357.24^{c} \pm 42.4$
κ-CN	$34.94^{a} \pm 3.4$	$97.73^{b} \pm 22.9$	$96.56^{b} \pm 18.2$	$96.74^{b} \pm 17.1$
β-Lg	$42.14^{a} \pm 0.3$	$17.38^{b} \pm 1.2$	$42.2^{a} \pm 0.2$	$42.0^{a} \pm 0.1$
β- Lg B	$5.44^{a} \pm 1.8$	$9.34^{b} \pm 3.8$	$10.76^{b} \pm 1.7$	$9.47^{b} \pm 1.4$
α- LA	$14.84^{a} \pm 0.1$	$6.05^{b} \pm 0.4$	$14.78^{a} \pm 0.02$	$14.68^{a} \pm 0.01$
IgM	$12.04^{a} \pm 0.3$	$4.95^{b} \pm 0.3$	$12.18^{a} \pm 0.3$	$11.97^{a} \pm 0.3$
BSA	$1.54^{a} \pm 0.7$	$4.38^{b} \pm 2.4$	$3.34^{b} \pm 0.9$	$3.06^{b} \pm 1.04$

[1]α- CN: α-casein; β- CN: β-casein; κ-CN: κ-casein; β-lg: β-lactoglobulin; β- Lg B: β-lactoglobulin B; α- La: α- lactalbumin; IgM: immunoglobulin M and BSA: bovine serum albumin.

[2]DMB: Dry Matter Basis

[a-d] Means within a row that do not share a superscript differ at $P<0.05$.

In NF, the whey protein fractions, β-lg, α-LA, and IgM decreased in concentration which might reflect the effect of high temperature and over filtration during the UF process to target the desired protein content for customers. However, the concentration of β-Lg B was greater in NF compared to raw milk possibly due to breakdown of β-Lg to peaks found in β-Lg B. The concentration of BSA was also greater in NF compared to raw milk. After drying of the product to MPC-80 and MPI-85, the concentration of the whey proteins was similar to that of raw milk except for greater in β-Lg B and BSA (Table 3.2).

An increased concentration of both α-CN and β-CN was observed through processing of raw milk to MPC-80 and MPI-85 (Table 3.2). α−Caseins (α_{s1}−, α_{s2}−) stabilize milk proteins through refolding properties and chaperone−like activity (Sakono et al., 2011). α−Caseins

are marginally influenced by temperature and strongly influenced by pH during processing (Post et al., 2012). α- CN, β- CN and κ-CN showed an increase in NF and powder concentrations. β-Caseins (β-CN) comprise about 45% of total caseins and differ from α-caseins by their strong temperature-dependent association.

Whey proteins such as α-LA, β-lg, and IgM are denatured by heat (Table 3.2) except β-Lg B and BSA which showed an increase of concentration in NF and powder samples. Moderate heat treatment (range of 60 to 70°C) generally results in structural unfolding of the proteins (Morr et al., 1968). At higher temperatures, depending on compositional factors, protein aggregation occurs (Dewit et al., 1981; Morr et al., 1968). The unfolding step involves molecular interactions such as hydrogen and hydrophobic bonding, whereas the aggregation step involves disulfide linkage and is mediated by calcium ions. The classical ranking of whey proteins in decreasing order of susceptibility to heat denaturation in milk is immunoglobulins, bovine serum albumin, β-lg, and α-LA (Schmidt et al., 1984). The extent of whey protein denaturation is usually determined by solubility measurements (Schmidt et al., 1984).

After the raw milk was pasteurized, UF and DF occurred to separate molecules in solution based upon size, shape, charge and affinity toward the membrane during filtration (Aimar et al., 1998; Bastian et al. 1991). Low molecular weight substances like water, lactose, soluble salts and vitamins pass through the membrane into permeate, while high molecular weight compounds like fat, if any, and proteins are retained and thus concentrated by the UF membrane (Cheryan, 1998; Mistry, 2011; Singh, 2007). The use of DF further reduces lactose while increasing protein content in the retentate (Guiziou, 2013).

Table 3.3. Differences of product least squares mean of concentrations of proteins found in milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85.

Protein[1]	Estimate ± SD	P-value
α- CN	16.58 ± 4.35	0.0011
β- CN	30.03 ± 12.12	0.0224
κ-CN	4.35 ± 5.83	0.4642
β-lg	0.51 ± 0.05	<0.0001
β- Lg B	0.83 ± 1.07	0.4465
α- LA	0.14 ± 0.01	<0.0001
IgM	0.02 ± 0.06	0.7595
BSA	0.57 ± 0.52	0.3294

[1]α- CN: α-casein; β- CN: β-casein; κ-CN: κ-casein; β-lg: β-lactoglobulin; β- Lg B: β-lactoglobulin B; α- LA: α- lactalbumin; IgM: immunoglobulin M and BSA: bovine serum albumin.

Comparison (Table 3.3) of the concentration of proteins in MPC-80 and MPI-85 by assessment of the differences of least square mean detected greater α- CN and β-CN in MPI-85 compared to MPC-80 as expected but slightly lower concentrations of β-Lg and α-LA. The other proteins measured were not different in concentration between MPC-80 and MPI-85.

3.3 Organic acids

The concentrations of OAs decreased through processing of MPC-80 and MPI-85 (Table 3.4). Most of the reduction in concentration occurred during UF/DF.

Table 3.4. Concentration (mg/100 ml) (mean ± SD (DMB[1]) of organic acids in raw milk, nanofiltration (NF) concentrate, and powder during processing of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85.

Organic acid	Raw	NF	MPC-80	MPI-85
Oxalic	$222.4^{a} \pm 69.9$	$56.4^{b} \pm 37.6$	$49.3^{b} \pm 3.5$	$51.1^{b} \pm 19.6$
Formic	$762.3^{a} \pm 127.5$	$118.1^{b} \pm 51.7$	$127.8^{b} \pm 18.7$	$95.7^{b} \pm 9.7$
Lactic	$810.3^{a} \pm 289.5$	ND[2]	$107.6^{b} \pm 49.2$	$116.5^{b} \pm 99.9$
Orotic	$56.8^{a} \pm 2.1$	$8.0^{b} \pm 1.5$	$10.2^{b} \pm 0.5$	$7.4^{c} \pm 0.7$
Citric	$1627.4^{a} \pm 711.7$	$597.0^{b} \pm 220.8$	$286.5^{c} \pm 72.2$	$269.4^{c} \pm 61.1$
Succinic	$280.9^{a} \pm 154.1$	ND	$92.8^{b} \pm 6.9$	$94.2^{b} \pm 16.0$

[1]DMB: Dry Matter Basis
[2] ND = not detectable
[a-c] Means within a row with different superscripts ($P<0.05$).

Citric, formic, succinic and lactic acids were the most abundant OAs across all processing steps (Table 3.4). Citric and succinic acids are intermediate products in tricarboxylic acid (TCA) cycle. The concentration of citric acid in raw milk was slightly higher than the findings of Ahmet et al. (2016), similar to those of Güler et al. (2014) and Garnsworthy et al. (2006), and slightly lower than that reported by Gadaga et al. (2001). The mean concentrations of lactic and formic acids in raw milk (Table 3.4) were greater than that reported (588 and 402 mg/L, respectively) by Ruas-Madiedo et al. (1998). Many different factors such as animal (e.g., genetics, stage of lactation, ruminal fermentation state, and mastitis) and feed (e.g., grain, dietary protein intake, seasonal, and regional effects) may account for variations in OA in raw milk (Garnsworthy et al, 2006; Islam et al, 2013; Walstra et al, 2006).

Orotic acid is a non-protein nitrogen (NPN) compound partly formed during protein metabolism of the animal (Dursun et al., 2016). The concentration of orotic acid in raw milk was slightly lower than the previous reports (Dursun et al., 2016, Gadaga et al., 2001).

Concentrations of all OAs were less in NF compared to raw milk (Table 3.4). In NF, the concentration of citric acid was similar to Ahmet et al. (2016). Heat treatment and filtration parameters such as time, temperature, and pore size might lead to a decrease in citric acid in NF (García-Martínez et al., 2010). The concentration of oxalic, formic, citric and orotic acids decreased in the NF samples which could be due to processing of raw milk samples through the heat treatments such as pasteurization and separation. Additionaly, UF decreases the concentration of organic acids. During heating of milk, lactose undergoes reactions that have significant impacts on OAs (Claeys et al, 2013; Nishimura et al., 2015). Compounds such as formic acid, acetic acid, pyruvic acid, hydroxyl methyl furfural, and furfuryl alcohol are formed. As a result of these factors, raw milk conditions and the technological process (i.e., severity and method of heat treatment, homogenization pressure and temperature) and storage conditions affect the concentration of OAs in processed milk (Claeys et al., 2013; Nishimura et al., 2015; Van Boekel et al., 1998). The concentrations of OAs in MPC-80 and MPI-85 were similar to those noted by Ahmet et al. (2016) in UHT milk. The concentration of oxalic, formic, and orotic acid in MPC-80 and MPI-85 were similar to those in NF (Table 3.4). Concentrations of citric acid were reduced in MPC-80 and MPI-85 compared to those in NF (Table 3.4). Concentrations of OAs were not different between MPC-80 and MPI-85 (Table 3.5) except for greater orotic acid in MPC-80 compared to MPI-85.

Table 3.5. Differences of product least squares mean for concentrations of organic acids in milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85

Organic acid	Estimate ± SE	P-value
Oxalic	6.26 ± 14.29	0.6661
Formic	27.83 ± 25.61	0.2901
Lactic	92.82 ± 54.09	0.1016
Orotic	1.78 ± 0.49	0.0015
Citric	5.74 ± 14.19	0.9689
Succinic	66.36 ± 26.51	0.0211

3.4 Effect of Storage Time and Temperature on the Functionality of Milk Protein Concentrates

3.4.1 Physical Properties

A change in color, texture, flavor, or aroma of MPC-80 and MPI-85 powder stored at 25 ℃ for 49 d was not observed by the researcher; however, powder stored at elevated temperatures (55 ℃) was noted to change after 7 d (Figure 3.1). Powder stored at 55 ℃ went from white and light with a milky flavor and aroma to a light yellow with milky flavor and aroma on d 7 to a light dark yellow color on d 14, 21, and 28 without change in flavor or aroma. The color shifted to dark yellow on d 35, 42 and 49 with a burnt taste and scent (Figure 2.1). These changes were observed at a similar temperature previously (Le et al., 2011). The changes in color and aroma could arise due to the Maillard reaction as Le et al. (2011) noted MPC powder was at risk for Maillard reactions when stored at temperatures between 25 to 50 °C.

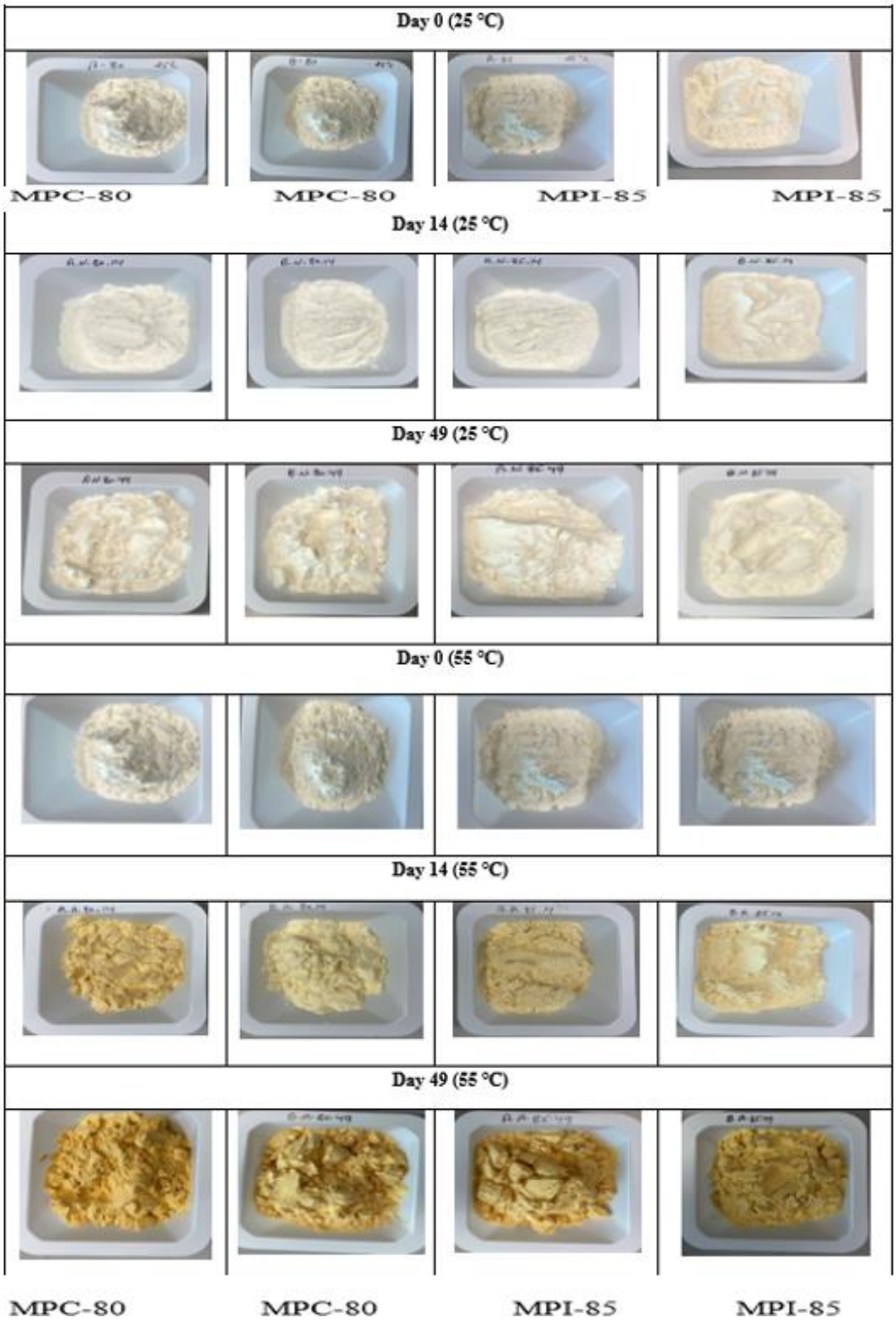

Day 0 (25 °C)
MPC-80
MPC-80
MPI-85
MPI-85
Day 14 (25 °C)
Day 49 (25 °C)
Day 0 (55 °C)
Day 14 (55 °C)
Day 49 (55 °C)
MPC-80
MPC-80
MPI-85
MPI-85

Figure 3.1. Photos of milk protein concentrate (MPC) and isolate (MPI) powders (MPC-80 and MPI-85) during storage at 25 ℃ and 55 ℃ over 49 d. The two left columns are replicates of MPC-80 and the two right columns are replicates of MPI-85. Color, aroma, texture, and flavor were observed by the researcher with images to support assessment of color.

3.4.2 Solubility

Solubility is one of the most important functional properties for proteins in fluid since insoluble proteins cannot perform useful functions and affect the expression of other properties (Uluko et al., 2016). An interaction of time by temperature (P = 0.01884) was detected with a greater decrease over time in solubility of either MPC-80 or MPI-85 stored at 55 ℃ compared to the decrease in solubility at 25 ℃ over time (Figure 3.2). The solubility of both MPC-80 and MPI-85 tended to differ (P = 0.07131) during storage at 25 ℃ and 55 ℃ with MPI-85 having reduced solubility compared to MPC-80 (Figure 3.2). Temperature affected solubility (P < 0.00001) with lower solubility of MPC stored at 55 ℃ compared to 25 ℃.

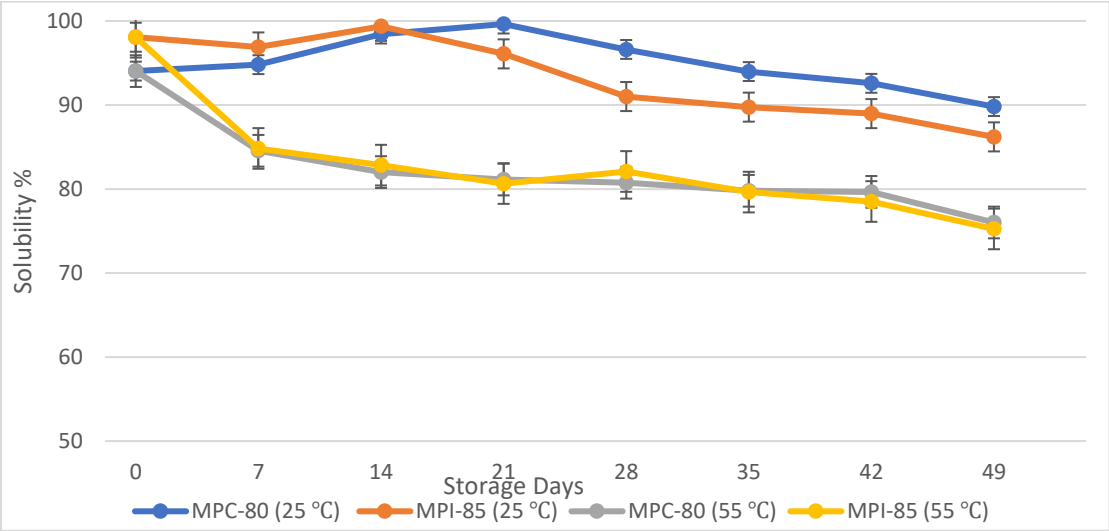


Figure 3.2. Effect of temperature and time of storage on solubility of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85 stored at 25 or 55 ℃ for 49 d.

At 55 °C, a decline in solubility for both MPC-80 and MPI-85 was apparent (P = 0.0003) on d 7 of storage (Figure 3.2). After production, MPC solubility decreases with storage time and storage temperature (Anema et al., 2006; Havea, 2006). Anema et al. (2006) concluded that the decrease in solubility of MPC-80 and MPI-85 from 20 to 50 °C for periods up to 60 d occurred by a similar mechanism at all temperatures, and that the rate of solubility loss was positively related to storage temperature. A possible mechanism for the loss of solubility in MPC powder involves protein-to-protein interactions on the surface of the powder particle (Meena et al., 2017). More specifically, the decrease in solubility of MPC could be related to hydrophobic association of casein micelles on the surface since caseins are insoluble at their isoelectric point and the insolubility range becomes wider with increasing temperature (Fox and McSweeney, 1998). Another factor negatively impacting solubility could be the Maillard reaction (Finto et al., 1981; Henle et al., 1991; Le et al., 2011; Ledl and Schleicher, 1990). The Maillard reaction is the main factor causing an increase in the surface hydrophobicity of milk protein powders mixed with glucose and lactose (Le et al., 2011). Mimouni et al. (2010) disputed that formation of insoluble material was behind the loss of solubility in stored MPC, and hypothesized the loss of solubility during storage was due to changes in rehydration kinetics. Protein cross-linking, the Maillard reaction, lipid oxidation, lipolysis, and proteolysis of MPC powders may occur during storage (Gaiani et al., 2007; Le et al., 2013). Protein unfolding and surface hydrophobicity increase when MPC powders were stored at high temperatures, which could lead to protein–protein interactions and solubility loss (Haque et al., 2010, 2011). In the current study, humidity of storage was not controlled. Le et al. (2011) and Haque et al. (2011) detected effects of humidity on the solubility of MPI-85 powder during

storage for 60 d. Future studies on the effect of temperature on solubility of MPC in storage should consider including humidity control in the research model.

3.4.3 Emulsion

The stability of an emulsion refers to the ability to resist changes in emulsifying properties over time (McClements et al., 2004). At 25 °C, no detectable separation or instability was observed in emulsions made from either MPC-80 or MPI-85 on d 7 of emulsion storage (top row, Figure 3.3), but separation of water and cream was observed on d 14 of emulsion storage. Stability of emulsions made from either MPC-80 or MPI-85 stored at 55 °C was poor with separation of lipid and water and sedimentation of powder on both d 7 and 14 of emulsion storage.

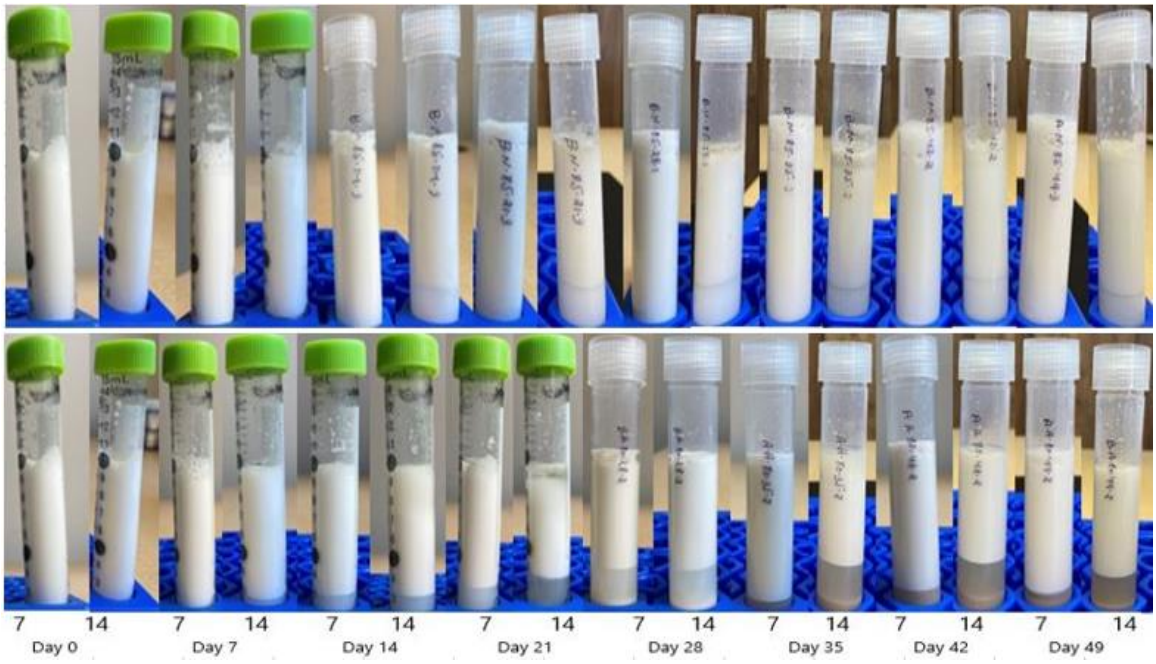


Figure 3.3. Photos of emulsions produced from milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85 after 0, 7, 14, 21, 28, 35, 42, and 49 d storage. Each emulsion made from the day of MPC-80 and MPI-85 storage was then evaluated after 7 and 14 d of storage at room temperature. The top row of tubes shows emulsions made from MPI-85 stored at 25 °C and bottom row shows emulsions made from MPI-85 stored at 55 °C. Only

photographs of emulsions made from MPI-85 are shown, but emulsions of MPC-80 showed a similar trend for stability.

A change in color and sedimentation was observed after 21 d of MPC-80 and MPI-85 kept at 55 °C with brownish particles settling out of solution and noted at the bottom of the tube. Sedimentation was also observed in both MPC-80 and MPI-85. McKenna et al. (1999) observed sludges and sediments in milk reconstituted from powders, using transmission electron microscopy. According to McKenna et al. (1999), the sediments are constituted of fused caseins englobing fat and result from the application of both shear stress and heat treatment during spray-drying. The sediments are insoluble, and their presence in reconstituted milk is linked to solubility loss of milk powders. Protein-protein interactions, at the origin of sediments, continue upon storage of milk powders with high protein content (Kenna et al., 2000). Another possible reason for sedimentation could be dissolution of stored MPC powders (Schuck, 2009). During storage, increased interaction occurs between and within micelles, leading to compaction of micelles and the formation of a monolayer skin of casein micelles packed close together. The combination of these micelles is likely responsible for the slow dissolution of stored MPC powders (Schuck, 2009).

3.4.4 Viscosity

Shear-dependent behavior to assess viscosity was evaluated by collecting shear rate sweeps (from 0.01 to 100 s^{-1}) at 25 °C. The viscosity range of 0.0014 to 0.0017 mPa was determined for powder (both MPC-80 and MPI-85) stored at 25 °C for 49 d and was similar between MPC-80 and MPI-85 (Figure 3.4). The solution of powders at 25 °C showed no

signs of air incorporation as no bubbles were found on either the cup or bob surface at the end of the experiment. Further, the flow curve showed no signs of hysteresis.

In MPC-80 and MPI-85 stored at 55 ℃, viscosity was greater (P = 0.0001) across all days of storage compared to MPC-80 and MPI-85 stored at 25 ℃ (Figure 3.4).

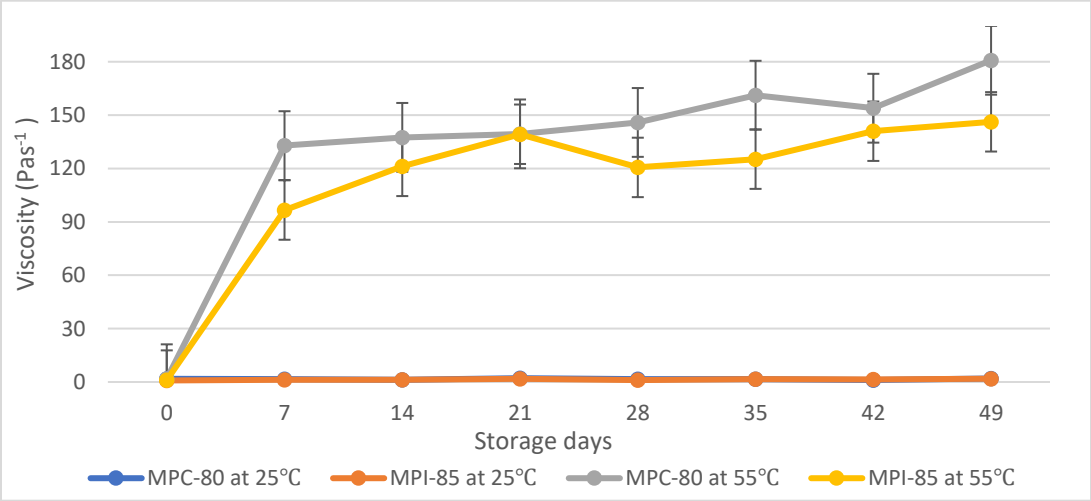


Figure 3.4. Effect of temperature and time on viscosity of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85 stored at 25 ℃ or 55 ℃ for 49 d.

Both MPC-80 and MPI-85 stored at 55 ℃ had viscosity graphs with a negative slope (Figure 3.5). Storage of either MPC-80 or MPI-85 at 55 ℃ resulted in sedimentation in the cup during testing. This could be due to the reduced solubility of MPC or MPI stored at 55 ℃. Low viscosity may be due to more compact protein structures in MPC-80 and MPI-85 (Rupp et al., 2018). Low viscosity is a valuable feature for high protein energy drinks and clinical formula with a high caloric density (Rupp et al., 2018).

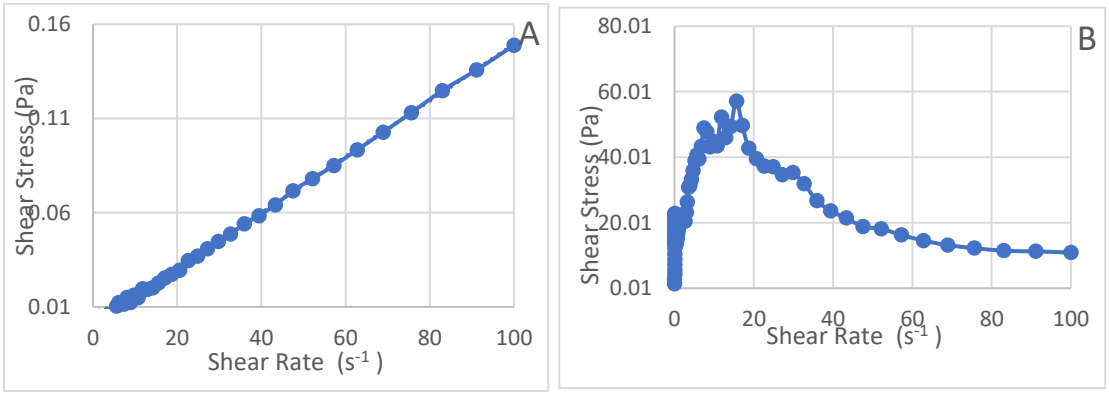
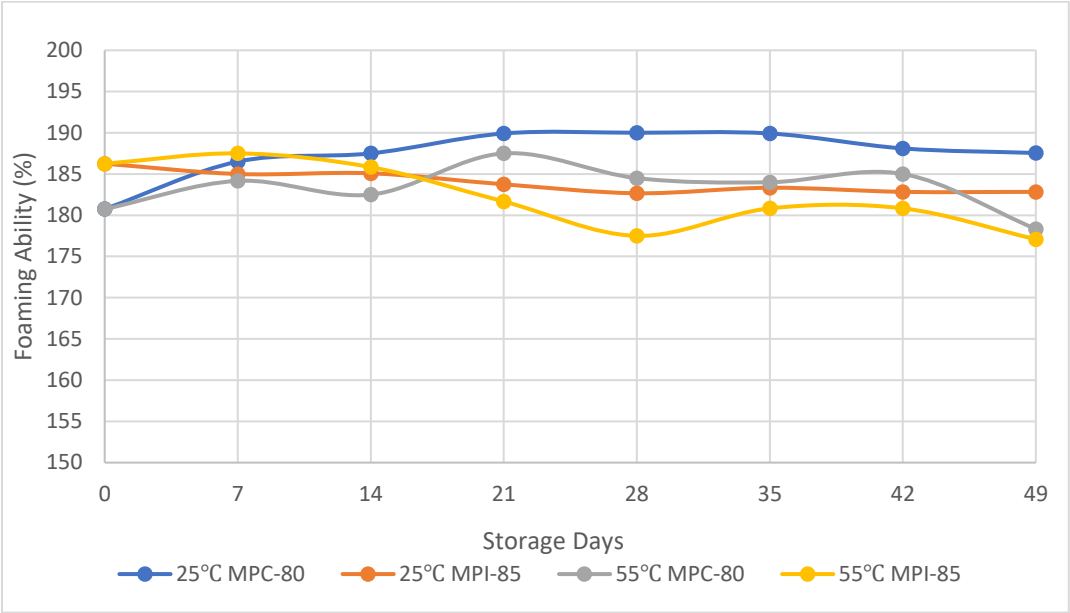


Figure 3.5. Viscosity graphs showing shear rate and shear stress of milk protein isolate (MPI)-85. Panel A represents the viscosity behavior of MPI-85 on d 35 of storage at 25 ℃. Panel B represents the behavior of MPI-85 on d 35 of storage at 55 ℃.

3.4.5 Foaming Ability and Stability

The ability of protein to entrap and retain air is known as foaming ability and is an important property in ingredients incorporated in ice cream, frozen desserts, bakery, and certain confectionary products. The foaming ability of MPC-80 and MPI-85 decreased (P= 0.002 and 0.05, respectively) during storage at 25 ℃ and 55 ℃ over 49 d of storage. In storage at 55 ℃ foaming ability decreased gradually according to time of storage (Figure 3.6).

At 25 ℃, MPC-80 had increased (P= 0.004) foaming ability over time but it did not differ in MPI-85. The slightly improved foaming capacity at 25 ℃ may reflect greater protein:protein interactions in the heterogeneous milk protein isolate that are not possible for casein or whey dispersions alone. These interactions have been shown to enhance bubble formation (Huppertz, 2010).



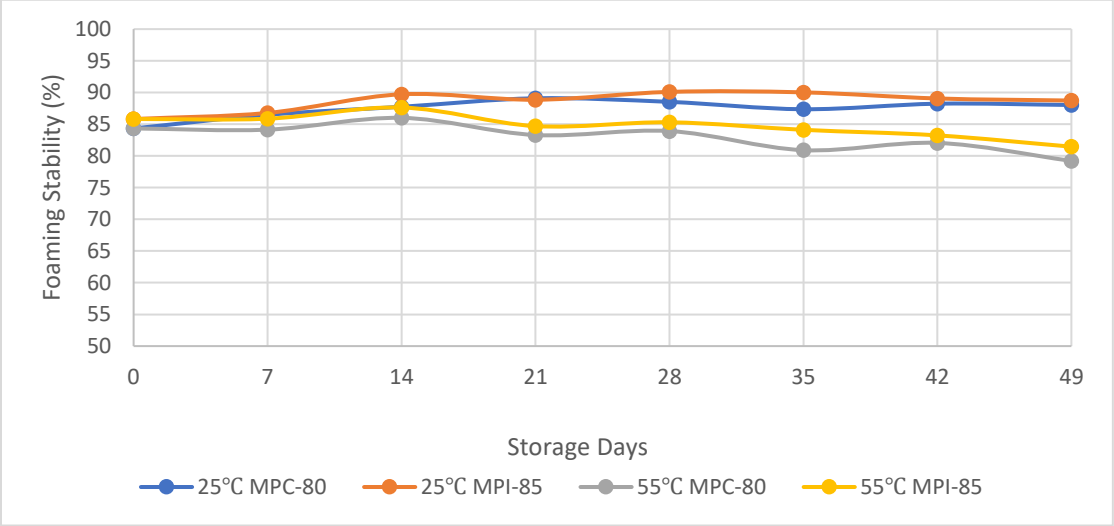


Figure 3.6. Foaming ability (top panel) and stability (bottom panel) of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85 stored at 25 °C and 55 °C over 49 d. The overall SEM for foaming ability of MPC-80 at 25 °C, MPC-80 at 55 °C, MPI-85 at 25 °C, and MPI-85 at 55 °C was 1.07, 0.99, 0.46, and 1.40%, respectively. The overall SEM for foaming stability of MPC-80 at 25 °C, MPC-80 at 55 °C, MPI-85 at 25 °C, and MPI-85 at 55°C was 0.52, 0.76, 0.54, and 0.66%, respectively.

Foaming stability for both MPC-80 and MPI-85 was reduced (P= 0.010 and 0.0001, respectively) during the storage period of 49 days (Figure 3.6) similar to that reported by Huppertz et al. (2015).

Foams are comprised of a discrete gas or bubble phase dispersed in either a liquid or solid continuous phase. Proteins play an important role in forming and stabilizing foams in aerated dairy products such as ice cream. Rupp et al. (2018) found foaming ability decreased after storage of MPC-80 at 30 °C for 6 mo, which could be related with the concurrent decrease in powder solubility during storage. High protein solubility is usually required for milk powders to exhibit good foaming (Huppertz et al., 2010). Greater foaming properties (foaming ability and stability) of milk as pH is increased during manufacturing above the natural pH of milk may be attributed to an increased availability of caseins (Augustin et al., 2008). Studies on the effects of heat treatments of caseinate and whey protein isolate solutions have shown that heat treatment improved foaming capacity (Schmidt et al., 1993). However, heat treatment of milk did not affect foaming capacity of milk (Samragy et al., 1993).

3.4.6. Gelation time

The initiation of gel formation is indicated by increase in storage modulus (Figure 3.7). No difference was observed in the storage modulus for either MPC-80 or MPI-85 at 25 °C through 49 d of storage.

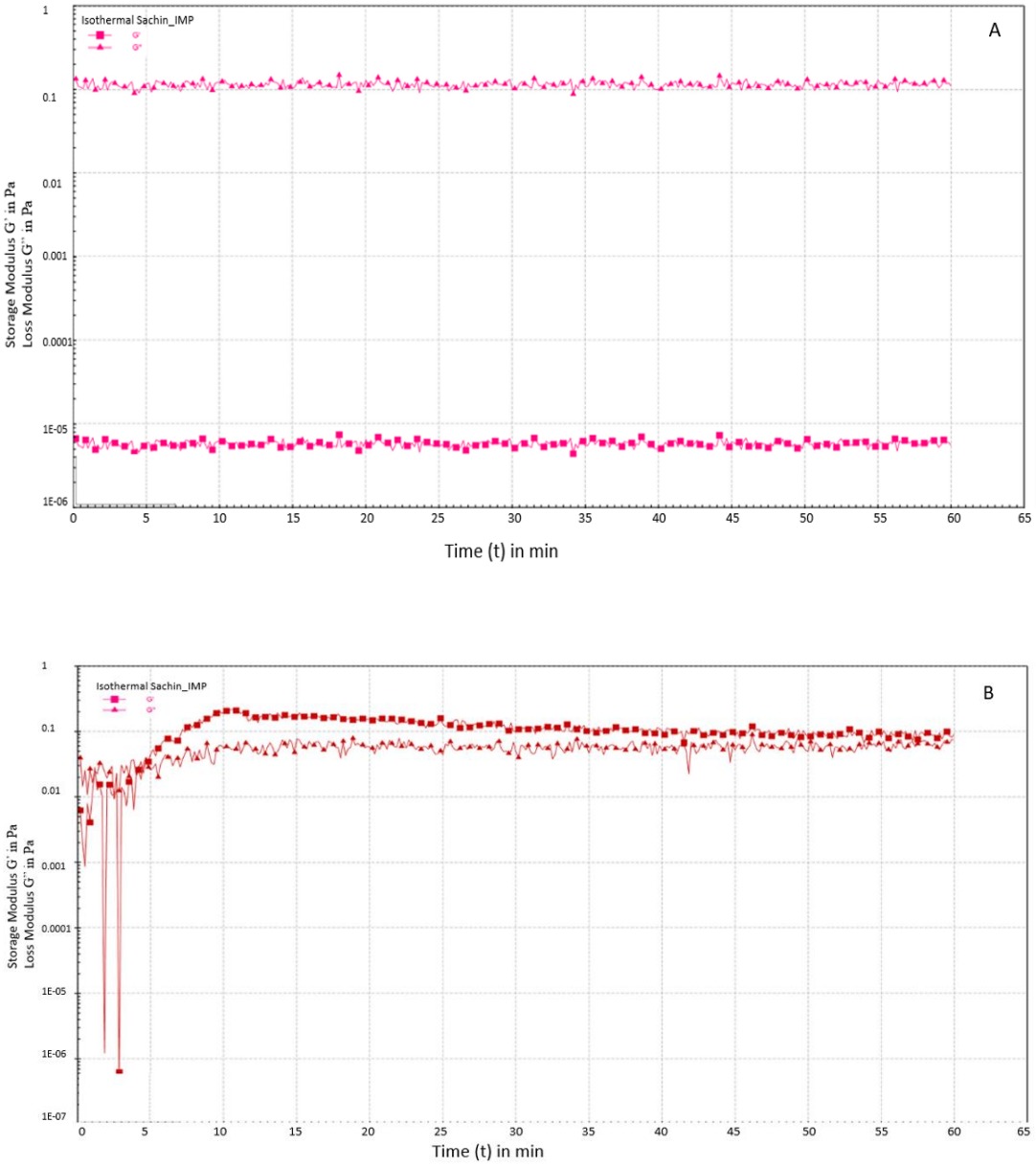


Figure 3.7. Gelation time of milk protein isolate (MPC)-85 after 35 d of storage. The top chart shows gelation time of MPI-85 stored at 25 ℃ and bottom chart shows gelation time of MPI-85 stored at 55 ℃. Gelation time is indicated by G" (loss modulus, ○) and G' (storage modulus, ■) over 60 min. Milk protein concentrate (MPC)-80 and MPI-85 showed a similar trend of gelation time across 0, 7, 14, 21, 28, 35, 42, and 49 d of storage.

Storage of MPC-80 and MPI-85 for 7 to 49 d at 55 ℃ resulted in increased gelation (Figure 3.7). Gelation started between 5 and 10 min in either MPC-80 or MPI-85 stored at 55 ℃ (Figure 3.8).

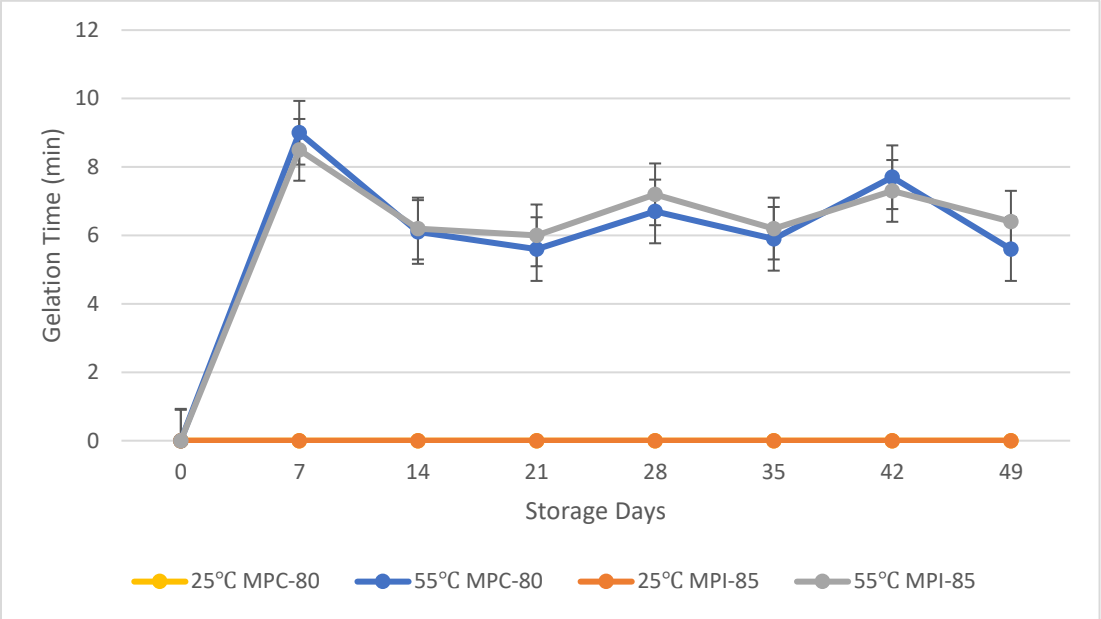


Figure 3.8. Gelation time of milk protein concentrate (MPC)-80 and milk protein isolate (MPI)-85 stored at 25 ℃ and 55 ℃ for 49 d.

Removal of minerals during UF and DF may be the reason (Martin et al., 2009) for the delay in gelation as minerals such as calcium helps gelation (Uluko et al., 2012). Alongside minerals, pH also plays a role in gelation. The importance of pH, minerals and salts in the gelation of MPC has been strongly established (Hussein et al., 2012). Lowering pH of milk from 6.7 to 5.5 boosts the gelation capability of MPC but increases membrane fouling and decreases UF flux (Luo et al. 2015). Enzymes such as protease, alpha-amylase, lipase, and cellulase may be added to overcome the problems of membrane fouling and UF flux (Yu et al., 2010). Acidification and partial mineral depletion might also compromise the integrity of the super molecular structure of the casein micelles and affect the gelation of

reconstituted milk protein concentrates (Eshpari et al., 2015). Removal of minerals during filtration also appears to prevent rennet coagulation of reconstituted MPC solutions (Martin et al., 2010).

Gelation temperature is a principle factor in the determination of gel properties (Lucey et al., 1998; Roefs and Van Vliet, 1990). However, other effects of adding $CaCl_2$, such as an increase in the ionic strength and a decrease in pH, could also have influenced the coagulation process (Martin et al., 2010). Supplementation with approximately 2 mM $CaCl_2$ is required for reconstitution of MPC lacking mineral after removal during UF/DF. Addition of sufficient calcium restored rennet coagulation kinetics and gel strength of reconstituted MPC to approximately that of raw skim milk (Martin et al., 2010). Mizuno and Lucey (2007) found that when gels are formed from MPC with addition of emulsifying salts, the resulting gel characteristics would depend on the pH and concentration of the added salts. Addition of 2 mM $CaCl_2$ and high temperature (60 °C) during reconstitution enabled rapid coagulation for MPC-80 stored at 55 °C (Martin et al., 2010).

During storage over 49 d, temperature reduced solubility of MPC stored at 55 °C compared to 25 °C but no difference was detected in solubility between MPC-80 and MPI-85. Emulsions made from either MPC-80 or MPI-85 showed no visibly detectable separation or instability at 25 °C but separation as cream and water and sedimentation of powder was visible for emulsions made from powder stored at 55 °C. Viscosity increased across all days of storage in MPC-80 and MPI-85 stored at 55 °C compared to that stored at 25 °C. Foaming ability and stability of MPC-80 and MPI-85 was reduced during storage at 55 °C, presumably due to the reduction in powder solubility. Gelation time increased in MPC-80

and MPI-85 stored at 55 °C. Over time, higher storage temperature adversely affected functionality of MPC-80 and MPI-85 with little difference between the milk powders.

Conclusion

Caseins were enriched in both MPC-80 and MPI-85. Concentrations of the major caseins (α-CN and β-CN) were increased in NF compared to raw milk and further in MPC-80 and MPI-85. The concentration of κ-CN was greater in NF compared to raw milk but not different in MPC-80 or MPI-85. Most whey proteins (α-lactalbumin, β-lactoglobulin, IgM) were similar in concentration on a dry matter basis between raw milk and MPC-80 as well as MPI-85. The major caseins (α-CN and β-CN) were greater in concentration in MPI-85 than MPC-80. The concentrations of OAs decreased through processing of MPC-80 and MPI-85 but no difference was detected between MPC-80 and MPI-85 (Table 3.5) except for greater orotic acid in MPC-80 compared to MPI-85. Over time, higher storage temperature adversely affected functionality of MPC-80 and MPI-85 with little difference between the milk powders. Preventing reductions in functionality of milk protein concentrates during storage at higher temperatures would likely aid in the economic value of these dairy ingredients.

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