A study on tailoring protein interactions to influence the functional properties of milk protein concentrate powders

by

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Abstract

Milk protein concentrate (MPC) powders are milk-derived ingredients that are highly functional and provide a wide range of nutritional and functional benefits in food product applications. High protein MPC powders are good for protein enhancement while giving a clean dairy flavor without the addition of lactose to product formulations. Besides protein enrichment and nutritional benefits, MPC powders provide functional properties like gelling, emulsification, foaming, and thickening to name a few. The functional properties of milk protein concentrates are correlated with their protein content and protein-protein interactions which in turn is a gateway for a wide range of product applications in the food industry. Various processing methods have been studied to improve the functionality of milk proteins. Heat treatment and pH adjustment separately have been widely used to modify and bring about changes in the functionality of milk proteins. As the initial step in this research study, MPC powders were manufactured from skim milk which was heated at varying pH. The protein fractions of the resultant MPC powders were analyzed using capillary electrophoresis to determine the protein-protein interactions at serum and micellar phases. Increased casein-whey protein interaction was observed at lower pH while higher levels of soluble whey proteins (bound and unbound) were found at a lower pH. This significantly ($p<0.05$) improved the heat stability of the resultant powders as well as a change in viscosity with respect to pH change. In the study that followed, the same MPC powders were used for gelation studies. The rheological characteristics and gel parameters of the MPC powders were studied with respect to the protein interactions observed in the previous study. MPC powders with heated skim milk bases showed early onset of gelation but were not significantly different ($P<0.05$) with respect to the varying pH. The G′ was lowest for samples at pH 6.5 leading to the formation of a weaker gel, while at pH 7.1 the gel structure was stronger. The network structure of the gel became denser

and less porous as the heating pH increased. Therefore, it can be concluded that tailoring protein-protein interactions by altering processing conditions during the manufacture of MPC powders brings about desirable changes in their functionality. These changes not only open possibilities for MPCs as a dairy ingredient but also in other untapped product applications.

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Dedication

This thesis work is dedicated to my beloved family and friends for their continuous support then, now, and always. To my mother, Mini M.K and my father, Dr. K.M.Dileep for always believing in me and giving me wings to fly. Also, to my little sister, Krishna Dileep for being a constant in my life.

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Chapter 1 - Introduction

Milk protein concentrates or MPCs are dairy proteins which contain both caseins and whey proteins in the exact ratio and form as found in milk, assuming that there is minimum heat impact during processing. Novel technologies like membrane separation help make protein ingredients that meets specific functional and nutritional needs. MPCs are manufactured by the process of ultrafiltration (UF) and diafiltration (DF) which concentrates the protein while removing majority of lactose and soluble minerals. The concentrate is further evaporated, and spray dried to create MPC powder (Agarwal et al., 2015). They are highly functional ingredients that can provide a range of benefits in food product applications.

Solubility/heat stability are very important properties during product processing. It is essential that the ingredient be able to remain in solution and maintain its structure throughout varying processing changes like heat, pH change or change in mineral level (eg: UHT and sterilized milk, infant formulas, beverages). The viscosity and gelation properties of MPCs that adds to structure development of products make it an ideal ingredient in yoghurts, puddings, custards, and confectionary. They are also known to have good emulsification properties which helps maintain stable emulsions in products like ice cream, salad dressings, dips etc. Foaming is a functional property that provides excellent whipping ability in products like ice cream, frozen desserts, whipped cream, and toppings. Extend of Maillard reaction during processing is the main determining factor for flavor and/or color development in many products especially bakeries and confectionaries. Therefore, depending on the application, bringing desirable changes to its functional properties would further expand possibilities for MPC as an ingredient in untapped food applications.

Modifications in functional properties of milk proteins can be brought about by physical, chemical and enzymatic modifications. Heat treatments are essential throughout the processing of products in the dairy industry. The functionality of MPC's is dependent on the type of protein interactions that occur in the protein system (casein-whey protein vs whey protein-whey protein) during heating. These interactions are governed by factors such as pH, ionic strength, whey protein to casein ratio and heating temperature (Donato and Guyomarc'h, 2009; Gaspard et al., 2017). Some non-thermal treatments used in modifying functional properties of milk proteins are ultrasonic processing; high-pressure processing; enzymatic modifications (eg., transglutaminase (TGase) cross-linking of proteins); chemical modifications (eg., succinylation, lactosylation and ligand binding). These tend to increase gel strength, viscosity, and water-binding capacity of acid gels as well as aids in maintaining stability of food emulsions. Preheating of milk prior to concentration is known to influence the heat stability of concentrated milks. While a lot of studies have focused on explaining the protein interactions in the skim milk at varying heating time, temperature, and pH, extremely limited studies have been conducted to evaluate the change in functional properties of MPC powders prepared from preheated and pH adjusted skim milk.

This thesis aims to investigate a pretreatment method on skim milk used for MPC manufacture by combining change in pH and heating to obtain protein denaturation prior to UF processing. Chapter 2 gives a background on studies relating to milk protein concentrates, their processing, functionality, and process modifications over the years. Chapter 3 will focus on identifying the protein interactions and changes in physicochemical properties MPC powders manufactured with skim milk pH adjusted and heat treated prior to UF and spray drying. While chapter 3 does go over a few functional properties, chapter 4 provides a detailed analysis of heat

stability of the newly manufactured MPC powders using acid gelation to determine the changes in gelation parameters with respect to pH and heat treatment.

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Chapter 2 - Review of Literature

Milk: composition & components

The US Food and Drug Association (FDA) defines milk as a mammary secretion free from colostrum and derived by the complete milking of healthy milch cows. It can include but not limited to concentrated milk, reconstituted milks, and dry whole milks. Two major parameters of milk are milk fat and milk solids-non-fat (SNF). While milk fat is self-explanatory, SNF or 'serum solids' consists of proteins, lactose, and minerals. The combination of SNF plus fat present in milk is termed as 'total solids. As per 21CFR131.110, the final packaged form of milk should be pasteurized or ultra-pasteurized with an SNF value not less than 8.25% and milkfat not less than 3.25% (Hui and Culbertson, 2006).

Milk is an emulsion of fat globules in an aqueous phase. It is considered a complete food which has approximately 87% moisture and containing several groups of beneficial nutrients. Proteins, carbohydrates, and lipids comprise of the organic components of milk. It also contains functional elements like traces of vitamins, enzymes, dissolved gases (CO2, N and O) and dissolved salts (Mourad et al., 2014).

Table 2.1: Quantitative composition of milk (THE CHEMISTRY OF MILK, 2015)

Main Constituent	Limits of variation	Mean value
Water	85.5 – 89.5	87.5
Total Solids	10.5 – 14.5	13.0
Fat	2.5 – 6.0	3.9
Proteins	2.9 – 5.0	3.4

Lactose	3.6 – 5.5	4.8
Minerals	0.6 – 0.9	0.8

From the industrial point of view, milk proteins and their distinctive properties are an important feature that helps in the manufacture of useful products from milk. Therefore, milk proteins have attracted significant attraction from researchers.

Common methods of heat treatment given to milk

A major problem faced by the dairy industry is microbial spoilage of products and therefore a direct negative impact on its shelf life. As temperature is an essential environmental factor that supports the growth of microorganisms, thermal processing of milk is an indispensable step in milk production adopted worldwide by the dairy industry to ensure extended shelf life as well as improved quality (Haq et al., 2013). While microbiological safety is an important application of heat treatment of milk, it can also be used to improve organoleptic properties of milk when used as a food ingredient in milk-based products. Thermal treatment is an important pre-treatment parameter that can also manipulate the functionality of milk proteins. Heat treatment of milk can be achieved through various methods depending on application.

Thermization and tyndalization

Thermization is the comprehensive description of a range of sub-pasteurization heat treatments of milk which significantly reduce the number of spoilage bacteria with minimal heat damage to milk components and no change in flavor. While it was developed originally for heat treatment of milk before cheesemaking, due to the widespread practice of cold storage of raw milk, thermization at 62-68 ℃ for 15 s became a widespread practice to prolong the shelf-life of raw

milk and cultured milk products like yoghurt and quarg. The treatment can be carried out similar to pasteurization using a plate heat exchanger with a standard holding time of 15 s (Stepaniak and Rukke, 2002; Rukke et al., 2011; Rukke et al., 2016; Deeth, 2022). While thermization achieves its goals, i.e., shelf-life extension, in cultured products its rationale is often questioned as it reduces the viable count of lactic acid bacteria substantially.

Another means of sterilization is the classic Tyndallization process which follows repeated boiling and incubation in which a medium is steamed for 30 minutes on 3 successive days. The process works on the principle of killing vegetative spores over an extended time at intervals and incubation at lower temperatures (Brown et al., 1979; Piqué et al., 2019).

While both thermization and tyndallization can be used to improve the microbial safety of milk, it is advisably more effective when used with other methods that follow a higher degree of heat treatment.

Pasteurization

Pasteurization is a mild heat treatment typically carried out at a temperature below the boiling point of water (Deak, 2014). The term pasteurization was derived from the French scientist Louis Pasteur (1860) who discovered that heating liquids at high temperature improves their keeping quality. The process that originated in Germany in the 1800s was initially intended for medical profession specifically in infant feeding. Following developments in the process, in 1933 Yale concluded that pasteurized milk can be considered safe milk (Lewis, 2003). Currently, International Dairy Federation (IDF, 1986) defines pasteurization as ‘a process applied to a product (milk) with the aim of minimizing possible health hazards arising from pathogenic micro-organisms associated with milk, by heat treatment which is consistent with minimal chemical, physical and organoleptic changes in the product.’

There are two main types of pasteurization for milk:

1. Low temperature long time (LTLT) or conventional batch method (63 ℃ for 30 min)
2. High temperature short time (HTST) method (72 ℃ for 15 s)

LTLT process involves filling the vessel, heating the milk to a temperature between 62.8 ℃ and 65.6 ℃, holding for 30 min and rapidly cooling before emptying the vessel and filling into containers (Lewis, 2006). In HTST pasteurization the milk is heated at 72 ℃ for a holding time of 15 s and then promptly cooled to 5 ℃ or below. This continuous operation saves cost for the industry through faster heating and cooling with a regeneration efficiency of more than 90%. Early research found survivors after batch pasteurization of milk which questioned the efficiency of LTLT process. Later, the HTST process was introduced which was commercially considered highly efficient due to continuous processing and energy regeneration.

Ultra-High Temperature processing

While pasteurization is an effective way to control microbial spoilage, it isn't sufficient to deactivate thermoresistant spores in milk. So, the concept of subjecting milk to temperature above 100 ℃ and packaging them in airtight containers was developed. Such processed milk is termed as 'sterilized milk'. The introduction of sterilized or UHT (ultra-high temperature) milk resulted in 'commercially sterile' milk, which is not essentially free of microorganisms, but those that might survive the heat treatment are not viable to proliferate and cause spoilage during storage (Lewis, 1994).

A continuous flow technique is used to manufacture UHT milk. In this process, the milk is heated to a temperature of 138 – 145 ℃ for 2 – 10s followed by aseptic packaging (Rosenberg, 2022). There are two methods for UHT processing:

1. Direct UHT (D-UHT) where the milk and heating medium is in direct contact due to the heat treatment being done either by steam injection or steam infusion.
2. Indirect UHT (ID-UHT) where there is no direct contact between the milk as the heat treatment is carried out by using tubular or plate-heat exchangers.

Unopened UHT milk products have a long shelf life of 6-12 months at ambient temperature and are expected to have better texture for long term with negligible adverse effects on the products nutritional value. But due to certain interactions of different compositional elements of the products, physical instabilities like age gelation, sedimentation and creaming can be observed over longer periods of storage.

Direct and Indirect Heating

The discussions so far have led to an understanding on the different types of heat treatments given to milk. Research has established that during any heat treatment the product quality is heavily dependent on the chemical changes occurring such as protein denaturation, flavor development especially cooked flavors due to Maillard reaction and vitamin loss. But in the dairy industry, these changes during heating can be controlled and/or modified to an extend by adjusting the time-temperature combination. Other factors that play a role in achieving the same are the efficiency of heat transfer, holding time and intervals between heating and cooling (Eisner, 2021). This is where the method of heating/cooling (direct and indirect) comes into effect.

A conductive system of heating where transfer of heat occurs between a medium (water) and product (wall in plate-and-frame or tube heat exchangers) is known as indirect heating. An important advantage of indirect heating is the energy recover rate of over 90%. Direct heating systems were introduced to ensure faster heating rates. Through direct contact with the product by using steam, direct heating provides very fast heating of product as the steam condenses and latent

heat is released. Over the years, significant differences in product properties have been observed in products prepared using different types of heating method. While no studies have found clear differences based on overall protein denaturation during direct and indirect heating, association of α-lactalbumin and β-lactoglobulin with casein micelles differ in their reaction based on the heating type since they are temperature and kinetic dependent. Most studies have also found sedimentation after direct heating at high temperatures in UHT processing. These sediments are attributed to κ-casein depleted casein micelles aggregating with each other and is dependent on pH and calcium ion activity, which is affected by indirect or direct heating, owing to the sedimentation. Based on the significant differences in product characteristics pertaining to the type of heating used, it is essential to determine which method of heating would be beneficial for the preferred product.

General heat induced changes in milk

Milk is a very heat-stable system that can sustain extreme heat treatments in comparison to other foods. Even so, several biological, chemical, and physical changes occur in milk during heating which could lead to changes in its nutritional, organoleptic and/or technological properties. These changes could be reversible or irreversible.

Biological changes

Bacterial growth rate. Bacterial inhibitors are inactivated during heating which greatly affects the growth rate of bacteria. The lactoperoxidase system which affects lactic acid bacteria (LAB) are denatured. Depending upon the intensity, *bacteriophages* which are important for lactic acid fermentation can be inactivated. Heating can also produce stimulants like formic acid which is good for the growth of LAB (Walstra et al., 2005).

Physico-Chemical changes

Creaming. Creaming of milk fat globules is a very common occurrence in any type of milk. Fat globules float to the surface to form a creamy layer depending upon the difference in densities between the fat and aqueous phase. Cow's milk undergoes a cryoglobulin dependent agglutination of fat globules. Moderate heating of milk (e.g., 70 °C × 15 min) causes denaturation of cryoglobulins and thereby preventing creaming of milk (Fox et al., 2015).

Changes in Milk Fat Globule Membrane (MFGM). During direct heat processing, there could be a change in globule size due to foaming during agitation. Heating milk at temperatures higher than 70 °C denatures membrane proteins, mainly whey proteins. These denatured whey proteins could lead to Maillard browning with lactose. At higher temperatures most of the proteins and phospholipids are lost from the membrane. But it has also been found that treatment of cream at high temperatures before butter making improves oxidative stability of the butter.

Maillard Browning. Also known as nonenzymatic glycation is a chemical reaction between amino group and carbonyl group. In case of milk, lactose and proteins react to form Maillard reaction products like melanoidins (browning compounds) during heat treatment of milk. Maillard browning is aesthetically undesirable in dairy products. It also alters the flavor of milk.

Milk salts. Calcium and carbonate are the only affected salts during heating. CO_2 is lost during heating which leads to an increase in pH. Calcium solubility decreases and is transferred to the colloidal phase. These changes are reversible on cooling depending on the heat intensity.

Proteins. Biologically active proteins like vitamin-binding proteins, immunoglobulins etc. are inactivated during HTST pasteurization and UHT treatment. These play significant importance in infant formulae. Thermal denaturation of whey proteins which are 20% of milk proteins are

important. The disulphide residues exposed on heating can form complexes of β-lg with κ -casein and other aggregates which could affect functionality of milk protein system.

Effect of pH changes on milk

Just like heating, pH is an important factor that can influence changes in milk. It is important as it alters the electrostatic repulsions to influence the interaction in proteins.

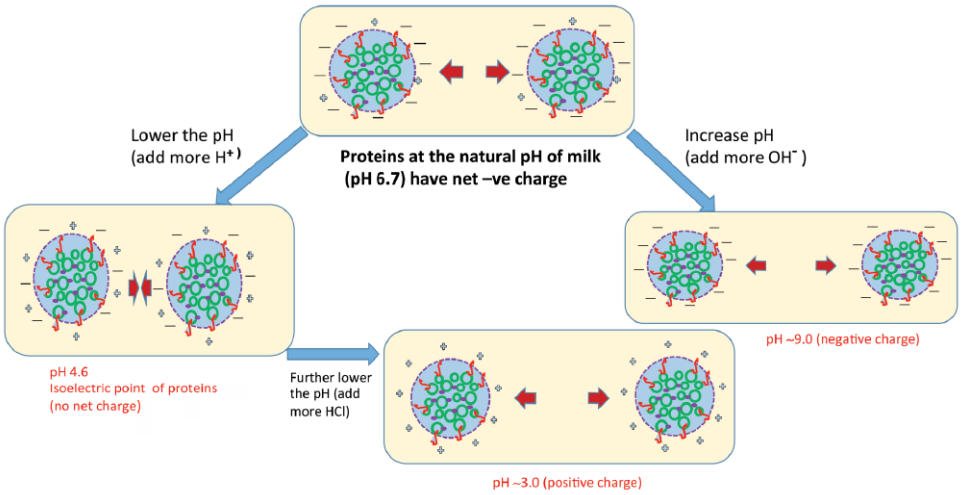

Figure 2.1: Schematic diagram showing effect of pH on the charges of protein molecules and protein interactions (Patel et al., 2015)

Acidification of milk is a very fundamental process in manufacturing of many dairy products such as yoghurts and cheese. As the pH is lowered from its natural pH of 6.7 there are two main changes in the milk system: 1) decrease in the surface charges of casein micelles which leads to release of calcium phosphate nanoclusters into the serum phase 2) collapse of κ-casein hairy layer due to neutralization of charge on proteins. This destabilizes the casein micelles and results in micelle aggregates as the proteins approach their isoelectric point (Graët and Gaucheron, 1999; Li and Corredig, 2020). The dissolution of colloidal calcium phosphate occurs at ~5.2 pH.

Further decrease of the pH to ~5.0 results in the collapse of the outer hairy layer and as the pH approaches ~4.6 the neutralization of charges happens, and dipole-dipole or hydrophobic attractions further lead to gel formation. Even with such loss in structure the caseins undergo little dissociation at lower pH at temperatures above 25 ◦C. In unheated milk the formation of gel with lowering pH occurs close to isoelectric point of caseins, ~4.8 pH, but in case of heated milk formation of whey protein complexes tends to determine the gel formation. The aggregation of proteins on heating with regards to pH is further explained.

Protein interactions in milk due to heating & varying pH

Numerous research has targeted the heat treatment of milk as well as pH and its influence on milk proteins. Most of the results are focused on changes to casein proteins and their interactions with whey proteins. Earlier research by Yoshino et al., 1964, reported that at temperatures above 100 °C due to dephosphorization of casein, the level of NPN increased and the liberation of NPN in casein fraction followed the following trend: κ- casein > αS-casein > β-casein. In addition, the formation of caseino-macro-peptide (CMP) like species during heating were observed by Alais (1967) and Hindle and Wheelock (1970). These research lead to significant results that proved hydrolysis of caseins on heating. Casein proteins has the property to self-associate largely through interaction of the hydrophobic regions with the hydrophilic/ charged regions. This is strongly dependant on temperature, pH, and ionic strength (Anema, 2021).

While earlier studies showed that prolonged heating of milk causes coagulation, it was determined in the later years that some dissociation or disaggregation of casein micelles would precede the coagulation. This would affect the distribution of casein and denatured whey proteins in the milk protein system. Kudo (1980) was the first to show the effect of pH on milk proteins

during heating and related it to heat stability of milk at 140 °C. Kudo proposed a theory where at low pH, whey protein coated casein micelles exist and that at high pH κ-casein (and whey protein) depleted micelles are present. This theory led to extensive research on pH-dependent dissociation of casein micelles. Singh and Fox (1985a,b; 1986; 1987a,b,c) determined that at temperatures above ~90 °C κ-casein dissociation increased with increasing pH and this dissociation seemed to be directly proportional to the levels of β-lactoglobulin (β-lg). Another factor identified was the involvement of electrostatic interactions. Increasing negative charges promoted dissociation while decreasing negative charges had the opposite effect. The concentration of milk at the time of heating at their natural pH influenced dissociation of κ-casein from casein micelles. Recent studies used ultracentrifugation to determine the levels of caseins in the supernatant. With higher temperatures and longer holding times, the κ-caseins (~80%) and denatured whey proteins in supernatant increased.

Whey protein, mainly β-lag plays an important role in κ-casein dissociation. From studies by Anema (2018) it was observed that the levels of κ-casein in the serum phase increased progressively from pH 6.5 to pH 7.1. These results identified κ-casein as being capable of stabilising high levels of denatured whey proteins to form serum phase aggregates. The presence of serum phase κ-casein and whey proteins had an influence on the stiffness of acid gels formed from heated milk (Anema, 2018). The interaction of denatured whey proteins with caseins (mainly κ-caseins) in milk at temperatures above 70 °C were studied. All the research done has led to believe that the distribution of denatured whey proteins between serum and colloidal phase can be correlated with the location of κ-casein and pH of the milk at heating. About 80% of denatured whey proteins were found to be associated with casein micelles when milk was heated at 90 °C and pH 6.5. At pH above 6.7, almost 30% association of denatured whey proteins with casein

micelles were observed. The percentage of association kept decreasing as the pH was increased resulting in almost all the denatured whey protein at the serum phase at pH above 7.1.

Whey protein denaturation is an irreversible result of heating of milk. Due to conformational changes in β-lg molecule a reactive thiol group is exposed upon heating. Through thiol group-disulfide bridge exchange reactions it could interact with other proteins which makes the denaturation irreversible. While α-lactalbumin (α-lac) lacks free thiol group to initiate polymerization, it still has four disulfide bridges which reacts with β-lg and gets irreversibly denatured (Mulvihill and Donovan, 1987). β-lg can also interact with casein micelles in a similar way resulting in casein-whey aggregates. The type of aggregates formed in heated milk was identified to be pH and temperature dependent. Vasbinder and de Kruif (2003) observed the protein-interactions of heated milk at varying pH. While there was a rather constant percentage of whey protein denaturation at pH 6.45, 6.55 and 6.7, at an increased pH of 6.9 α-lac showed slight increase in denaturation and β-lg showed more denaturation at 6.35. Both fractionation study done by Vasbinder et al. (2003) and ultrafiltration study done by Oldfield et al. (2000) showed that at lower pH an increased association of whey proteins with casein micelles are observed and at higher pH there was evidence of formation of whey protein aggregates. α-lac was determined to have a higher affinity for aggregates while β-lg has a higher affinity for casein micelles.

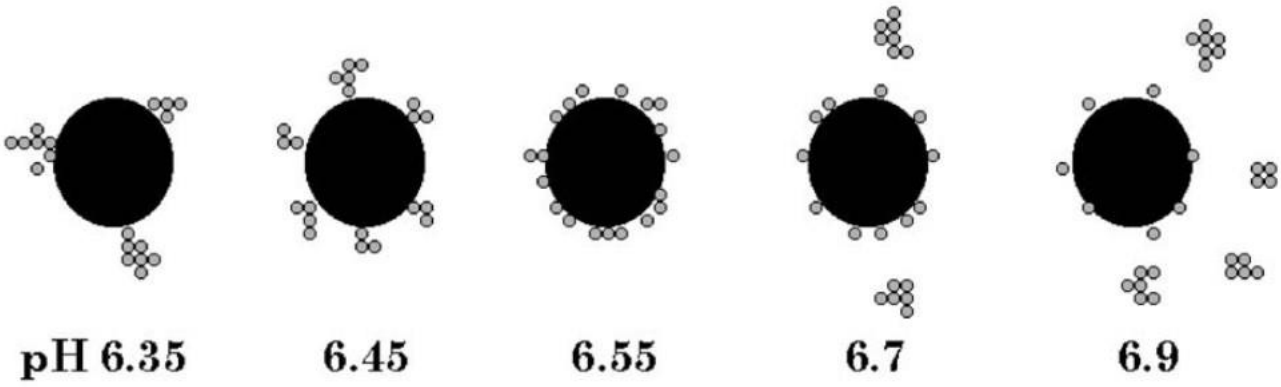

Figure 2.2: A schematic representation of the interactions between casein micelles and whey proteins occurring in milk during heat treatment for 10 min at 80 °C at pH values ranging from 6.35 to 6.9. The small circles represent denatured whey proteins, the large circles the casein micelles. The whey proteins are either present in aggregates or covalently associated with the casein micelle. Native whey proteins are not included in the figure. (Vasbinder & de Kruif, 2003)

Milk Protein Concentrate

Milk protein concentrate (MPC) are functional ingredients which have been gaining popularity among high protein dairy ingredients in recent years. They could be loosely described as spray-dried protein enriched forms of skim milk (Kelly, 2011). MPCs are manufactured using technological innovations and development in ultrafiltration (UF). While the UF membranes selectively concentrate larger molecular size constituents like milk proteins, addition of water also known as diafiltration (DF) is required to attain higher protein concentrations (>80%). MPCs are thus available in a varying range of protein concentrations (42 to 85%) and their lactose content inversely proportional to the protein concentration (O'Kennedy, 2009).

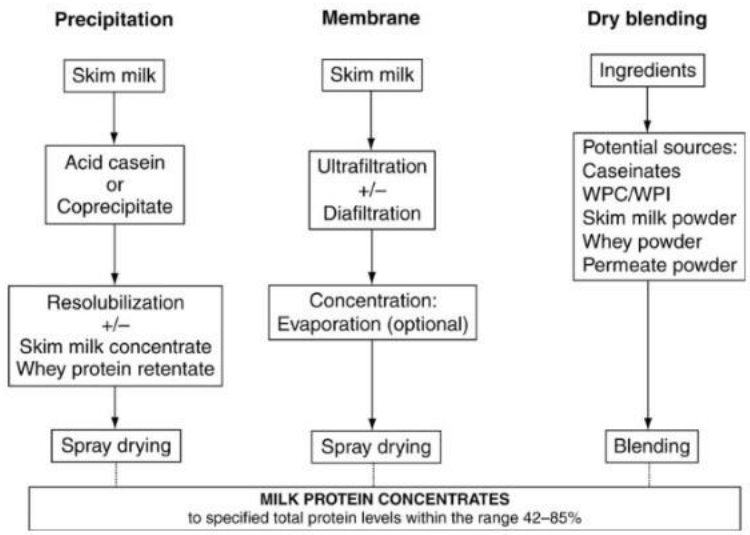

Figure 2.3: Processing options for the manufacture of milk protein concentrates (MPCs) based on membrane separation, protein precipitation-initiated processes, and ingredient dry blending. Note that only membrane separation is likely to retain the casein in its original micellar state. WPC, whey protein concentrate; WPI, whey protein isolate. (Kelly 2011)

Table 2.2: Typical gross composition (%) of milk protein concentrates (MPCs) with protein concentrations ranging from 42 to 85% **(**Kelly 2011**)**

Ingredient	***Moisture***	***Fat***	***Protein***	***Lactose***	***Ash***
MPC-40	3.5	1.0	42.0	46.0	7.5
MPC-70	4.2	1.4	70.0	16.2	8.2
MPC-75	5.0	1.5	75.0	10.9	7.6
MPC-80	3.9	1.8	80.0	4.1	7.4
MPC-85	4.9	1.6	85.0	1.0	7.1

MPCs are typically manufactured under modest levels of heat treatments mostly during the pre-processing stages and surviving microorganisms, spores or enzymes are retained during UF treatment at a temperature between 10 – 15 °C. The molecular weight cut-off (MWCO) ranges from 1 to 200 kDa with ~0.01 μm pore size and <10 bar operational pressure (Rosenberg, 1995). The resulting retentate is then subjected to preheat treatment and then spray dried in a spray dryer followed by an 'after-drying' and cooling in an external fluid bed.

Functionality of MPC

The functional properties of milk protein concentrates are correlated with its protein content. Interfacial properties of milk proteins and milk powders are key properties for their use as food ingredients (Uluko et al., 2016). An example would be that at higher protein content MPCs are good for protein enhancement while giving a clean dairy flavor without the addition of lactose to product formulations. Interfacial properties are very important for their use as food ingredients. Here we will discuss a few important functional properties in relevance of dairy products.

One of the most important properties of MPC is solubility. Since the dissolution of powdered milk proteins is necessary for the expression of their functional properties, the solubility of MPC is regarded as a critical property by the manufacturers and end users of MPC (Agarwal et al., 2015). This is because solubility is considered as a prerequisite to certain functional properties, namely gel formation or stable foams and emulsions. While MPC have been recognized to have poor solubility at ambient temperature, research and studies have made progress in solving this issue. Solubility of MPC increases with increasing temperature (Baldwin and Truong, 2007) and decreasing protein concentration (Sikand et al., 2011). At higher concentrations of protein, the solubility becomes an even greater challenge, but according to a recent study by Babu et al., (2018), MPC at 70, 80 and 90 percent concentrations showed better particle morphology and flow behavior at 40°C. Addition of monovalent salts to ultrafiltered retentate (Carr, 2002) or removal of calcium ions before drying (Bhaskar et al., 2003) were found to improve solubility of high-protein MPCs.

Gelation is another important functional property of MPC and is crucial in yoghurt applications. Matia-Merino and Singh (2007) studied acid-induced gelation of MPC and partly concluded that there is a possibility to manipulate the generation of different types of gel matrices, by controlling rate of acidification, casein micelle integrity, calcium levels and pectin

concentrations to obtain different synergistic or antagonistic effects (Uluko et al., 2016). Heat treatment of milk has been widely studied to influence acid gel properties. Studies on the formation of soluble and micelle-bound why protein/κ-casein complexes in heated milk and their physico-chemical properties have shown that these complexes tend to support early gelation and increased gel strength. They are believed to bring new functionalities to casein micelles and enhance gel network formation (Morand et al., 2011, Anema et al., 2018, Rathod et al., 2022, Lucey et al., 2022).

Heat stability is another property that can be defined using the concept of heat coagulation time (HCT). It is usually documented at 140 °C and is considered as the time at which onset of heat coagulation of the sample is observed. A higher HCT is preferred for dairy ingredients as it makes it suitable for processing at higher temperatures and better protein stability (Meena et al., 2017). One way to achieve this is through shift in ionic equilibrium between serum and colloidal phase during UF of skim milk. While UF of skim milk is expected to reduce heat stability, DF was used to enhance it by Sweetsur and Muir (1985). Research have also concluded that lowering spray drying temperature resulted in MPCs with better functionality.

Milk proteins are known to be excellent emulsifiers. Emulsifying properties are of three mainly: the emulsifying capacity, the emulsifying activity, and the emulsifying stability of a protein. And the main contributing factors are - type of oil, protein concentration and homogenization. Studies have been done on the effects of thermal processing on emulsifying abilities of protein and have shown that heat treatment has a different effect on whey protein functionality and that exhibited by caseins. Heating had an adverse effect on emulsion stability in whey protein systems and in mixed protein systems. This was associated droplet flocculation arising from protein-protein interactions. But addition of small amounts of sodium caseinate

improved the heat stability of a whey-protein based emulsion systems (Raikos, 2010). While research have been making headway in tailoring functionality of a milk protein system, stabilizing emulsions in mixtures of whey and protein are proved to be challenging.

Foaming is a typical property of ice-creams. Proteins play a major role in formation and stabilization of foams in such aerated products. Several studies have shown that heat treatment, pH and ionic environment are some important factors that govern this property (Uluko et al., 2016). To further increase its functionality, it was observed that the presence of EDTA in ice cream increased the stability of the ice cream emulsion to shear and greatly decreased the proportion of fat globules involved in air serum interfacial adsorption (Zhang and Goff, 2004). MPC as an ingredient has received very little attention so far in this area.

Methods used in modification of functional properties of MPC

Various processing methods have been studied to improve the functionality of milk proteins. As seen earlier, heat treatment has been a widely used modification to bring about changes in functionality of milk proteins. Solubility of MPCs improved by increasing the reconstitution temperature (Uluko et al., 2016). While emulsions formed from unheated proteins were found to be less stable, Dybowska (2008) found that increasing the preheat temperature resulted in ~50% more stable emulsions in his research the effect of preheat treatment on O/W emulsions (30% rapeseed oil) stabilized with MPC at 60°C and homogenization pressure of 10 MPa and 2 MPa. Udabage et al., (2011) found a beneficial combination of heat and pressure (40 °C and 200 MPa) to apply to concentrates before spray drying that can improve the MPC powder solubility. Improved gelation of MPC has also been observed by various studies through heating of skim milk or UF concentrate. Addition of at least one monovalent salt prior to drying of MPC

ultrafiltrate enhances solubility of the final product and is an advantage in cheese making (Carr et al., 2004; Schuck et al., 1999, 2000; Mao et al., 2012; Sikand et al., 2013). Schokker (2011) concluded that by adding sodium caseinate to UF retentate prior to spray drying can influence properties of the dried powder. Partial calcium depletion using chelating agents, cation-exchange chromatography, or acidification of skim milk results in dried MPC or MPI with improved physical and functional properties (Bhaskar et al., 2007).

Protein interaction during heat treatment is a niche field which is currently being studied by researchers. There have been many upcoming studies that have been able to shed light on the type of protein interactions that happens during heat processing and its effect on heat stability of final dried product. The effect of pH on the type of protein interactions is an interesting area of study which provides lots of opportunities for MPC as a functional ingredient. Blazey (2000) developed a processing method for MPC powders where the skim milk was heated after pH adjustment and then ultrafiltered. The selection of alkali and acid used for pH adjustment helps provide a retentate with desirable calcium to protein ratio without extra addition of insoluble calcium salts and thereby naturally providing desired nutritional compositions. This method is considered as an efficient way to improve the functionality of MPCs and thereby improving their efficiency as a functional ingredient.

Chapter 3 will focus on identifying the protein interactions and changes in physicochemical properties MPC powders manufactured with skim milk pH adjusted and heat treated prior to UF and spray drying. While chapter 3 does go over a few functional properties, chapter 4 provides a detailed analysis of heat stability of the newly manufactured MPC powders using acid gelation to determine the changes in gelation parameters with respect to pH and heat treatment.

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Chapter 3 - Determination of protein interaction in milk protein concentrate powders manufactured from pH adjusted and heat-treated skim milk

ABSTRACT

Milk protein concentrate (MPC) powders are widely used as a food ingredient due to its functional and nutritional benefits. Depending on the application, bringing desirable changes in its functional properties would further expand the possibilities for MPC as an ingredient. However, the effects of pretreatments on the skim milk base prior to UF/DF on the structural and functional properties of the resultant MPC powders is highly understudied. In this study, the protein fractions of MPC powders manufactured from heat treated and pH adjusted skim milk was investigated to determine the level and type of protein interactions happening in the final ingredient developed. The pH of skim milk was adjusted to 6.5, 6.8, or 7.1 and subsequently divided into 2 parts. The first part was heated at 90°C for 15 min and the remaining part was used as control. After heating, the pH of skim milk was readjusted to 6.8. The skim milk was ultrafiltered and spray dried to manufacture MPC powders (84.4% average protein in DM basis). Capillary electrophoresis was used to determine the distribution of caseins and whey proteins in the soluble and micellar phase. The MPC was reconstituted to 5% protein solution and characterized in terms of particle size, apparent viscosity, and heat coagulation time (HCT). A high heat treatment on skim milk at 90 °C for 15 min resulted in a greater amount of unbound whey proteins in samples with a heated skim milk base at pH 7.1; while most whey proteins were found to be bound to casein micelles in samples with a heated skim milk base at pH 6.5. A larger median diameter was observed in particles of

MPC powders with heated skim milk base at pH 6.5 which correlates with an increase in viscosity as well. Heat stability was observed to have improved in samples with pH adjusted to 6.5 and 7.1. These findings create a base for further research on understanding changes in functional behavior of MPC powders due to processing factors. This in turn opens possibilities for MPC powders through tailoring protein interactions to achieve desired ingredient functionality in untapped product applications.

Key words: MPC powders, heating, pH, whey protein, caseins, physicochemical properties

INTRODUCTION

Increasing demand for food proteins can be observed in the consumer market due to the world's population growth and changing lifestyles. A huge part of the rising demand for proteins is met by the dairy industry. Milk protein concentrate (MPC) powder is an important dairy ingredient that provides high-quality milk protein in the same ratio as in milk with added benefits of excellent nutritional and functional properties in a variety of food applications (Sunkesula et al., 2021). MPCs are manufactured widely using a method known as ultrafiltration (UF) which does not subject the skim milk to harsh processing conditions like high heat treatments or pH adjustments. The technology uses porous membranes with specific pore sizes that use pressure to separate whey and casein proteins from smaller milk components (Blazey et al., 2000). The skim milk is concentrated at varying volumetric concentration factors from 2 to 5 depending on the amount of lactose and minerals that need to be removed with respect to desired final protein content. As the concentration factor increases, the viscosity of the retentate tends to increase. In such cases, for efficient removal of lower molecular weighted materials, a process known as diafiltration (DF) is used wherein a diluent (generally water) is added to the concentrated retentate. The UF/DF

retentate can be further spray dried to manufacture MPC powders varying in protein content (35-90% w/w) depending upon the concentration factor achieved.

Thermal treatment is an important processing step in the manufacture of dairy products. Various factors like ionic strength, mineral composition, protein concentration, and pH affect heat treatment's impact on the final product's physicochemical properties (Singh et al., 2015; Clodagh M Kelleher et al., 2020). Heat treatment of proteins leads to denaturation or aggregation of proteins. At temperatures greater than 70 °C, an irreversible unfolding of whey proteins occurs which causes them to denature and expose reactive functional groups such as the free thiol groups in β-lactoglobulin (β-LG). These functional groups can thereafter aggregate through hydrophobic bonding and thiol/disulphide exchanges with corresponding denatured whey proteins, caseins, (CN), or κ-caseins (κ-CN) resulting in the formation of whey protein aggregates, whey protein-CN aggregates or whey protein/κ-CN complexes (Anema, 2007; Donato and Dalgleish, 2006; Donato and Guyomarc'h, 2009). These protein interactions play an important part in influencing the functional properties of MPCs. Numerous studies on skim milk have investigated the protein interactions due to heating and have associated pH at heating to be a major contributing factor in the distribution of proteins and type of interactions. Denatured whey proteins were found to form filamentous appendages at the casein micelle surface on heating at higher temperatures and below pH 6.7, whereas serum-phase whey protein aggregates were found at higher pH (Creamer and Matheson, 1980; Anema and Li, 2003). In a study of heated skim milk system (Oldfield et al., 2000), association of whey proteins with casein micelles at lower pH were determined to be more than 80% while only about 20% were found at skim milk pH above 6.8 and reminder of the denatured whey proteins existed in the serum phase.

In comparison to other protein rich powders in the market, MPC powders fall short in their functional properties. MPC powders with higher protein and calcium contents have reported poor solubility and functionality which have eventually limited their use in various food formulations (Meena et al., 2018). Even though it has been recognized that MPC powders have high functional potential there have been fewer research focused on improving their functional properties. Studies by Ho et al., (2018,2019) showed that pH adjustment and heating of liquid MPC had significant effects on viscosity, Ca^{2+} activity and heat stability of the concentrates. Another study demonstrated that MPC powders manufactured from liquid ultrafiltration concentrate heat treated at ≥ 100 °C proved challenging to disperse and solubilize (McSweeney et al., 2022). The earliest study on preheat treatment of skim milk during manufacture of MPC powders was done by Carr (1999) on MPC85 powders to characterize the rheology of the concentrate and functional properties of the powder. Lin et al., 2018 manufactured MPC powders from skim milk that was heat treated at 72 °C for 15 s (LHMPC) or 85 °C for 30 s (MHMPC) and studied the functionality of the corresponding reconstituted MPC dispersions. Meena et al., 2018 investigated the effect of change in pH of skim milk prior to ultrafiltration on the final MPC70 powders and concluded that there were significant changes in the functional properties of the treated MPC70 powders.

It is evident from the available research that thermal treatment or pH adjustment of skim milk prior to manufacture of MPC powders significantly affect the functional properties of the resulting MPC powders. But no studies have been conducted so far to evaluate the effect on MPC powders prepared from preheated and pH adjusted skim milk. Therefore, the objective of this study was to investigate the effect of pH adjustment and heat treatment of skim milk prior to UF/DF to tailor protein interactions that could affect the pysico-chemical and functional properties of MPC powders.

MATERIALS AND METHODS

Experimental Approach

Fresh milk procured from SDSU Dairy Research and Training Facility (DRTF), Brookings, SD was separated and pasteurized to make skim milk. The skim milk was divided into 3 equal parts and pH was adjusted to 6.5, 6.8, and 7.1, respectively. Each pH adjusted skim milk was further divided into 2 equal halves where one half was heat treated at 90 ℃ for 15 minutes and the other half was used as is. The pH of heated and unheated samples was readjusted to 6.8. Further, the skim milk was ultrafiltered to 5x concentration and spray dried to manufacture MPC powders (3% moisture, 82% true protein). Physicochemical properties of each powder were analyzed and compared. The whole experiment was replicated thrice from independent lots of skim milk.

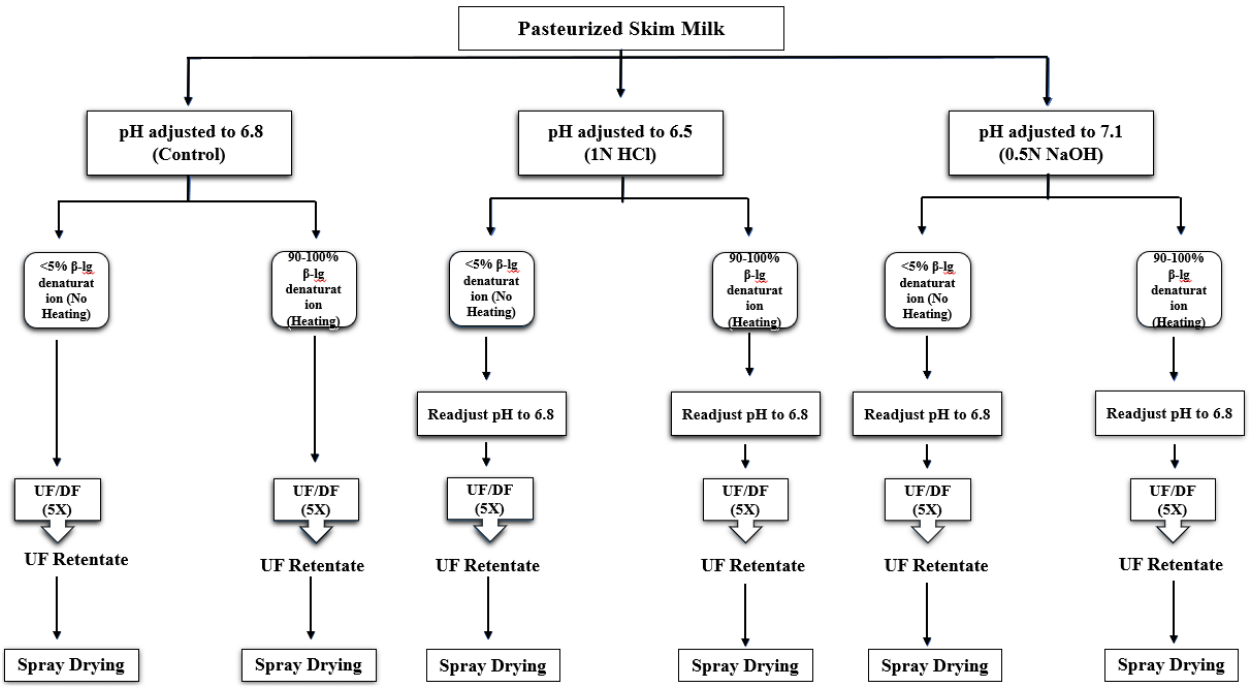


Figure 3.1: Experimental design for the manufacture of MPC powders from heated and pH adjusted skim milk

Manufacture of functionally modified MPC powders

Milk supply and treatment

Fresh milk was procured from SDSU Dairy Research and Training Facility (DRTF), Brookings, SD. The freshly obtained milk (3500 lbs) was separated to form skim milk and then pasteurized at 71.7 ℃ for a hold time of 15 s. Skim milk was then divided into three equal parts each amounting to 1100 lbs and their pH adjusted to 6.8, 6.5 and 7.1 respectively using 1 N HCl or 0.5 N NaOH. Each pH adjusted samples were further divided into equal halves of 550 lbs. One half was left unheated while the other half was heat treated at 90 ℃ for 15 minutes using a temperature-controlled circulatory water bath (Cole Parmer, Vernon Hills, IL) to achieve 90 – 100% β-lg denaturation. Both heated and unheated samples were readjusted to 6.8 pH and kept for cooling.

Ultrafiltration of treated skim milk

Ultrafiltration (UF) was performed on each treated milk samples using two parallelly arranged 10 kD polyether sulfone spiral wound membranes (3838 element format, Dominick Hunter Filtration Divison—N.A, Parker Hannifin Corporation, Oxnard, CA, USA). Each membrane element had a diameter of 97 mm and length of 965 mm with a 1.1-mm feed spacer and a total surface area of 5.7 m². Ultrafiltration was performed at 15° C. The inlet and outlet pressures were maintained at 50 and 20 psi, respectively, resulting in a transmembrane pressure of 30 psi. Diafiltration water was added to concentrate the skim milk to a volumetric concentration factor of 5 by optimal removal of lactose and minerals. The heated samples required approx.. 75% DF water while the non-heated samples only required approx.. 65% DF water.

Spray drying

The UF retentates were spray dried in a pilot scale dryer fitted with a Niro centrifugal disc atomizer (ASO 412E, Niro Inc., Columbia, MD). The inlet and outlet temperatures were maintained at 185

°C and 105 °C, respectively. The powders were packed in airtight containers and stored at -21°C until further analysis was completed.

Analysis of protein interaction in the functionally modified MPC powders

Separation of serum phase to determine whey protein denaturation. Serum phase was separated from MPC powder reconstituted to 5% protein dispersion using the method defined by Lucey (1998). MPC dispersions were ultracentrifuged at 90000 g and 20 °C for 60 min to determine the extent of denaturation of whey proteins and their association with casein micelles. The carefully collected supernatant was used for capillary gel electrophoresis. The extent of denaturation was determined by comparing the amounts of β-lg and α-lac present in the ultracentrifuged supernatant of heated samples with that of unheated control samples. The difference in the amounts of β-lactoglobulin (β-lg) and α-lactalbumin (α-lac) in the ultracentrifuged supernatant of heated and unheated MPC dispersions were determined as the percentage of whey proteins associated with casein micelles (Lucey et al., 1998).

Capillary Gel Electrophoresis (CGE). The centrifuged and separated serum were analyzed using CGE to determine individual protein fractions present in them. A Beckman P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA, USA) equipped with a UV detector set at 214 nm was used to carry out CGE. The separation was performed using a 50- μm bare fused silica capillary (20.2 cm effective length from the inlet to the detection window) following the method suggested by Salunke (2021). The peaks in the capillary electropherogram were identified and compared. Using a valley-to-valley approach, area of each identified individual CN fraction (αS1-CN, αS2-CN, β-CN, κ-CN, and γ-CN), serum protein fraction (α-lac, β-lg, peptides (peaks

between 10 kDa and 20 kDa), and NPN fraction (all positive peaks below 10 kDa)) was calculated as percentage of total area (positive peaks).

Characterization of heat induced changes in modified MPC powders

Mean particle size and zeta potential

Mean particle size and zeta potential were analyzed using a dynamic light scattering analyzer (DelsaMax Assist, Beckman Coulter). Samples were prepared for measurement by diluting to 1/100. Disposable cuvettes were used for the analysis of particle size and for zeta potential, samples were injected into the flow cell using a syringe at room temperature.

Apparent Viscosity

The apparent viscosity was measured at a varied shear rate between 10 s^{-1} to 300 s^{-1} at 25 °C using a stress-strain-controlled rheometer (MCR-92 Anton Paar, Vernon Hills, IL) fitted with a 50-mm diameter stainless steel cone with 1° angle and 101-μm gap (Rathod and Amamcharla 2021). Using the power law model, the consistency coefficient and flow behavior index were also calculated.

Heat Coagulation Time (HCT)

Heat-resistant screw-cap test tubes (8 mL, 17 mm × 63 mm; DWK Life Science, Millville, NJ) containing three milliliters of sample were inserted on the stainless-steel rack. The rack with the tubes was immersed in an oil bath (Narang Scientific Works Pvt. Ltd., New Delhi, India) maintained at 140°C and placed on the rocker. The time was recorded from the moment the tubes were immersed in the oil bath till the onset of coagulation was observed in the samples. This time was noted as the heat coagulation time. (Crowley et al., 2014; Ho et al., 2018).

Statistical analysis

Statistical analysis was performed using PROC GLIMMIX and Tukey's test in SAS Version 9.4 (SAS Institute Inc.) to determine significant differences based on treatments. The significance was established when $P \leq 0.05$.

RESULTS AND DISCUSSION

The approximate composition of skim milk used to make the individual lots of MPC powders provided by the manufacturer are as follows: Fat – 0.07%, Protein – 3.31%, Total Protein – 3.51%, Lactose – 4.88%, Total Solids – 9.27%, SNF – 9.19%. The MPC powders made from unheated skim milk had a total protein range of 82.72 – 83.29 % and those made from heated skim milk had a total protein range of 81.08 - 82.09 % (Table 3.1). The non-protein nitrogen (NPN) content of all samples was comparable. While it can be observed from Table 3.1 that there was a decrease in the non-casein nitrogen (NCN) values on heating, statistical analysis showed no significant difference ($P > 0.05$). The total solids (TS) content of all powders was within the range of 96.36 – 97.74 %

Table 3.1: Protein and total solids (TS) content (%) of MPC powders manufactured from unheated and heated skim milk

Attribute	pH 6.5		pH 6.8		pH 7.1	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
Total Protein (%)	83.15 ± 1.12	81.08 ± 1.71	82.72 ± 0.20	81.95 ± 0.67	83.29 ± 0.43	82.09 ± 1.36
Non-Casein Nitrogen (%)	9.12 ± 5.56	4.42 ± 0.17	11.03 ± 3.63	5.39 ± 0.24	11.43 ± 3.09	4.40 ± 0.21
Non-Protein Nitrogen (%)	1.37 ± 0.39	1.89 ± 0.59	1.98 ± 1.03	1.30 ± 0.35	1.26 ± 0.21	1.50 ± 0.29
Total Moisture (%)	97.45 ± 0.72	96.36 ± 0.53	97.74 ± 0.31	97.45 ± 0.31	97.16 ± 0.70	96.63 ± 0.82

a-b Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD.

Analysis of protein interaction in the modified MPC powders

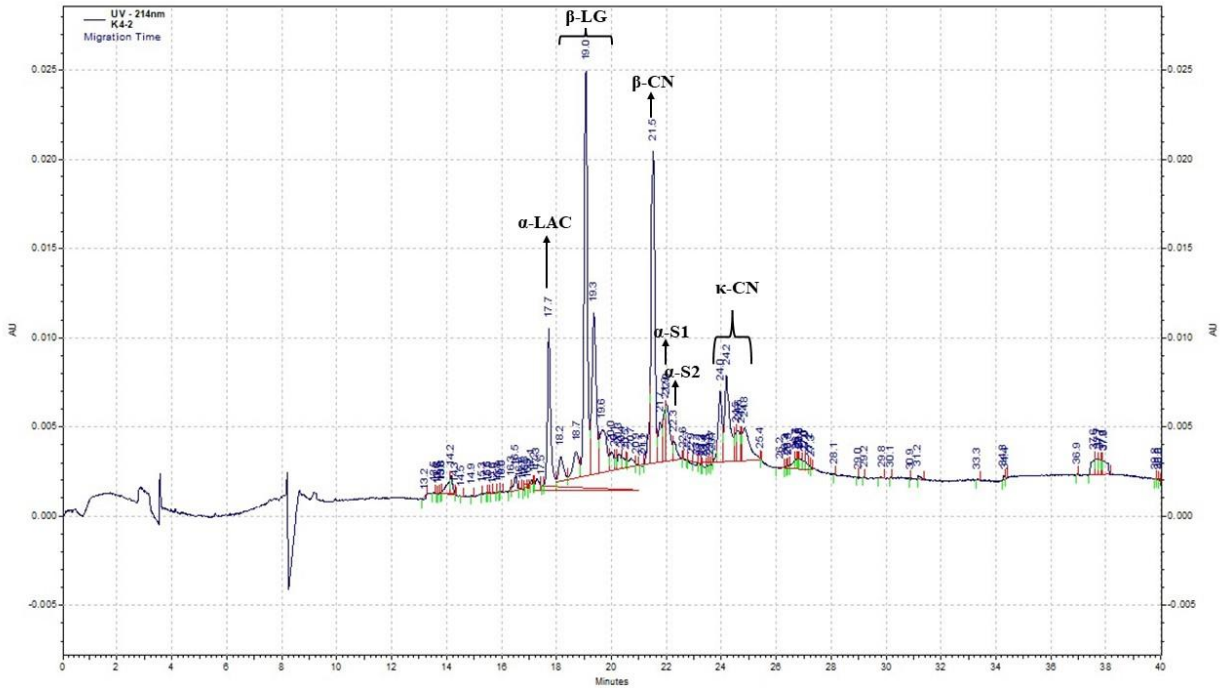
Table 3.2: Percentage milk protein fractions in serum phase of MPC dispersions of MPC powders manufactured from unheated and heated skim milk

Attribute	pH 6.5		pH 6.8		pH 7.1	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
α-LA	9.40 ± 0.11^{b}	1.30 ± 0.31^{c}	9.30 ± 1.36^{b}	6.90 ± 0.34^{b}	15.70 ± 0.07^{a}	7.00 ± 0.00^{b}
β-LG	41.70 ± 2.80^{b}	3.80 ± 0.08^{d}	43.40 ± 0.34^{b}	27.90 ± 0.53^{c}	50.10 ± 0.12^{a}	41.80 ± 0.10^{b}
β-CN	17.60 ± 0.37^{c}	43.80 ± 0.46^{a}	17.00 ± 0.43^{c}	24.40 ± 0.70^{b}	17.50 ± 0.38^{c}	16.90 ± 0.66^{c}
αS1-CN	8.50 ± 0.02^{c}	32.20 ± 1.01^{a}	6.20 ± 2.36^{c}	18.50 ± 1.04^{b}	6.20 ± 0.64^{c}	10.80 ± 1.85^{c}
αS2-CN	1.20 ± 0.24^{b}	6.30 ± 1.24^{a}	3.00 ± 2.51^{ab}	4.50 ± 1.18^{ab}	1.00 ± 0.08^{b}	2.40 ± 0.94^{ab}
κ-CN	17.80 ± 2.48^{a}	5.60 ± 0.99^{b}	17.90 ± 0.77^{a}	15.40 ± 2.65^{a}	4.40 ± 0.93^{b}	17.40 ± 2.59^{a}

a-d Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD.

The supernatant obtained after centrifugation was analyzed using capillary electrophoresis to quantify the level of whey protein denaturation in the samples. Previous literature assumes that the difference in quantity of α-lac and β-lg between heated samples and unheated control samples will determine the amount of whey proteins associated with the casein micelles. Table 3.2 shows the percentages of whey proteins present as a function of the pH at heat treatment in the separated serum phase of reconstituted MPC powders. The control is the MPC dispersions made from unheated skim milk base. The presence of caseins was detected in the serum phase. A decrease in pH of skim milk to 6.5 prior to UF/DF did not show any significant difference among the protein fractions. But samples at pH 7.1 showed a significantly increased levels of whey protein content (α-lac & β-lg) and significant decrease in κ-Casein in the soluble phase in comparison to the control sample.

(A)


UV - 214nm
K4-2
Migration Time
β-LG
β-CN
α-LAC
κ-CN
α-S1
α-S2
AU
Minutes

(B)

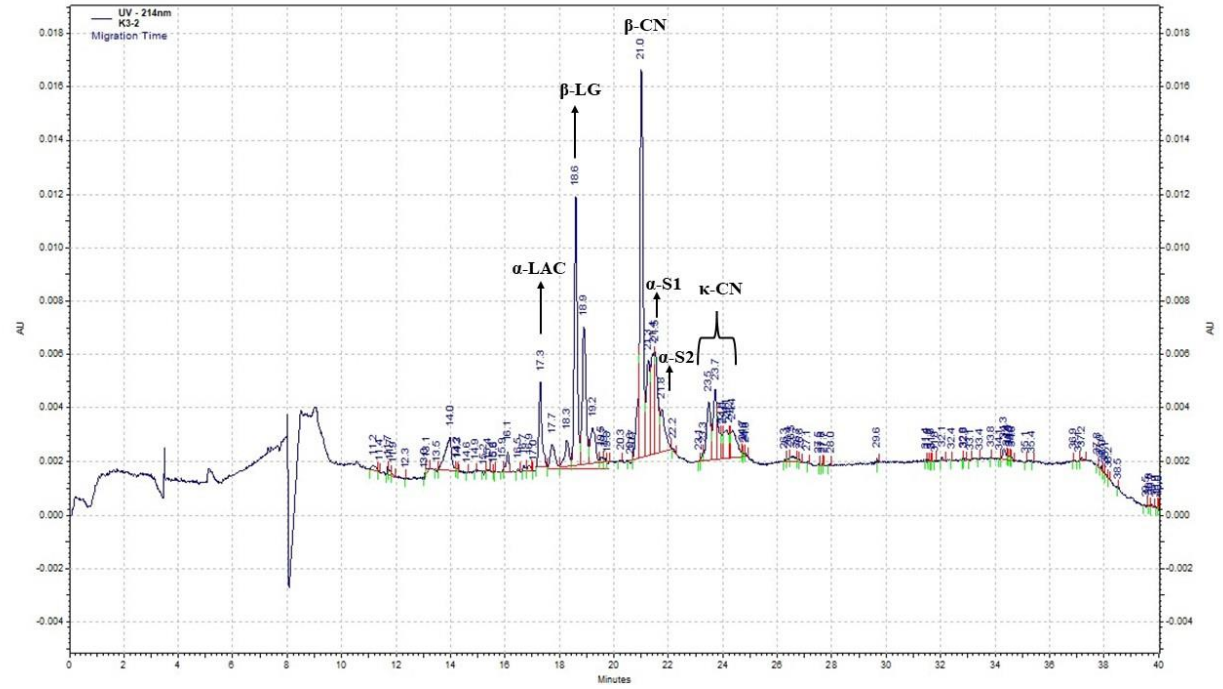

UV - 214nm
K3-2
Migration Time
β-CN
β-LG
α-LAC
α-S1
κ-CN
α-S2
AU
Minutes

Figure 3.2: Capillary gel electrophorohram of serum phase of (A) MPC dispersions of MPC powders manufactured from unheated skim milk at pH 6.8 (B) MPC dispersions of MPC powders manufactured from heated skim milk at pH 6.8

A considerable decrease in the amount of whey proteins can be observed after heat treatment at all pH. Formation of soluble aggregates increased with increase in pH. After heat treatment at 90 °C for 15 min, more interactions happened in the soluble phase at a higher pH than at a lower pH – approximately 10% of β-lg at pH 6.5 and 83% of β-lg at pH 7.1. A similar result was observed by Vasbinder & de Kruif (2003) while using a rennet fractionation method to quantify the protein fraction. A significant increase in κ-casein was observed in the samples heated at 7.1. This could be because heat treatment has been found to cause whey protein association with κ-casein. Around 90% of the whey proteins were found to have coated on casein micelles at pH 6.5 among which β-lg (~75%) was the major whey protein associated. This agrees with the claim by Vasbinder & de Kruif (2003) that β-lg has a higher affinity for casein micelles. But comparing with the protein fractions in unheated supernatant at 6.5 pH there was an 86% reduction in α-lac in the heated sample. This is comparable with 90% reduction of β-lg after heating. Therefore, it can be argued that even though β-lg might be higher in quantity, the ratio of denaturation of both α-lac and β-lg is comparable at a lower pH. With an increase in pH from 6.5 to 7.1, the amount of bound whey protein showed a steep decrease from ~90% to ~25% ($P<0.05$) in heated skim milk base. This observation is similar to that by Qi et al., 2022 where the decrease was from 95.0% to 21.4% at the same pH range. An increased amount of αS2-casein was detected in the heated samples at 6.5. This can be attributed with formation of whey protein and casein aggregates (Vasbinder et al., 2003). As the pH increased, even though the trend of association of whey proteins with casein micelles were the same, a significant amount of whey proteins remained soluble especially at pH

7.1 where 75% of whey proteins were retained in the supernatant. It can also be noted that at pH 7.,1 55% of α-lac was found to be associated with casein micelles and only 16% β-lg. Overall the results show that at higher pH most of the aggregation happens between whey proteins. The result of this study is in line with the observation by Anema (2018) that the levels of κ-casein in the serum phase increases progressively from pH 6.5 to pH 7.1.

Mineral equilibrium and state of colloidal calcium phosphate (CCP) maintains the casein structure (Walstra, 1990). Studies have shown that pH is an important factor that modifies the structure of casein micelles (Gonzalez-Jordan et al., 2015). The result from this study further strengthens the claim. Anema (1998) demonstrated that in reconstituted skim milk of 10% TS, at pH lower than 6.7, low levels of casein were solubilized and when pH was increased from 6.7 to 7.1 there was an increase in the levels of solubilized casein, regardless of temperature. In the current study it can be observed that in unheated samples, soluble caseins were present in the supernatant (serum). But the level of distribution was comparable for all types of caseins except κ-casein at pH 7.1, which showed a drastic decrease in comparison with pH 6.8 and 6.5. Samples after heating showed an increase in the levels of soluble caseins in the serum phase. 50% of the soluble caseins found in samples heated at 6.5 were β-casein while the levels of κ-casein were lowest. The lower levels of κ-casein and β-lg in samples heated at 6.5 proves that higher levels of β-casein are associated with casein micelles at pH 6.5. Samples heated at 6.8 and 7.1 showed a rather uniform distribution of both β-casein and κ-casein. As the pH was decreased from 7.1 to 6.5 the level of total soluble caseins was observed to increase. The results indicate that while heating did cause dissociation of caseins from the micelles, increasing the pH to 7.1 resulted in more stable casein micelles in the colloidal phase and more whey protein aggregate formations in the serum phase. It was also observed that the amount of κ-casein in the serum phase increased with increase in pH at heating.

This result obtained from reconstituted modified MPC powders is the opposite of what was observed by Anema (1998) on reconstituted skim milk. But Renan (2006) reported that soluble aggregates in milk heated at pH 7.2 were smaller in size and richer in κ-casein. Singh and Fox (1985a,b; 1986; 1987a,b,c) determined that at temperatures above ~90 °C κ-casein dissociation increased with increasing pH and this dissociation seemed to be directly proportional to the levels of β-lactoglobulin (β-lg), which is in line with the results of the current study. This could be attributed to the manufacturing process that the initial skim milk had to go through in the making of MPC powder. Further research to analyze the samples at each step of the processing could shed light on reasons behind this change.

Mean particle size and zeta potential

Table 3.3: Mean particle size (nm) of MPC dispersions of MPC powders manufactured from unheated & heated skim milk at varying pH

Attribute	pH 6.5		pH 6.8		pH 7.1	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
Diameter (nm)	184.12 ± 18.08^{b}	247.70 ± 32.62^{a}	181.97 ± 3.48^{b}	199.17 ± 6.78^{ab}	173.40 ± 9.90^{b}	181.85 ± 23.41^{b}
D10 (nm)	53.67 ± 7.26^{a}	65.92 ± 7.76^{a}	59.38 ± 5.01^{a}	57.47 ± 9.34^{a}	55.54 ± 3.75^{a}	58.99 ± 3.17^{a}
D50 (nm)	82.54 ± 12.70^{a}	110.82 ± 27.30^{a}	89.43 ± 5.47^{a}	95.91 ± 11.49^{a}	84.87 ± 8.25^{a}	89.68 ± 8.69^{a}
D90 (nm)	127.17 ± 39.83^{a}	212.04 ± 52.35^{a}	158.00 ± 11.33^{a}	187.90 ± 45.80^{a}	140.11 ± 12.31^{a}	139.95 ± 24.73^{a}
Zeta Potential (mV)	24.27 ± 2.50^{a}	24.10 ± 2.17^{a}	23.59 ± 3.02^{a}	23.29 ± 3.76^{a}	26.73 ± 1.40^{a}	26.32 ± 0.92^{a}

a-b Means with different superscripts are significantly different ($P < 0.05$).
D10, D50, and D90 are the diameters (μm) where 10%, 50% and 90% of all powder particles have smaller size, respectively.
All values are expressed as mean ± SD.

The particle diameter increased as the pH was decreased from 7.1 to 6.5. It can be observed from Table 3.3 that the mean particle diameter of the heated samples increased in comparison to their unheated counterpart. There was no comparable difference between unheated samples at varying pH. But at a pH of 6.5, the particle diameter of the MPC dispersions showed significant increase on heating which indicates that the particle size of heated MPC dispersions is heavily pH dependent (Anema & Li, 2003 a,b). The largest median particle size (D50) was also observed in samples heated at 6.5 pH. which can be correlated to the high viscosity of the mentioned

samples. The size of the particles of samples heated at pH 7.1 was the smallest. The previous result regarding protein fractions in serum phase of samples showed whey protein aggregate formations and increase in proportion of κ-caseins in heated samples at pH 7.1. It could be assumed that the size of the aggregates could be inversely proportional to the proportion of κ-casein present (Renan et al., 2006). Li (2014) in his research on yak milk concluded that the decrease in mean particle size with increase in temperature is similar to bovine milk and therefore may be attributed to the κ-casein dissociation from casein micelles. The serum analysis of the samples in the current study (Table 3.2) also showed an increase in κ-casein content in samples heated at pH 7.1 which supports the theory. The particle diameter of both heated and unheated samples at pH 6.8 and 7.1 was not significantly different ($P > 0.05$) from each other.

Zeta potential is a possible analysis that can explain the estimate surface charge of particles. The stability of casein micelles has been associated with the net negative charge on the surface of the micelle. Hence, any changes in the zeta potential value can be an indication of possible aggregation. The zeta potential values of both heated and unheated MPC dispersions were not statistically significant ($P > 0.05$) at any pH considered in this study. Wade & Beattie (1997) reported that for a commercial skim milk sample suspension the zeta potential was -18 mV, and it increased with decreasing pH. Wade used a method that determined zeta potential using electroacoustic effect because the common method of diluting samples to observe them through light scattering could alter the environment of the casein micelles. Since the MPC dispersions were diluted before analysis, the alteration in the natural environment of particles might be the reason the final values showed little to no difference.

Apparent Viscosity

Table 3.4: Apparent viscosity, n and K value of MPC dispersions of MPC powders manufactured from unheated & heated skim milk at varying pH

Attribute	pH 6.5		pH 6.8		pH 7.1	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
Viscosity at 112 mPa.s	2.32 ± 0.95^{b}	4.82 ± 0.90^{a}	2.34 ± 0.16^{b}	3.29 ± 0.43^{b}	2.13 ± 0.21^{b}	2.97 ± 0.63^{b}
Fluid Index, n	0.95 ± 0.02^{a}	0.82 ± 0.12^{a}	0.92 ± 0.05^{a}	0.81 ± 0.23^{a}	0.93 ± 0.02^{a}	0.92 ± 0.05^{a}
Consistency Index, K (mPa.s)	0.0030 ± 0.00^{a}	0.0145 ± 0.01^{a}	0.0037 ± 0.00^{a}	0.0145 ± 0.01^{a}	0.0030 ± 0.00^{a}	0.0046 ± 0.00^{a}

a-b Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD.

The trend in apparent viscosity changes at pH 6.5, 6.8 and 7.1 for both unheated and heated samples at a shear rate of 112 s^{-1} can be compared in Table 3.4. The results for the unheated and heated MPC dispersions at pH 6.5 indicates that there was a significant increase in the heated samples. The apparent viscosity value which was at 2.32 mPa.s for unheated samples at a shear rate of 112 s^{-1} increased to 4.82 mPa.s after heating which shows approximately 50% increase in viscosity at a heating pH of 6.5. Anema (2004) had also observed increase in relative viscosity of reconstituted skim milk by about 15% on heating at 90 °C for 30 min. Liquid MPC also showed increase in viscosity at lower pH post heat treatment (Ho et al., 2018). This pH dependent increase in the viscosity value during heating was associated with higher levels of aggregation of whey proteins with casein micelles. When pH is adjusted with HCl there is increase in Ca^{2+} activity in the serum phase which can considerably increase viscosity at lower pH (Ho et al., 2018). While the apparent viscosity

changes between unheated and heated samples at pH 6.8 and 7.1 showed an increase, there was no statistically significant difference observed ($P > 0.05$). These observations can be related to the results of mean particle size of the samples (Table 3.3) discussed above which followed a similar trend. The consistency index (k) and fluid index (n) are functions of protein content. Shear-thinning ($0<n<1$), Newtonian ($n=1$) and shear-thickening ($n>1$) behavior are predicted by power law model (Kieferle et al., 2019).

The significant changes in both viscosity and particle diameter on heating is an indication of the influence of heating pH which is in line with previous literature.

Heat Coagulation Time (HCT)

Table 3.5: Heat coagulation time (min) of MPC dispersions of MPC powders manufactured from unheated and heated skim milk base

	Heat Coagulation Time (sec)	
pH	**Unheated**	**Heated**
6.5	5.06 ± 0.05^{b}	14.32 ± 0.09^{a}
6.8	6.10 ± 0.02^{b}	7.26 ± 0.02^{b}
7.1	4.86 ± 0.01^{b}	14.20 ± 0.06^{a}

a-b Means with different superscripts are significantly different ($P < 0.05$).
All values are expressed as mean ± SD.

Earlier research has attributed variation in heat stability of milk with two major factors – one, the differences in composition of milk salts and two, presence or absence of the CCP (O'Connell and Fox, 2003). Heat stability of the MPC dispersions made from unheated and heated skim milk at varying pH is shown in Table 3.5. The unheated samples at all pH showed no significant difference ($p>0.05$) in their heat coagulation time. This leads to the conclusion that pH changes alone did not influence the heat stability of the final product. But the heated samples showed a different pattern. While no significant difference ($p>0.05$) was observed among unheated and heated samples at pH

6.8, a statistically significant ($p<0.05$) increase was observed in the heated samples at pH 6.5 and 7.1.

The heat induced destabilization of milk can be attributed to various factors. The main factors worth considering are Ca-ion activity in certain pH regions, heat-induced protein interactions (whey protein-whey protein / casein-whey protein) at different pH regions and heat-induced dissociation of κ-caseins (Crowley et al., 2014). Also, Sweetsur and Muir (1980) demonstrated that UF concentration produced more stable concentrates due to reduction in lactose and soluble Ca. The major factor for reduced heat stability has been identified as Ca-ion activity. Protein interactions on heating contributes to stabilization, which is the reason why heat stability of heated samples at 6.5 and 7.1 increased. According to literature, a reduced extent of κ-casein denaturation increases the heat stability. Previous results from the current study have shown reduced levels of κ-casein in the serum phase of samples heated at 6.5. As this indicates stability of casein micelles in this pH region, an increased heat stability was observed in these samples. Another possible reason for increased heat stability in samples heated at pH 6.5 could be related to readjustment of skim milk pH during the manufacturing process as there is a possibility of reversing certain physico-chemical changes. This theory can be supported by the results obtained by Ezeh and Lewis (2011). They observed that when pH of skim milk was readjusted from 5.45 to 6.47 (i.e., from a lower pH to higher pH), an improvement in heat stability was observed due to restoration of CCP. Pre-acidification of skim milk prior to UF declined the heat stability of MPC, but pH-restoration improved it (Liu et al., 2019).

CONCLUSION

This study illustrated that the combination of heat treatment and minor pH adjustments on skim milk base before manufacturing high protein MPC powders can influence the physicochemical

properties of the resulting powders. A high heat treatment on skim milk at 90 °C for 15 min at pH 6.5, 6.8, and 7.1 resulted in protein-protein interactions. Results were similar to the previous literature done on skim milk alone. In the resulting MPC powders, a greater amount of unbound whey proteins was found in samples with a heated skim milk base at pH 7.1; while most whey proteins were found to be bound to casein micelles in samples with a heated skim milk base at pH 6.5. These results indicate that while heating did cause the dissociation of caseins from the micelles, increasing the pH to 7.1 resulted in more stable casein micelles in the colloidal phase and more whey protein aggregate formations in the serum phase. Differences in pH during heating therefore effected the physicochemical properties, mainly viscosity which showed a significant increase in samples with a heated skim milk base at pH 6.5. Heat stability was observed to have improved in samples with pH adjusted to 6.5 and 7.1. Further study on the change in viscosity and heat stability by using acid gelation can help gain more understanding of the potential of the functionally modified MPC powder in new or improved product applications.

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Chapter 4 - Acid gelation properties of milk protein concentrate powder manufactured from heat-treated and pH adjusted skim milk

ABSTRACT

Whey protein denaturation and its association with casein micelles and/or other denatured whey proteins have been determined to improve the functional properties of milk protein concentrate (MPC) powders. High protein MPC powders were manufactured from unheated and heated skim milk bases at varying pH (6.5, 6.8, and 7.1). The control for both unheated and heated products was considered to be the samples at pH 6.8. The current study was aimed at analyzing and understanding the rheological characteristics and gel parameters of the MPC powders with respect to the protein-protein interactions. MPC powders with heated skim milk bases showed early onset of gelation but were not significantly different ($P<0.05$) with respect to the varying pH. While samples that were unheated formed stronger gels, highest storage modulus was detected at pH 7.1 in both unheated (811.76 ± 297.01) and heated (555.33 ± 80.93) samples, indicating that increasing the pH of skim milk prior to ultrafiltration resulted in the increase of gel strength. Therefore, it can be concluded that selective pre-treatment of skim milk during the manufacture of high protein MPC powders can improve gelation characteristics of products that are acid gel based.

Key words: milk protein concentrate, heat treatment, pH, protein interactions, acid gelation

INTRODUCTION

Milk protein concentrates (MPC) are concentrated forms of milk proteins containing both caseins and whey proteins in the same ratio as found in milk (Ann Augustin et al., 2011). They are valuable ingredients in processed and prepared foods based on their excellent nutritional and functional properties (Lagrange et al., 2015). The fact that MPC has less milk fat, lactose and mineral content

makes it a quality ingredient in food products (Meena et al., 2017). MPC powders are manufactured using ultrafiltration (UF) of skim milk, occasionally followed by diafiltration (DF) with water to remove minerals and lactose (Mistry and Hassan, 1991; Getler et al., 1997). The ultrafiltered retentate is further dried into powder form through spray drying (Chen and Patel, 2008; Agarwal et al., 2015).

MPC has high potential as an ingredient due to its unique merits in functionality which can tailor various properties in food products. In various such food applications, MPC powders are exposed to heat treatments after reconstitution. Therefore, heat stability is an important functionality that can prevent product quality losses due to heat induced destabilization (Crowley et al., 2014; Luo et al., 2016).

Milk proteins are the building blocks of dairy gels like cheese or yoghurt. The two major types of milk proteins accounting to gel formation are globular-soluble whey proteins and casein micelles which are found in colloidal assemblies (Morand et al., 2011; Lucey, 2016). Usually, casein micelles play the major role in building up the gel network. Yoghurt formation follows destabilization of casein micelles by lowering the milk pH from 6.8 to well below 4.5 using bacterial cultures or fermentative production of lactic acid from lactose. Using high-protein MPCs as an active ingredient in high protein yoghurts have various benefits. One, it prevents formation of high acidity in high-protein yoghurts caused by large amounts of lactose. Two, promotes better growth of starter bacteria. And three, improves quality by maintaining casein to whey protein ratio (Qi et al., 2022). Adding glucono-d-lactone (GDL) can mimic the role of bacteria in lowering the pH and is widely used as a method to recreate gel formation to obtain similar results in research studies (Kruif et al., 1995; Matia-Merino and Singh, 2007). Whey protein denaturation through heating at high temperatures (> 75 °C) have been determined to significantly alter the behavior of

acid gels (Lucey et al., 1998). This is either because whey proteins form soluble whey protein aggregates or whey protein coated casein micelles through association with casein micelles (Lucey et al., 1999; Vasbinder et al., 2004). Koutina & Skibsted (2015) also observed that stability of the casein micelles can be influenced by the alteration in the distribution of calcium and phosphorus between the serum and micellar milk phase through pre-heating of milk followed by acidification.

While studies have shown the effect of high heat treatment of milk on acid gels, there have been fewer research on the effect of heat treatment of milk which was previously pH adjusted. The previous chapter studied the protein interactions in milk protein concentrate (MPC) powders manufactured from heat treated and pH adjusted milk and changes in their functional properties. In the current study, the MPC powders were used for gelation studies. The objective was to analyze and understand the rheological characteristics and gel parameters of the MPC powders with respect to the protein-protein interactions.

MATERIALS AND METHODS

Experimental Approach

Skim milk for treatment was separated and pasteurized from fresh milk obtained from South Dakota State University (SDSU) campus farm. The pH was adjusted to 6.5, 6.8 and 7.1 on three equal proportions of skim milk. Each adjusted portion of the skim milk was divided into equal halves where one half was heat treated at 90 °C for 15 minutes and the other half was not heat treated. After readjusting all the samples to 6.8 pH, the treated skim milk samples were ultrafiltered to 5x concentration using diafiltration water, and spray dried to manufacture modified MPC powders (97% moisture; 82% true protein). The spray dried MPC powders were analyzed for buffering capacity and acid gelation. The whole experiment was replicated twice from independent lots of skim milk.

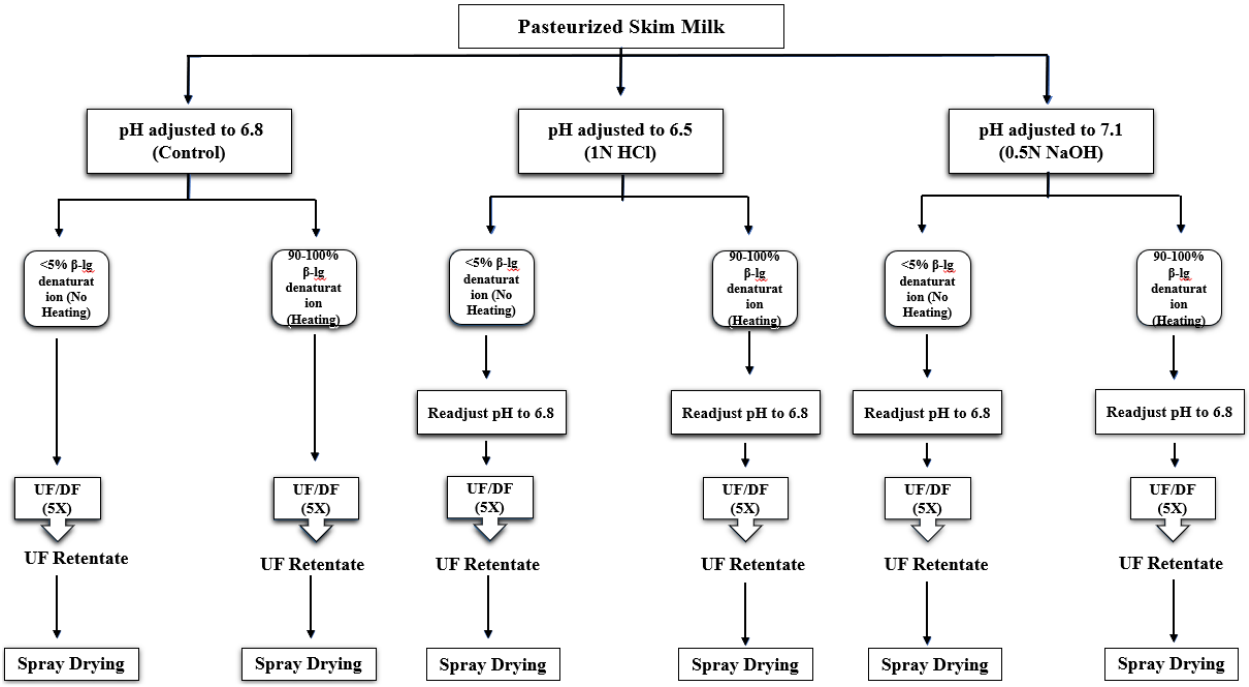


Figure 4.1: Experimental design for the manufacture of MPC powders from heated and pH adjusted skim milk

Manufacture of modified MPC powders

Pretreatment. Skim milk for treatment was separated and pasteurized at 71.7 °C for a hold time of 15 s from fresh milk (3500 lbs.) obtained from SDSU Dairy Research and Training Facility (DRTF), Brookings. Skim milk was then divided into three equal parts (1100 lbs. each) and their pH adjusted to 6.8, 6.5 and 7.1 respectively using 1 N HCl or 0.5 N NaOH. Each pH adjusted samples were further divided into equal halves. One half was left unheated while the other half was heat treated at 90 °C for 15 minutes using a temperature-controlled circulatory water bath (Cole Parmer, Vernon Hills, IL). Both heated and unheated samples were readjusted to 6.8 pH and kept for cooling.

Ultrafiltration. The treated skim milk was ultrafiltered at 15° C using two parallelly arranged 10 kD polyether sulfone spiral wound membranes (3838 element format, Dominick Hunter Filtration Divison—N.A, Parker Hannifin Corporation, Oxnard, CA, USA). The inlet and outlet pressures were 50 psi and 20 psi respectively, and the transmembrane pressure was 30 psi. To reach a concentration factor of 5, diafiltration (DF) was added. The heated samples required approx.. 75% DF water while the non-heated samples only required approx.. 65% DF water.

Spray Drying. The UF retentates were spray dried in a pilot scale dryer fitted with a Niro centrifugal disc atomizer (ASO 412E, Niro Inc., Columbia, MD) at an inlet temperature of 185 °C and outlet temperature of 105 °C. The powders were packed in airtight containers and stored at -21°C until further analysis was completed.

Acid-base titration

Acid-base titration curve of non-heated and heated MPC dispersions (protein 5%, w/w) were determined using the method described by Meletharayil et al. (2015). Both non-heated and heated samples were titrated from their initial pH to pH 3.0 by adding 0.5 N HCl in increments of 0.2 ml under constant stirring. Subsequently, 0.5 N NaOH was added at 0.2 ml increment to the same sample to titrate it back to pH 7.0 under constant stirring. The pH was recorded after every 0.2 ml addition of 0.5 N HCl or NaOH using an Accumet™ AP110 Portable pH Meter (Fisher Scientific, Waltham, MA).

Acid Gelation

Acid gels were prepared from both non-heated and heated MPC dispersions using the method described by Rathod et al., 2022. The MPC powder samples were reconstituted to 5% protein

dispersions. The MPC dispersions were heated for 90 °C for 10 min and rapidly cooled to 30 °C. 2% glucono-δ-lactone (GDL; Fisher Scientific, Waltham, MA) of 90 mL sample were added to the MPC dispersion and stirred continuously using a magnetic stirrer for 2 min. A part of the mixed sample (40 mL) was immediately transferred to a temperature-controlled water bath (Cole Parmer) maintained at 30°C for continuous pH measurements using an Accumet™ AP110 Portable pH Meter (Fisher Scientific, Waltham, MA). The remaining sample (50 mL) was monitored using a stress-strain-controlled rheometer (MCR-92 Anton Paar) with a cup-and-bob geometry consisting of a coaxial cylinder (bob length, 60 mm; bob diameter, 38.7 mm, and cup diameter, 42 mm) for its rheological properties throughout the acid gelation process. The sample was maintained at 30°C in the rheometer. For minimal disruption of the gel network of the sample during acid gelation, an oscillation of constant frequency of 1 Hz with an applied strain of 0.5% was maintained. The pH, elastic modulus (G′) and loss modulus (G″) of the acid gels were measured every 5 min. The point when G′ of gels was greater than 1 Pa was arbitrarily defined as the gelation point (Meletharayil et al., 2015). The analysis was carried out in duplicates.

Confocal microscopy to determine the gel structure

Acid gels were prepared from the MPC dispersions prepared out of MPC powders manufactured form unheated and heated skim milk at pH 6.5, 6.8 and 7.1 using the method described by Rathod et al., 2022. The MPC dispersions were heated at 90°C for 10 min, followed by rapid cooling to 30°C. The heated sample (30 mL) was transferred to a beaker. A precalculated amount of GDL was added to it in order to reach the final acid gel pH of 4.6. The samples were stirred for 2 min using a magnetic stirrer to ensure complete mixing of GDL. Then, 100 µL of the mixture was transferred to an Eppendorf tube and a few drops of fast green FCF stain (5 mg dye

in 5 mL water) was added to it. After mixing, 50 μL of sample was mounted on a glass slide and covered with a coverslip. The glass slide with the sample-GDL mixture was incubated at 30°C for 4 hours for a pH drop to 4.6 resulting in gel formation.

A confocal laser scanning microscope (LSM 5 Pascal: Zeiss, Thornwood, NY) version 3.2 SP2 was used to view the stained gels. The Axioplan 2 upright microscope captured the image using a Zeiss Axiocam HR digital camera at Plan Neoflour objective 40x/0.75. The fast green which detects proteins in green color was excited using the 543 nm Helium-Neon LASER line and a primary dichroic HFT 543. The emission signal was collected with Channel 1 using a secondary dichroic NFT 635 and an LP 650 nm filter (Impa et al., 2020). The 8-bit images obtained were analyzed with ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD) with a color pixel counter plugin to measure the green color area.

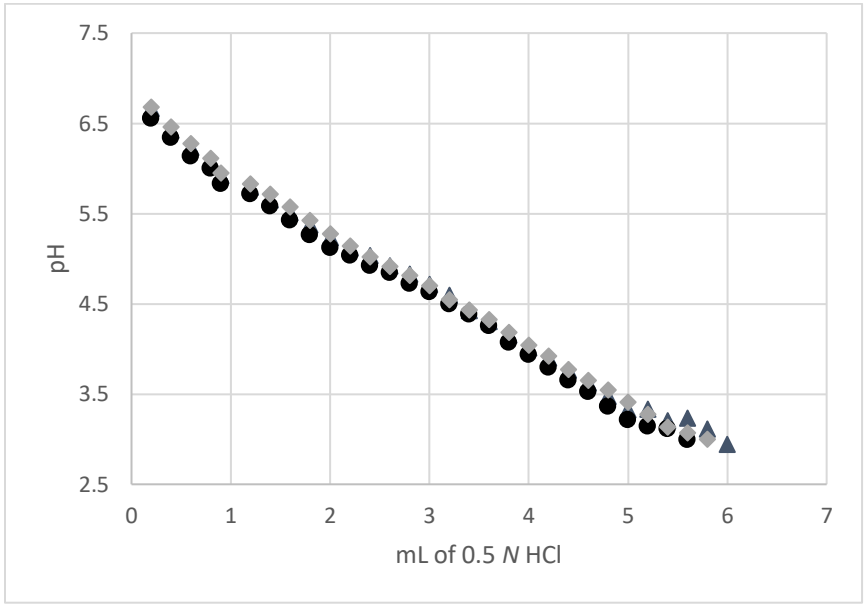
Statistical analysis

Statistical analysis was performed using PROC GLIMMIX and Tukey's test in SAS Version 9.4 (SAS Institute Inc.) to determine significant differences based on treatments. The significance was established when $P \leq 0.05$.

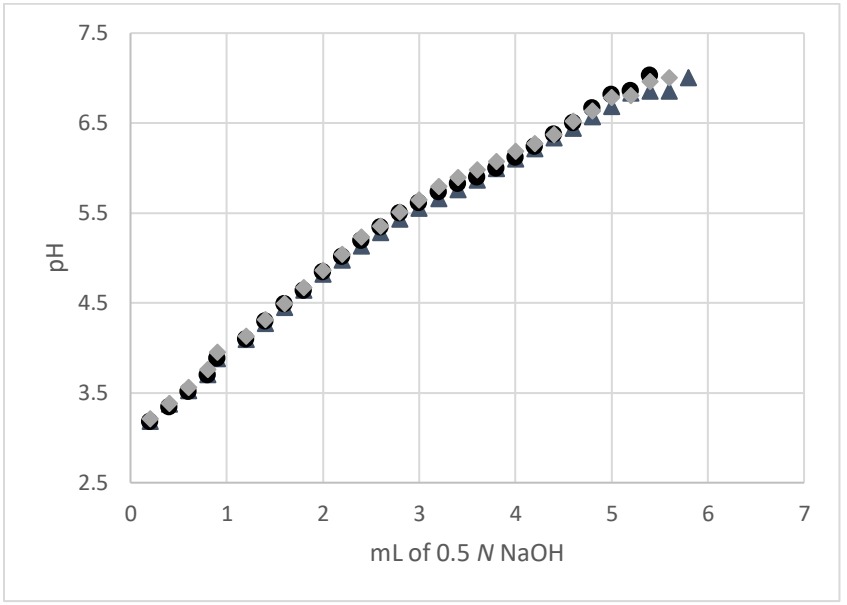
RESULTS AND DISCUSSION

Acid-base titration

(A)



(B)



(C) (D)

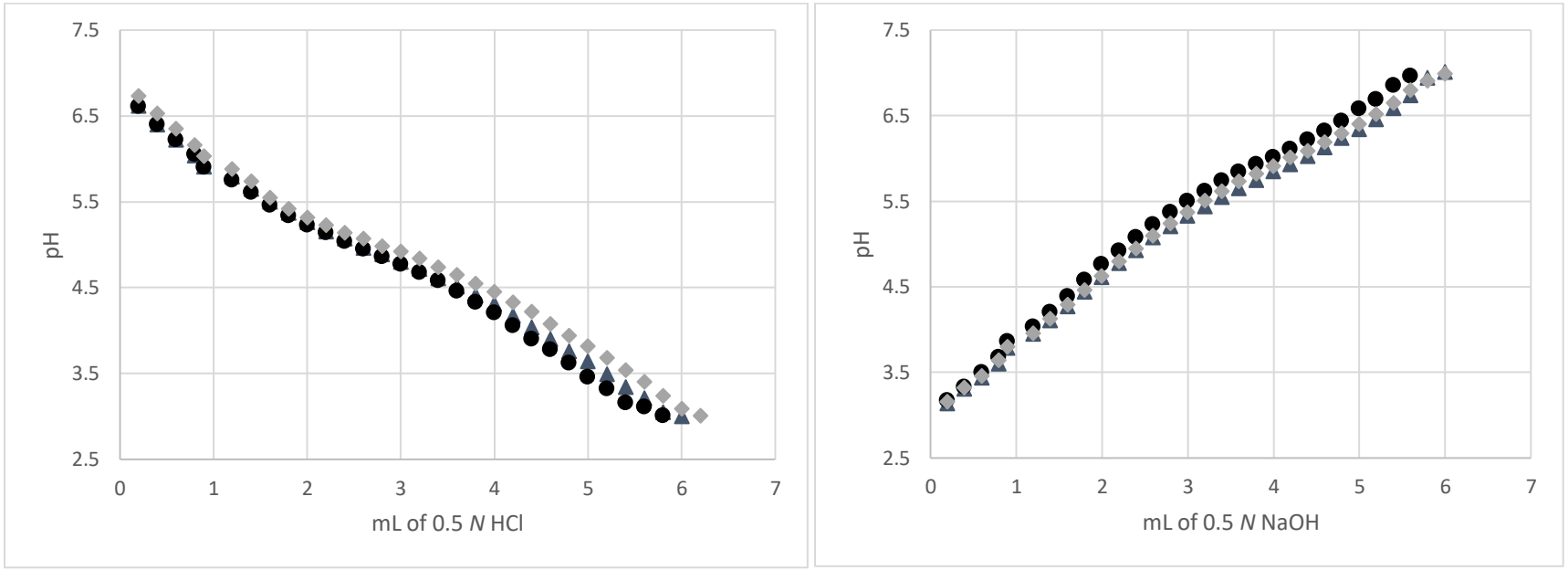


Figure 4.2: Acid-base titration curves. (A) Acidification of MPC dispersions made of MPC powders manufactured from unheated skim milk (B) Neautralization of MPC dispersions made of MPC powders manufactured from unheated skim milk (C) Acidification of MPC dispersions made of MPC powders manufactured from heated skim milk (D) Neautralization of MPC dispersions made of MPC powders manufactured from heated skim milk (▲ =pH 6.5; ● = pH 6.8; ♦ = pH 7.1)

The acid-base titration curves for MPC dispersions (5% protein w/w) made from unheated and heated skim milk are shown in Figure 4.2. The amount of acid required for acidification of the unheated dispersions varied with respect to pH. Samples at 6.5 pH required 0.2 ml less acid than samples at pH 6.8, while samples at 7.1 required 0.2 ml more acid than samples at 6.8. These results indicate that the buffering capacity of MPC powders are directly proportional to the increase or decrease of pH at pretreatment of skim milk. In case of MPC powders made form heated skim milk, during the initial stages of acidification samples at both 6.5 and 6.8 pH required

approximately the same amount of acid (3.6 ml) to reach a pH of 4.51. On reaching a pH ≥ 4.6 there is a visible increase in the buffering capacity of heated sample at 6.5 from the sample heated at 6.8. Sample heated at a higher pH of 7.1 showed the maximum buffering capacity increase among the three pH. Unlike the unheated samples, the heated samples showed an increase in buffering capacity when the pH was lowered to 6.5 (by 0.2 ml) and when the pH was increased to 7.1 (by 0.4 ml). Heating skim milk prior to MPC manufacture resulted in better buffering capacity of the final powder. During forward titration of unheated samples from pH 3.0 to 7.0 an increase in the amount of base used was observed at pH 6.5 and 7.1. But throughout the base curve it can be observed that there wasn't a significantly different change in the buffering capacity. After heating, contrary to the acid curve the highest buffering capacity was observed at pH 6.8. Both pH 6.5 and 7.1 samples required the same amount of base (6 ml) to reach a pH of 7.0 but according to the Figure 4.2 it can be observed that 7.1 had a slightly greater buffering capacity than 6.5 throughout the alkalization process.

Meletharayil (2015) associated a reduced buffering capacities at high protein MPCs with lower phosphate and citrate content. A possible reason for this is their removal during the UF/DF process. At lower pH there is greater destabilization of casein micelles which would lead to increase of phosphate in serum phase. Therefore, after DF most of the phosphate is lost in the permeate for the samples at a lower pH. This could be a reason why samples at pH 6.5 showed decreased buffering capacity. Studies have shown that diafiltration of milk changes ionic composition of the serum phase leading to mineral imbalance and thereby causes casein dissociation due to loss of CCP. In the current study heated samples required 10% more DF water in comparison to unheated samples. Any changes in the Ca and phosphate equilibrium during heating can also affect the buffering capacity (Li and Corredig, 2014).

For dairy products like cheese and yoghurt that demands acidification and formation of acid gels, buffering capacity of high protein powders is of great importance.

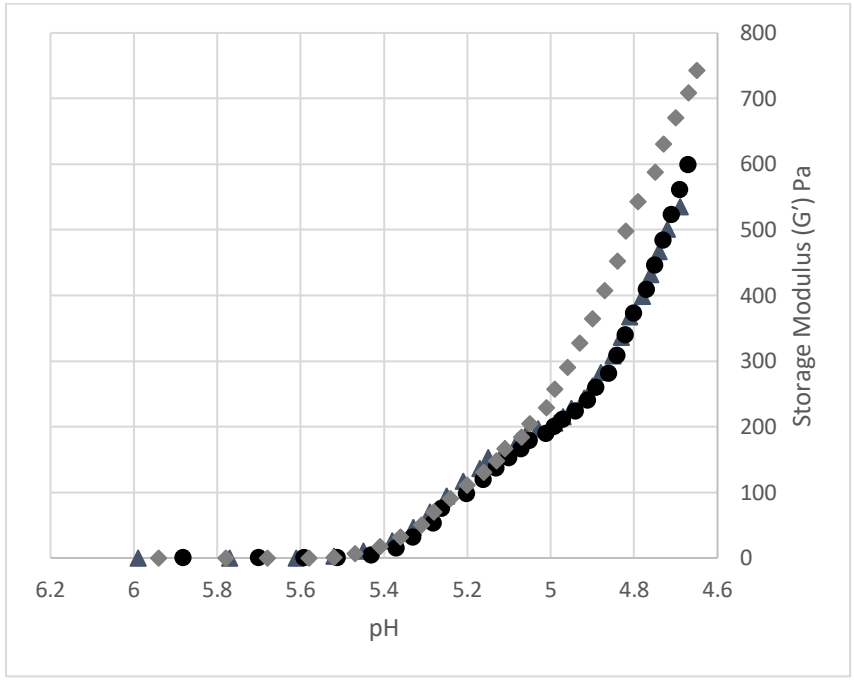
Acid gelation

Figure 4.3 shows the gelation behavior of MPC dispersions made form unheated and heated skim milk at varying pH. For decreasing pH, the storage modulus (G′) which is an indication of gel strength showed an initial lag phase prior to gelation pH which was followed by a sharp increase that was maintained in both unheated and heated samples (Ercili Cura et al., 2009; Li and Corredig, 2020).

From the plots of tan δ as a function of pH (Figure 4.3) an inflection point (peak) (Lin et al., 2018) can be observed at an approximate pH of 5.0 in all the heated samples after which there was a decline as the pH decreased and then became constant as the pH neared 4.6. Similar peaks are observed in unheated samples as well, but the pH of the inflection point differed with respect to the pretreatment pH of skim milk. The peak was observed at a pH of 4.92 in samples at 6.5. Samples at 6.8 showed an increased peak at 4.94 and samples at 7.1 showed the maximum peak at 5.05 pH. The maximum of tan δ can be associated with solubilization of CCP (Vliet et al., 1997). Li and Corredig (2020) showed that tan δ decreased at the onset of the gelation point in unheated retentates with higher protein content. The same can be observed in Figure 4.4 C. It can also be observed that for both unheated and heated samples the graph maintained a plateau for a while after reaching the inflection point before decreasing. The inflection point indicates that the bonds or strands in the gel are highly sensitive to break or facilitate further rearrangement of the gel. Studies have shown that the maximum in tan δ does not occur in unheated milk because the gelation occurs at a low pH value (<5.2) but in the current study the peak can be observed in both

unheated and heated samples. A possible reason for this observation can be the influence of pH change on skim milk prior to the MPC manufacture which caused early on protein-protein interactions. This is a behavior that has been previously observed in studies regarding heated acidified milk. It is an indication of structural change of protein network (protein-protein interaction) because of the gelation point being at an earlier pH than the isoelectric point of caseins (Li and Corredig, 2020). Lucey et al., 1998 associated this occurrence with formation of heat induced aggregates.

(A)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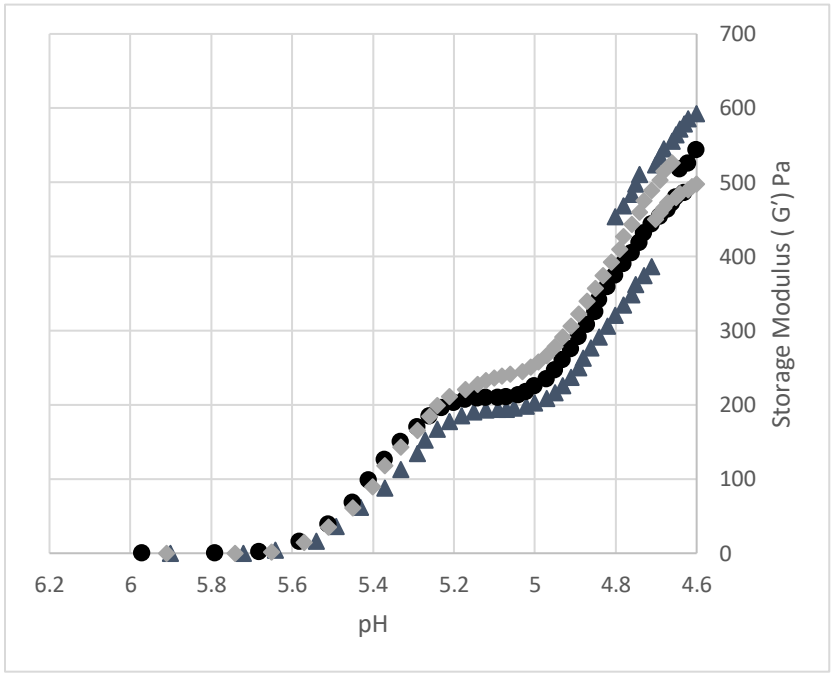
pH

(B)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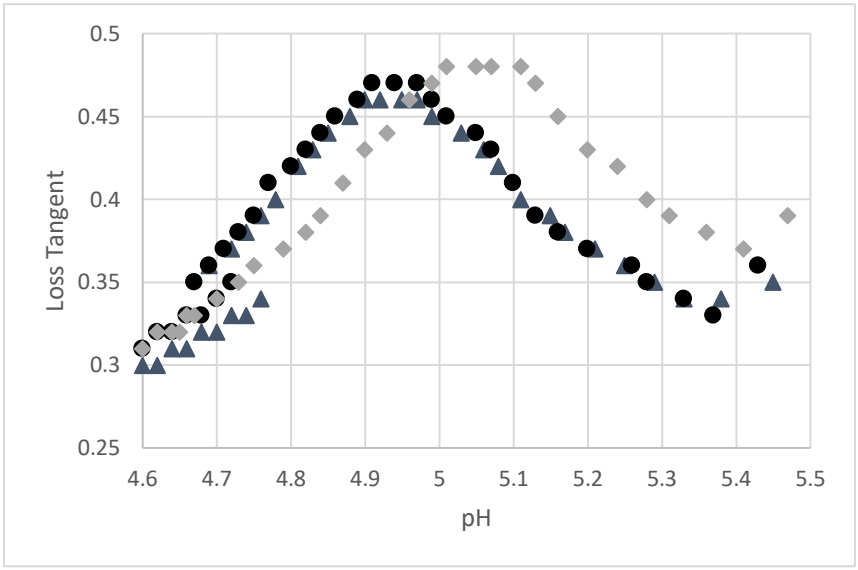
(C)



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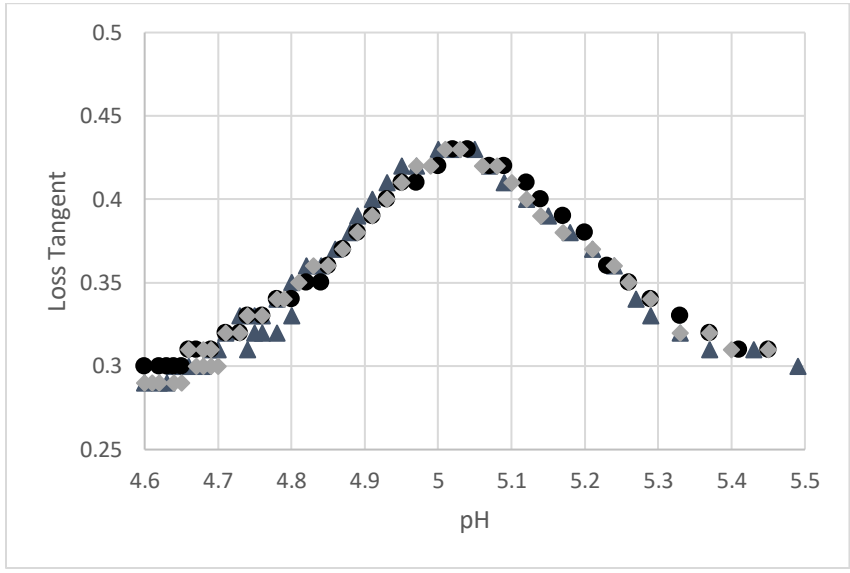


Figure 4.3: Storage modulus (G′) versus pH during gelation: (A) MPC dispersions of MPC powders manufactured from unheated skim milk (B) MPC dispersions of MPC powders manufactured from heated skim milk; Loss tangent versus pH during gelation: (C) MPC dispersions of MPC powders manufactured from unheated skim milk (D) MPC dispersions of MPC powders manufactured from heated skim milk (▲ =pH 6.5; ● = pH 6.8; ♦ = pH 7.1)

Table 4.1: Rheological parameters obtained during gelation of acid gels of milk protein concentrate powders (MPC) powders manufactured from unheated and heated skim milk at varying pH (MPC; means ± SD)[1]

Sample		6.5	6.8	7.1
Unheated				
Gelation point	**Time, min**	17.50 ± 3.54^{ab}	20.00 ± 0.00^{a}	22.50 ± 3.54^{a}
	pH	5.48 ± 0.13^{a}	5.43 ± 0.07^{a}	5.49 ± 0.08^{a}
Maximum storage modulus	**G´, Pa**	628.99 ± 143.53^{a}	714.20 ± 199.70^{a}	811.76 ± 297.01^{a}
	pH	4.60 ± 0.00	4.60 ± 0.00	4.60 ± 0.00
	Time, min	167.50 ± 31.82^{a}	167.50 ± 24.75^{a}	160.00 ± 14.14^{a}
Heated				
Gelation point	**Time, min**	10.00 ± 0.00^{b}	10.00 ± 0.00^{b}	10.00 ± 0.00^{b}
	pH	5.64 ± 0.06^{a}	5.68 ± 0.08^{a}	5.65 ± 0.01^{a}
Maximum storage modulus	**G´, Pa**	464.55 ± 181.66^{a}	503.21 ± 57.04^{a}	555.33 ± 80.93^{a}
	pH	4.60 ± 0.00	4.60 ± 0.00	4.60 ± 0.00
	Time, min	205.00 ± 49.50^{a}	207.50 ± 10.61^{a}	217.50 ± 31.82^{a}

a-b Means within a row with different superscripts differ (P < 0.05)
[1] From the plot of storage modulus (G′) versus pH (Figure 4.3)

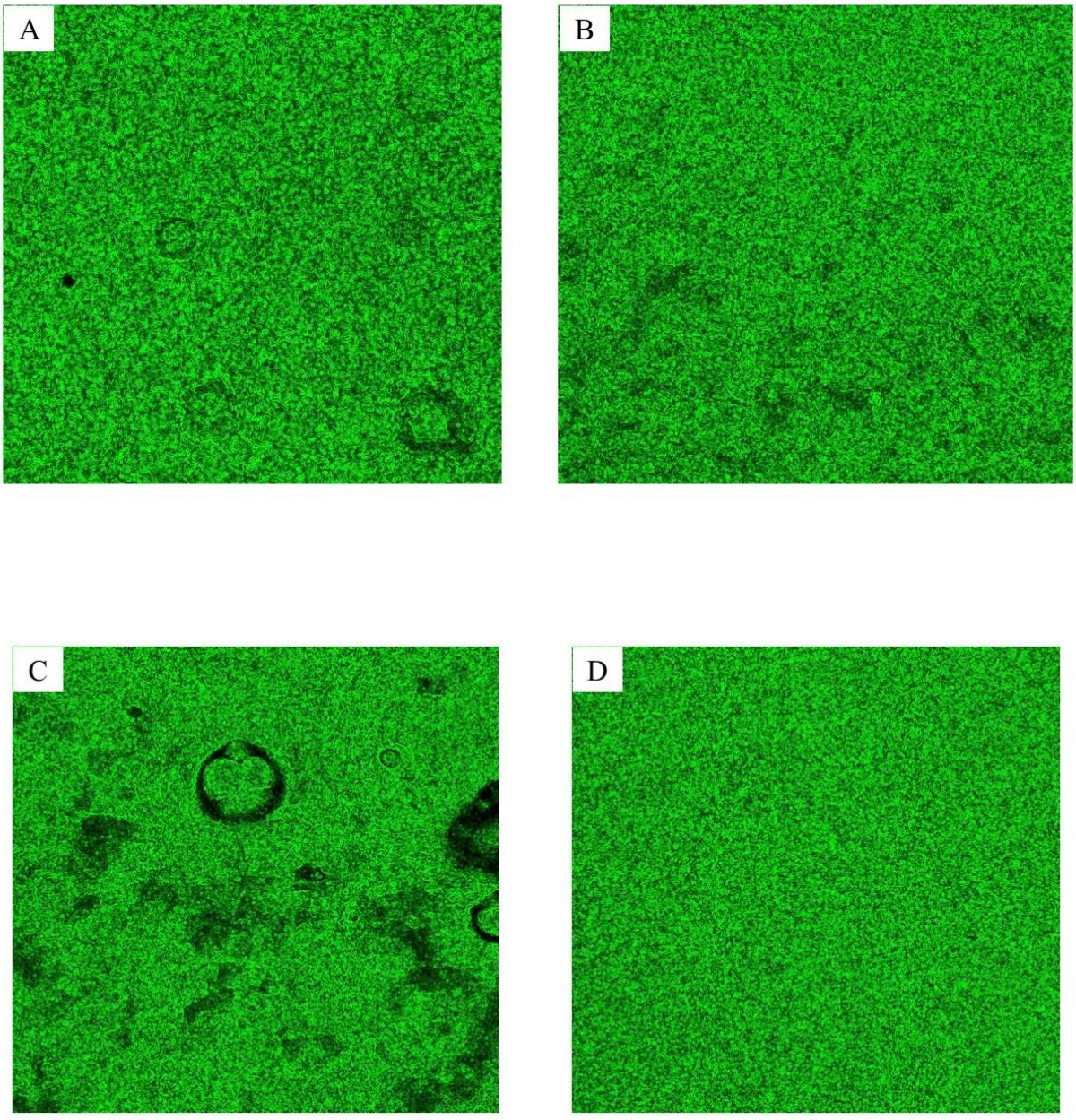
Table 4.1 shows the gelation parameters of MPC dispersions made from both unheated and heated skim milk at different pH. Significant difference (P>0.05) was observed between the gelation time of unheated and heated samples, but no significant difference (P<0.05) was observed in the gelation pH. Unheated samples at 6.5 showed the maximum variability of gelation time (17.50 min) among the unheated samples. All unheated samples took more time to reach gelation point than the heated samples. Among the unheated samples 6.5 was observed to have reached gelation point significantly (P>0.05) earlier than 6.8 and 7.1. A possible reason for this early gelation could be the increased level of casein-whey interactions in the samples (Rathod et al., 2022). The previous results (Figure 4.3) shows that samples at 6.5 showed a lower buffering capacity which can be related to the shorter time samples at 6.5 pH took to reach the gelation point (Célia et al., 2017; Qi et al., 2022). Although all the gelation pH were statistically significant (P<0.05), a visible increase in gelation pH can be observed in heated samples. Heating induces denaturation of β-lg. The isoelectric pH of β-lg is ~5.3 that causes a decrease in electrostatic repulsion which would explain the high gelation pH and earlier start of gelation in heated samples (Lucey, 2016). Lucey

(1997, 1998) reported that acid gels made from heated skim milk have a higher gelation pH and form firmer gels. The current study shows that acid gels made form MPC dispersions manufactured from unheated skim milk were firmer (greater G′) than those made form heated skim milk. One of the reasons for this change could be because the milk used in the previous literature had the presence of fat which could also be a contributing factor. In the previous chapter it was discussed that after heating at pH 6.5 and 7.1 denaturation of whey proteins were determined. At pH 6.5 almost 90% of the denatured whey proteins were found to have either formed casein micelles coated with whey proteins or casein-whey aggregates while those heated at 7.1 were found to have formed aggregates between whey proteins. These aggregates can be determined as the main reason for the decrease in gel strength of dispersions made form heated skim milk. Caseins are established to play a major role in the formation of the gel network. It is likely that due to the whey proteins coating the surface of caseins, a proper aggregation of caseins was not possible in heated samples. This explains why the lowest G′ was observed in samples heated at pH 6.5. But Lucey (2016) claims that in such cases cross-linking of aggregated particles may occur through the denatured whey proteins associated with the casein micelles and therefore such “bound” whey proteins are essential contributors to gel formation in heated samples.

In conclusion, the gelation time of heated samples was significantly lower than the unheated counterparts. The pH had no influence in determining the onset time of gelation. Thus, we can conclude that during the manufacture of MPC powders, the adjustment of skim milk from its original pH to a slightly acidic or basic pH has no influence on the gelation time or gelation pH during heat treatment. Powders made from skim milk that did not undergo heat treatment made the strongest gels. Thus, it can be concluded that heat treatment is an effective way to lower the gel strength. While the maximum storage modulus did not show any statistical difference

corresponding to pH change, it can be visibly inferred from data that samples at pH 7.1 produce the strongest gels.

Confocal microscopy to determine the gel structure



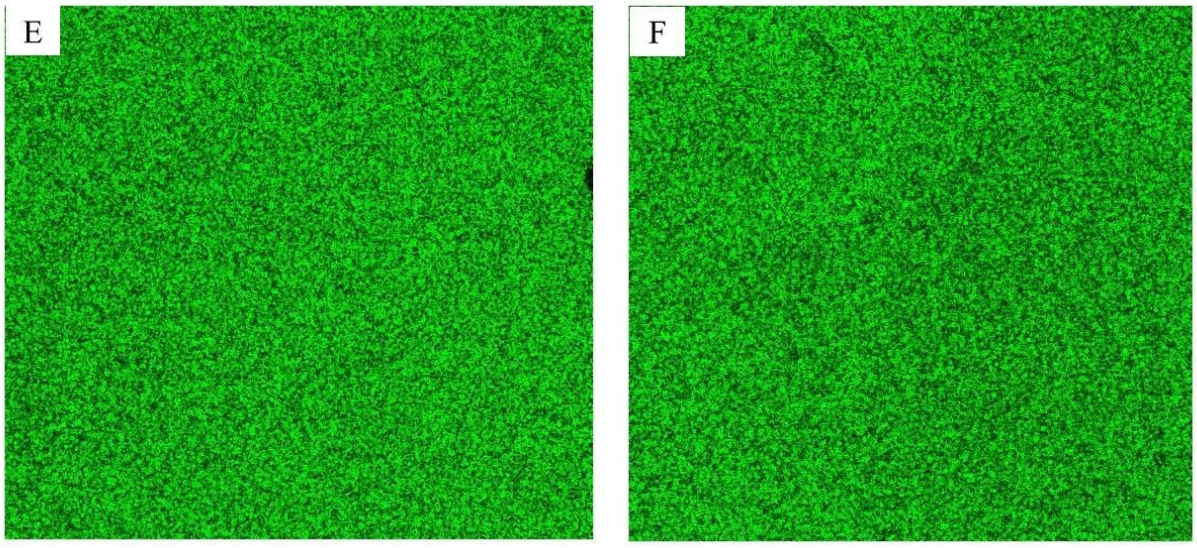


Figure 4.4: Confocal laser scanning microscopic images of acid gels made from MPC dispersions of MPC powders manufactured from: (A) heated skim milk at pH 6.5 (B) unheated skim milk at pH 6.5 (C) heated skim milk at pH 6.8 (D) unheated skim milk at pH 6.8 (E) heated skim milk at pH 7.1 (F) unheated skim milk at pH 7.1

The microstructure of acid gels prepared from MPC powders manufactured from unheated and heated skim milk bases are shown in Figure 4.5. The unheated samples were observed to have densely packed continuous gel structure with less porosity. The heated samples showed presence of larger pores with faint green or dark black backgrounds. While considering the pH differences it can be observed that in both unheated and heated samples, the most loosely connected gel network structure was observed at pH 6.5 attributing to a weaker gel. Samples at pH 7.1 showed the strongest and homogenous gel network attributing to a stronger gel.

Casein micelles are the main building blocks of the gel network. But the strength of the gels can be more or less associated with denatured whey proteins. During gel formation, the binding of denatured whey protein to casein micelle surface will sterically hinder their direct cross-linking and therefore leading to the formation of a weaker gel network. But an opposite effect is possible due to the dissociation of κ-casein from the surface of the micelles. This will lead to a reduction in

steric hindrance which will allow a direct cross-linking of the micelles resulting in the formation of a stronger gel network (Ye et al., 2019; Qi et al., 2022).

These results are in correspondence with the observations made form the rheological analysis of acid gels discussed earlier as well as with the protein fraction analysis discussed in the previous chapter.

CONCLUSION

The heating and pH changes to the skim milk base during the manufacture of MPC powders significantly affected the acid gelation properties of the final high protein MPC powders. While heating significantly lowered the gelation time, the onset of gelation was at the same time for heated samples at all three pH. The G′ was lowest for samples at pH 6.5 leading to the formation of a weaker gel, while at pH 7.1 the gel structure was stronger. The network structure of the gel became denser and less porous as the heating pH increased. Heating at high temperatures at a slightly acidic pH can result in the formation of a desirably softer and smoother gel; while heating at a slightly higher pH can promote the formation of a denser and stronger gel. The results conclude that depending on the type of protein interactions happening, the gel strength and onset of gelation can be tailored to meet product specifications. This opens a wide range of opportunities for MPC powders as functional ingredients.

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Appendix A - SAS Code

All the data was analyzed using SAS Studio 5.1, Copyright © SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513, USA. Given below is the example of the SAS data arrangement and code used for the analysis.

```
data MPC83;
input heattrt $ pH $ Lot $ GelationTime GelationpH SMmax SMpH SMtime;
datalines;
H 6.5 1 10 5.68 593 4.6 240
H 6.5 2 10 5.6 336.09 4.6 170
H 6.8 1 10 5.62 543.54 4.6 215
H 6.8 2 10 5.73 462.87 4.6 200
H 7.1 1 10 5.64 498.1 4.6 240
H 7.1 2 10 5.66 612.55 4.6 195
NH 6.5 1 15 5.57 730.48 4.6 190
NH 6.5 2 20 5.39 527.5 4.6 145
NH 6.8 1 20 5.48 855.41 4.6 185
NH 6.8 2 20 5.38 572.99 4.6 150
NH 7.1 1 25 5.43 601.74 4.6 150
NH 7.1 2 20 5.55 1021.77 4.6 170
;
proc print; run;

proc glimmix data=mpc83;
class heattrt pH Lot;
model GelationTime = heattrt|pH;
random lot lot*heattrt lot*pH(heattrt);
lsmeans heattrt/ pdiff lines adjust=Tukey;
lsmeans pH/ pdiff lines adjust=Tukey;
lsmeans heattrt*pH/ pdiff lines adjust=Tukey;
run;

proc glimmix data=mpc83;
class heattrt pH Lot;
model GelationpH = heattrt|pH;
random lot lot*heattrt lot*pH(heattrt);
lsmeans heattrt/ pdiff lines adjust=Tukey;
lsmeans pH/ pdiff lines adjust=Tukey;
lsmeans heattrt*pH/ pdiff lines adjust=Tukey;
run;

proc glimmix data=mpc83;
class heattrt pH Lot;
```

```
model SMmax = heattrt|pH;
random lot lot*heattrt lot*pH(heattrt);
lsmeans heattrt/ pdiff lines adjust=Tukey;
lsmeans pH/ pdiff lines adjust=Tukey;
lsmeans heattrt*pH/ pdiff lines adjust=Tukey;
run;

proc glimmix data=mpc83;
class heattrt pH Lot;
model SMpH = heattrt|pH;
random lot lot*heattrt lot*pH(heattrt);
lsmeans heattrt/ pdiff lines adjust=Tukey;
lsmeans pH/ pdiff lines adjust=Tukey;
lsmeans heattrt*pH/ pdiff lines adjust=Tukey;
run;

proc glimmix data=mpc83;
class heattrt pH Lot;
model SMtime = heattrt|pH;
random lot lot*heattrt lot*pH(heattrt);
lsmeans heattrt/ pdiff lines adjust=Tukey;
lsmeans pH/ pdiff lines adjust=Tukey;
lsmeans heattrt*pH/ pdiff lines adjust=Tukey;
run;
```