FUNCTIONALITY AND STABILITY OF MICELLAR CASEIN CONCENTRATES DURING HEAT TREATMENT AND STORAGE

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FUNCTIONALITY AND STABILITY OF MICELLAR CASEIN CONCENTRATES DURING HEAT TREATMENT AND STORAGE

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The use of micellar casein concentrates (MCC) obtained by microfiltration is receiving increasing interest from the food and dairy industry, which creates the need to understand their functionality and stability during processing and storage. This work focused on the rheological characterization and evaluation of heat treatments and storage behavior of MCC across a range of concentrations, pH and temperature conditions.

MCC preparations of 2.5-12.5% casein concentration displayed shear thinning behavior, which was more pronounced as concentration increased. The apparent viscosity of MCC increased exponentially with casein concentration and decreased with temperature in the temperature range 0-80°C, following an Arrhenius relationship. These dependencies were incorporated into a modified Arrhenius model that was able to accurately predict MCC viscosity under a range of shear, temperature and concentration conditions.

MCC with casein concentrations of 5-10% were subjected to continuous-flow UHT treatment and in-container retorting, and stored for 8 weeks at 25°C. UHT-treated MCC preparations showed aggregation and sedimentation during heat treatment and storage, while the retorted preparations were relatively stable.

A systematic investigation of the effects of pH and temperature in the sterilization range on heat stability of MCC showed that at pH < 6.7 all heat treated samples were visibly aggregated or even

coagulated. At pH 7.1 or higher only little or no changes in particle size were observed after heat treatment. Casein dissociation, evaluated by LC-MS/MS analyses of the supernatants obtained by ultracentrifugation, was higher at increasing pH, at all temperatures. Overall, it was concluded that increasing the pH and lowering the processing temperature increased stability of MCC. MCC subjected to sterilization after targeted modifications – which includes increased pH and lower processing temperatures, at equivalent lethality, showed a significant reduction in particle size and no coagulation or aggregation, after both retorting and UHT treatment.

The knowledge generated as a result of this work will provide the dairy and food industry with data necessary for effectively stabilizing micellar casein preparations and developing practical applications for this valuable ingredient, such as the production of innovative shelf-stable protein beverages.

BIOGRAPHICAL SKETCH

Anne Sauer was born and raised in Dresden, Germany and drawn to life sciences and technology early on. She earned a B.S./M.S. degree in Food Technology from Technische Universität Berlin, Germany during which time she was an active member of the international student organization AIESEC. In 2006/07 Anne spent one year as a graduate exchange student at Cornell University doing research on inactivation of *E. coli* using Pulsed Light. In that time she became involved with the Cornell Food Science Club and the Institute of Food Technologists (IFT). Back in Germany, she interned with Unilever's foodservice division in R&D for six month, reformulating instant soups and developing dressings and product applications.

After the completion of her M.S. degree, Anne returned to Cornell University to pursue a Ph.D. in the field of Dairy Process Engineering under the supervision of Dr. Carmen I. Moraru. She has worked part-time as an intern for the "Innovation Interface" start-up at the Johnson School of Management, consulting for an international food company.

Anne has been active in several roles with the Food Science Club and the Nonthermal Processing Division (NPD) of IFT. Additionally, she enjoys volunteering in workshops and science demos to share her passion of Food Science. She has attended and presented her research at meetings of several professional societies, such as IFT and the American Dairy Science Association (ADSA). She was awarded graduate scholarships from IFT and from the International Dairy Foods Association and won 1st place in the graduate student poster competition at ADSA's 2010 Annual Meeting. In 2012, Anne was the recipient of IFT NPD's Outstanding Volunteer Award.

Upon the completion of her Ph.D., Anne will join the food industry to pursue a career in the field of Research & Development.

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β-lg = beta lactoglobulin

CCP = colloidal calcium phosphate

DLS = dynamic light scattering

HCT = heat coagulation time

HSD = honestly significant difference

IR = infrared spectrophotometry

LC-MS/MS = liquid chromatography coupled with tandem mass spectrometry

MCC = micellar casein concentrate

MF = microfiltration

R-MCC = reconstituted micellar casein concentrate

SP = serum protein

UF = ultrafiltration

UHT = ultra high temperature

WI = whiteness index

LIST OF SYMBOLS

$\dot{\gamma}$= shear rate (s^{-1})

$\eta_{(\dot{\gamma},T,C)}$ = apparent viscosity at any shear rate ($\dot{\gamma}$) > 0 s^{-1}, for temperature (T) and concentration (C)

$\eta_{\dot{\gamma}}$ = apparent viscosity at any shear rate ($\dot{\gamma}$) > 0

η_{100} = viscosity at shear rate = 100 s^{-1} (Pa·s)

η_{app} = apparent viscosity (Pa·s)

n = flow behavior index

σ = shear stress (Pa)

σ_0 = yield stress (Pa)

κ = consistency coefficient ($Pa \cdot s^n$)

CHAPTER 1

INTRODUCTION: THE CASEIN MICELLE AND THE EFFECTS OF PH AND HEAT ON ITS STABILITY

Functional food and beverage product introductions worldwide grew from 1,566 products in 2005 to 5,753 products in 2009, forecasted to reach a sales potential of $10.3 billion in the U.S. market in 2014 (Mintel International Group Ltd., 2009). Companies are often looking to dairy proteins to add value to their products, due to the unique functionality and excellent nutritional properties. Recently, the use of micellar casein concentrates obtained by membrane separation started receiving increased interest from the food and dairy industry, particularly for the development of high protein foods and beverages. This creates the need to understand their functionality and stability during processing and storage.

Micellar casein concentrate as emerging food ingredient

Casein and caseinates are effective food ingredients because of their nutritive value as well as unique physico-chemical and functional properties. Due to their water-binding, emulsifying, whipping, foaming and texturizing properties, casein concentrates are used in a range of commercial applications, including protein fortification of dairy foods, ingredients for beverages, bakery, or meat products (Mulvihill and Ennis, 2003). The number of new products containing casein or caseinates launched in the U.S. has grown by about 22% per year in 2000-2008 (Affertsholt, 2009).

Traditionally, caseinates and caseins were prepared by either isoelectric precipitation or rennet coagulation (Fox, 2001). Recently, the use of casein preparations obtained by membrane filtration started receiving increased interest from the food and dairy industry. In the membrane filtration process, casein is separated from serum proteins based on their difference in molecular size using microfiltration (MF) (Brans et al., 2004). Additional diafiltration of the resulting retentate can lead to

serum protein removal from the casein concentrate of up to 97% (Hurt and Barbano, 2010). In casein concentrates obtained by membrane separation, the casein micelle is closer to its native state, which is why these products are typically called micellar casein concentrates (MCC). MCC have different functionality than traditional casein ingredients, which opens the field for new possible applications, such as premium shelf-stable, low fat nutritional beverages (Nelson and Barbano, 2005; UBIC Consulting, 2010). The manufacture of shelf stable beverages involves a sterilization step. Thus heat stability of MCC, defined as the ability to withstand high processing temperatures without visible flocculation, gelation or protein separation (Fox, 1982), under a range of pH and temperature conditions, is crucial.

Casein micelles in milk are remarkably stable systems that can withstand the rigorous conditions applied during commercial processing (Fox, 1982). However under certain conditions of temperature and pH, the colloidal integrity of the casein micelles can be disrupted, resulting in decreased stability of the system. The mechanism and the pH-dependent, heat-induced instabilities of the casein micelles have been subject of a considerable amount of research, but most of this work has been conducted in milk, where the casein micelles are in the presence of lactose and serum proteins and also a different mineral concentration as compared to MCC. All of these differences are expected to have a significant effect on the heat stability of casein micelles, which prompted the need for this work.

Structure and properties of the bovine caseins

About 80% of the proteins in bovine milk are represented by caseins, which are associated into casein micelles. Caseins are made up of four sub-groups of α_{s1}-, α_{s2}-, β- and κ-casein, present in milk in the ratio of roughly 4:1:4:1.3 (Walstra, 1990). The primary structures of the four caseins and most of their genetic variants have been identified through amino acid sequencing, which allows drawing conclusions about some of their specific physicochemical characteristics.

All caseins undergo post-translational modifications in which they are phosphorylated to varying extent; hence they are referred to as phosphoproteins. Additionally, κ-casein is glycosylated (Swaisgood, 2003). The different genetic variants, caused by either minor differences in the amino acid sequence or different post-translational modifications, cause large heterogeneity amongst caseins, leading to challenges in their identification and quantification (Wake and Baldwin, 1961).

α_{s1}-casein

The α_{s1}-casein, in which the "s" stands for calcium-sensitive, consists of 199 amino acids and a molecular weight of about 23,000 Da. Currently eight genetic variants are known, which contain 8-9 phosphate groups (Farrell Jr. et al., 2004). The phosphate groups are not distributed equally, but rather are clustered, leading to a very polar domain along amino acids 41-80, while the rest of the molecule has essentially no net charge at pH 6.6 (Swaisgood, 1982). α_{s1}-caseins exhibit consecutive self-association from monomers to dimers to tetramers etc., with the degree of association being strongly dependent on pH and ionic strength of the solution. At pH 6.6 the monomers exist in an equilibrium with oligomers (Schmidt, 1970). As calcium binds primarily to the phosphoseryl residues (Ono et al., 1980), α_{s1}-casein precipitates at Ca^{2+} concentrations of 3-8 mM (Aoki et al., 1985).

α_{s2}-casein

α_{s2}-casein, consisting of 207 amino acids, contains the highest degree of phosphorylation with 10-13 phosphate groups, depending on the genetic variant, of which four are currently known. Consequently this protein is the most hydrophilic of the caseins, resulting in stronger electrostatic repulsive forces and thus less extensive association of monomers than in the case of α_{s1}-casein (Snoeren et al., 1980). Unique for the α_{s2}-casein is its dipole character. The C-end of the molecule has a high positive net charge, while the N-end has a very high negative net charge at pH 6.6 (Swaisgood, 2003). α_{s2}-casein does also contain two cysteine residues at position 36 and 40, which form both intra- and

intermolecular disulfide bonds (Rasmussen et al., 1994). Given the high number of phosphoseryl residues, α_{s2}-casein is also the most calcium-sensitive of the caseins, precipitating at Ca^{2+} concentrations of less than 2 mM (Aoki et al., 1985).

β-casein

β-casein, of which twelve genetic variants are known, consists of 209 amino acids, and contains 4-5 phosphate groups. All phosphoseryl residues are clustered in one small region, leaving the N-terminal highly charged. The large C-terminal has essentially no net charge and is very hydrophobic, making β-casein the most hydrophobic of the caseins (Farrell Jr. et al., 2004). Unlike α_s-caseins, the self-association of β-caseins is extremely temperature dependent. At 0-4°C only monomers are observed, while at higher temperatures their tendency to associate is increasing (Payens and van Markwijk, 1963). β-casein can be proteolytically degraded by plasmin (EC 3.4.21.7), an endogenous milk protease, splitting it up into smaller segments. The segments are called γ_1-, γ_2- and γ_3-casein, which are identical to β-casein's amino acid fractions 29-209, 106-209 and 108-209, respectively (Eigel, 1981). The N-terminal fragments of the plasmin-hydrolyzed β-casein are called proteose peptones (Eigel et al., 1984). β-casein is the most hydrophobic of milk proteins, and it has a lower sensitivity to Ca as compared to α_s-casein.

κ-casein

The primary structure of κ-casein consists of 169 amino acids, and the molecular weight of monomeric κ-casein is around 19,000 Da. Currently there are eleven known genetic variants of κ-casein, though variants A and B are the two major variants that occur at almost equal frequencies (Farrell Jr. et al., 2004). Two unique characteristics of κ-casein are the absence of clusters of phosphoseryl residues (both κ-casein variants contain only one phosphoseryl residue) and the post-translational glycosylation. The glycosylation happens at sites of the amino acid threonine, at the positions 131, 133, 135 and 142; those can differ slightly depending on the genetic variant. In most milks the carbohydrate moiety is

composed of three monosaccharides: N-acetylneuraminic acid (AcNeu), galactose (Gal) and N-acetylgalactosamine (GalNAc), which have been found to be present mainly as either trisaccharides or tetrasaccharides (Saito and Itoh, 1992). These two unique features of κ-casein make it unable to bind calcium to the same extent as the other casein proteins, rendering it insensitive to calcium.

From its structure it can be inferred that κ-casein is extremely amphipathic, meaning it possesses distinct regions of hydrophilic and hydrophobic character. The highly hydrophobic N-terminal domain corresponds to the amino acid sequence 1-105 (called *para* κ-casein) and the hydrophilic C-terminal domain is comprised of the amino acids 106-169. The C-terminal end is called glycomacropeptide (GMP); it contains all the glycosylated threonines (Hill and Wake, 1969) as well as the phosphoseryl residue in position 149.

The peptide bond between Phe 105-Met 106 is specifically attacked and hydrolyzed by the enzyme chymosin (EC 3.4.23.4, often referred to as "rennet"), which cleaves κ-casein into the *para* κ-casein and GMP portions. While the hydrophobic *para* κ-casein remains associated with the casein micelle, the polar C-terminal end, containing the post-translational modifications as well as the genetic variations, is removed, eliminating the electrostatic and steric stabilization of the micelle.

κ-casein also contains two of the amino acids cysteine, which allow for disulphide bonding of κ-casein via free sulfhydryl groups. They are located at position 11 and 88, in the *para* κ-casein portion. However research remains unclear about whether the disulphide bonds lead to intra-, intermolecular and/or binding between micelles (Groves et al., 1992). Some work suggests that in the native state κ-casein exists as monomers, as well as polymers with up to ten κ-casein molecules linking randomly (Rasmussen et al., 1992).

Colloidal calcium phosphate

In bovine milk, about two thirds of the calcium and half of the inorganic phosphate are present in the colloidal form, with the remainder in soluble form. Within the casein micelles, the main inorganic constituent is colloidal calcium phosphate (CCP). The nature of CCP is complex as these salts can have many compositions, such as tricalcium phosphate ($Ca_3(PO_4)_2$) or calcium brushite ($CaHPO_4$), or exist in amorphous or different crystalline structures (Lucey and Horne, 2009). The main binding sites of calcium phosphate in the casein micelle are the phosphate groups of the casein phosphoserine residues. Corresponding to the phosphoserine content of the caseins, the capacity for CCP binding is decreasing in the order α_{s2}- > α_{s1}- > β > κ-casein (Gaucheron, 2005).

CCP is an important constituent in maintaining the integrity of casein micelles. It is recognized that CCP participates in the changes of casein micelles in dairy processing such as heating, cooling and rennet coagulation (Aoki, 1991). The solubility of calcium phosphates present in the serum decreases at high temperatures. Upon severe heat treatments, changes in the structure and composition of the original micellar calcium phosphate have been suspected (Visser et al., 1986; Aoki et al., 1990). Acidification of milk leads to solubilization of CCP, with its extent depending on both the pH and the temperature of acidification (Dalgleish and Law, 1989; Singh et al., 1996). The exact nature of CCP, its interactions with casein molecules, and the effects on the heat stability of casein micelles are still unresolved.

Casein micelle structure

About 95% of the casein in milk exists as casein micelles, which are roughly spherical particles with diameters ranging from 50-500 nm. Casein micelles are made up of α_{s1}-, α_{s2}-, β- and κ-caseins and about 6% of low molecular mass, mostly inorganic salts, especially calcium and phosphate (Walstra, 1990; Fox, 2003). The micelles are highly hydrated, have porous structures and bind higher levels of CCP then would otherwise be able to be dispersed in an aqueous solution. The main biological function of the

casein micelle is the delivery of CCP from the mammary gland to the neonate, to cover the high demand of calcium in the young animal. The casein micelle is a very stable system that can withstand processing operations, such as pumping, homogenization, drying, ultracentrifugation and reconstitution.

The properties and functions of the casein micelle have been the topic of research for a long time, yet still no consensus has been reached about its structure. As more information has become available over time, some principal features have been elucidated that must be met by any micelle model:

1. κ-casein is surrounding the micelle in a surface layer, in which it is able to stabilize the calcium-sensitive α_{s1}-, α_{s2}- and β-caseins. This arrangement is supported by the fact that chymosin hydrolyzes κ-casein rapidly when added to casein suspensions. Furthermore, it has been shown that the serum protein β-lactoglobulin interacts with κ-casein via the formation of disulfide bonds to form complexes upon heating, which also indicates that κ-casein is located at the surface of the micelle.
2. The casein molecules are held together by CCP, which is supported by the observation that the micelles disintegrate upon removal of CCP. Calcium phosphate-free micelle systems are unstable and sensitive to low levels of calcium.
3. At lower temperatures β-casein is dissociating from the micelle and migrating into the serum. This dissociation is reversible at increasing temperatures and indicates the role of hydrophobic bonds in the casein micelle structure.

Various micelle models have been proposed in the past and some were dismissed quicker than others, based on meeting the features of the micelle or being unable to explain some of its properties.

One of the longest lasting models has been the sub-micelle model, which pictured the casein micelle as being composed of sub-micelles (Figure 1.1). Each sub-micelle was thought to have a size of about 10-15 nm and around 15-25 sub-micelles are linked together by colloidal calcium phosphate, which gives it the open and porous structure (Schmidt, 1982). The sub-micelles were considered to

follow a core-coat model, with the hydrophobic α_s- and β-caseins in the center of the micelle and hydrophilic κ-casein on the outside; sub-micelles that contain more κ-casein would be located on the outside of the micelle aggregate, allowing for the hydrophilic C-terminal end of the κ-caseins to provide steric hindrance against flocculation.

While the model was able to explain the main features of the casein micelle, it was dismissed after more advanced electron microscopy images showed a rather inhomogeneous internal structure with particles in the size of 8-10 nm (McMahon and McManus, 1998).

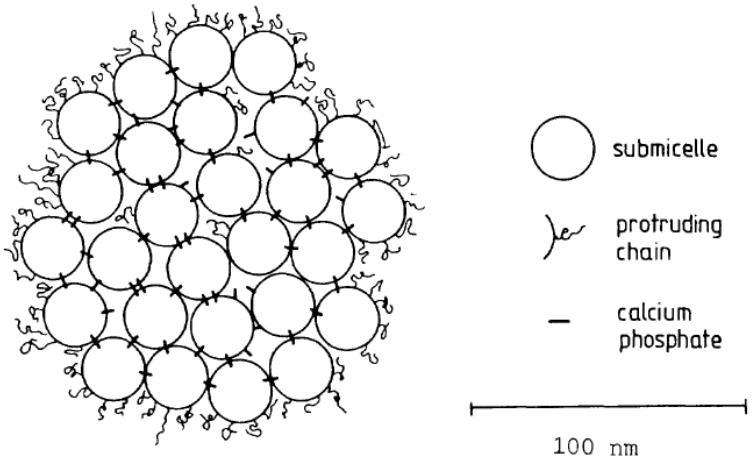

Figure 1.1. Sub-micelle model of the casein micelle (from Walstra et al., 1984).

One of the most recent casein micelle models is the so called interlocking lattice model and was introduced by McMahon and Oommen (2008). The model proposes that caseins form linear and branched chains interlocked by casein-stabilizing calcium phosphate nanoclusters (Figure 1.2). The structure is irregular and different caseins act as chain extenders (β- and α_{s1}-casein), chain branch points (α_{s1}- and α_{s2}-casein) or chain terminators (κ-casein). Hydrophobic interactions, as well as calcium bridging, are thought to be the predominant forces between caseins (McMahon and Oommen, 2008). Some chains can extend outwards, placing κ-casein on the surface of the structure.

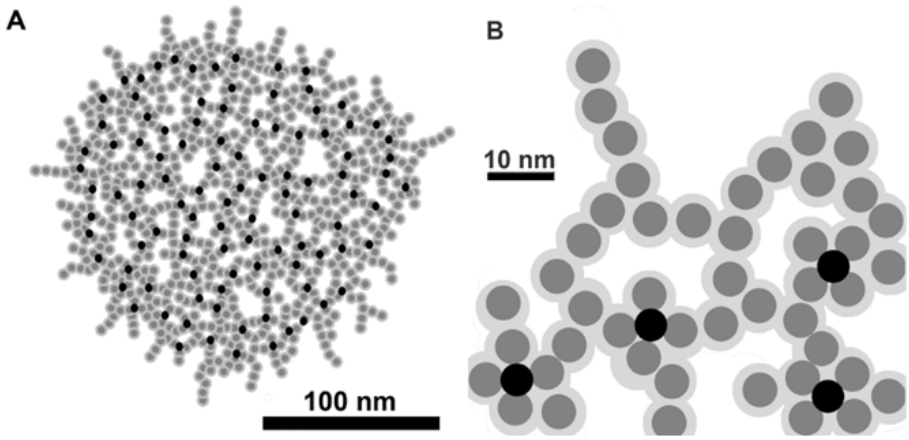

Figure 1.2. Schematic diagram of an interlocking lattice model of the casein micelle. Calcium phosphate nanoclusters are shown as black spheres and caseins are shown as grey spheres (from McMahon and Oommen, 2008).

While the authors were able to support their proposed model by high-resolution transmission electron micrographs (TEM), the model has already been challenged and the disagreement about the actual structure of the casein micelle continues (Horne, 2010).

The effect of heat treatment on the casein micelle

Heat-induced changes in milk happen as a function of temperature. At temperatures up to about 90°C, reactions occur at a relatively slow rate and are mostly reversible, with the exception of serum protein denaturation, while at higher temperatures, reactions happen rapidly and irreversibly (O'Connell and Fox, 2003).

Dairy products that are shelf-stable undergo sterilization, a high heat-treatment performed at temperatures above 110°C (O'Connell and Fox, 2003). Under certain conditions, this process causes flocculation, gelation or protein separation in milk products (Fox, 1982), which has led the topic of pH-dependent heat stability of casein micelles in milk to become the subject of a considerable amount of research. Most of the research has been done on milk and concentrated milk products and only few studies are available on micellar casein systems devoid of serum proteins and lactose.

pH-dependent heat stability of concentrated and normal milks

Studies on the effect of pH on the heat stability, often measured as heat coagulation time (HCT), of fresh and reconstituted milks have shown that the HCT-pH profiles of most milks show a minimum around pH 6.9 and a maximum around pH 6.7 (Rose, 1961) (Figure 1.3). The minimum in heat stability was attributed to the dissociation of κ-casein after forming complexes with serum proteins, which results in reduced zeta potential and steric hindrance, and thus a destabilization of the casein micelle (Singh and Fox, 1985; Singh and Fox, 1986). The heat-induced dissociation of casein micelles was shown to be dependent on the pH of the milk. Low levels of casein dissociation were reported to occur when the initial milk pH is below 6.8, while at higher pH values, increasing levels of casein dissociate from the casein micelles during heating (Kudo, 1980). As the pH of milk is decreased, CCP dissolves (Singh et al., 1996), causing dissociation of caseins, particularly β- and κ-casein (Holt et al., 1986; Aoki, 1988). Micellar integrity, however, is largely maintained upon CCP depletion (Dalgleish and Law, 1989). As the phosphoserine charge is neutralized by acidification, protein net charge decreases, allowing for aggregation of casein molecules as well as increased hydrophobic interactions (Singh et al., 1996).

Tessier and Rose (1964) showed that adding κ-casein to a minimum/maximum HCT-pH profile milk (Type A milk) reduces or eliminates the minimum in the heat stability curve, while adding β-lactoglobulin will convert a Type B milk (milk for which heat stability increases with increasing pH) into a Type A milk. They suggested that the ratio of κ-casein and β-lactoglobulin are responsible for the pH-dependent heat stability of milk (Tessier and Rose, 1964).

In concentrated milks the heat stability is reduced and the heat stability is shifted to lower pH values as compared to normal milk (Figure 1.3). More κ-casein is dissociated with increasing concentration of milk solids and increased heating times, which is more pronounced at higher pH (Singh and Creamer, 1991a; Singh and Creamer, 1991b). Rose and Tessier (1959) found progressively less calcium and phosphate in the soluble phase on increasing temperatures up to 110°C, with phosphate

decreasing stronger at the higher temperatures and calcium decreasing relatively uniform throughout the temperature range. This is in agreement with reports of a gradual reduction of soluble calcium and phosphorus, with a corresponding increase of the amount of these minerals bound to the colloidal phase throughout sterilization treatments of concentrated milks (Hardy et al., 1984).

The reasons for the dissociation of κ-casein, however, have not yet been fully explained on the molecular level and heat stability problems remain, especially with concentrated milk products. One study suggests that the micellar dissociation is induced by an increase in electrostatic repulsive forces as the milk pH is increased above the critical value of 6.7 and the temperature is raised. Under those conditions, CCP and the hydrophobic forces are insufficient to maintain micellar integrity and the micelles progressively disintegrate (Anema and Li, 2000).

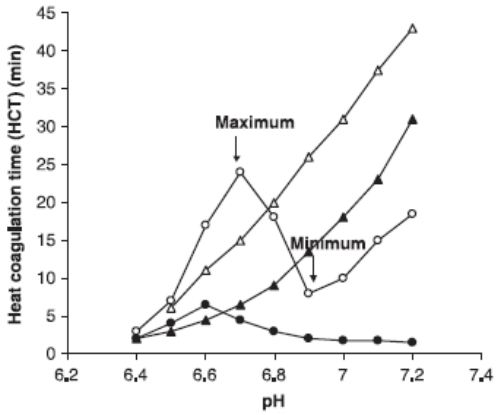

Figure 1.3. Heat coagulation time (HCT) vs. pH profile for different skim milks heated at 140°C. Type A milk (○), Type B milk (Δ), serum protein-free casein micelle dispersions (▲) or concentrated milk (20% total solids) (●) (from Singh, 2004).

The effect of heat treatments on serum protein-depleted casein concentrates

Serum proteins, specifically β-lactoglobulins (β-lg), have been identified as one reason for the typical Type A heat stability-pH profile with a pronounced minimum and maximum (Fox and Hoynes,

1975; Schmidt and Poll, 1986). When heating serum protein-depleted suspensions of casein micelles in milk ultrafiltrate, the heat stability was found to increase with increasing pH (Figure 1.3), while the addition of 0.3% β-lg lead to a maximum heat stability around pH 6.8 and a minimum around pH 7.0 (Schmidt and Poll, 1986). Increasing concentrations of added β-lg both increased the maximum heat coagulation time, and extended the minimum over a wider pH range (Fox and Hoynes, 1975; Singh and Fox, 1987). It has been concluded that β-lg protects the casein micelle against heat-induced coagulation through the association with κ-casein in the pH range 6.5-6.7, but destabilizes the micelle by promoting dissociation of κ-casein at pH-values > 6.9 (Singh and Fox, 1987).

A study by Aoki et al. (1974) showed that when heating serum protein-depleted milk at temperatures of 110°C and higher, the soluble casein increased with increasing heating time and concentration, while amounts of colloidal Ca and P increased only slightly (Aoki et al., 1990). Above 140°C the amount of soluble casein in supernatant from concentrated serum protein-depleted milk was found to increase significantly and coagulation was observed within 5 min of heating (Aoki et al., 1974). This was confirmed by results showing that the content of casein cross-linked by CCP decreased from 51.9% to 46.1% on heating serum protein-free milk at 135-140°C for 15-75 s, suggesting that the binding between CCP and casein was weakened due to the formation of a different type of calcium phosphate upon heating (Aoki et al., 1990; Singh, 1994). Using gel electrophoresis and laser densitometry, Anema and Li (2000) found that increasing the heating temperatures between 20-90°C, and increasing the pH from 6.5 to 7.1, led to increased dissociation of α_s-, β- and κ-casein (Figure 1.4). At any particular temperature or pH, the levels of κ-casein dissociation were higher than dissociation of the other two casein groups (Anema and Li, 2000).

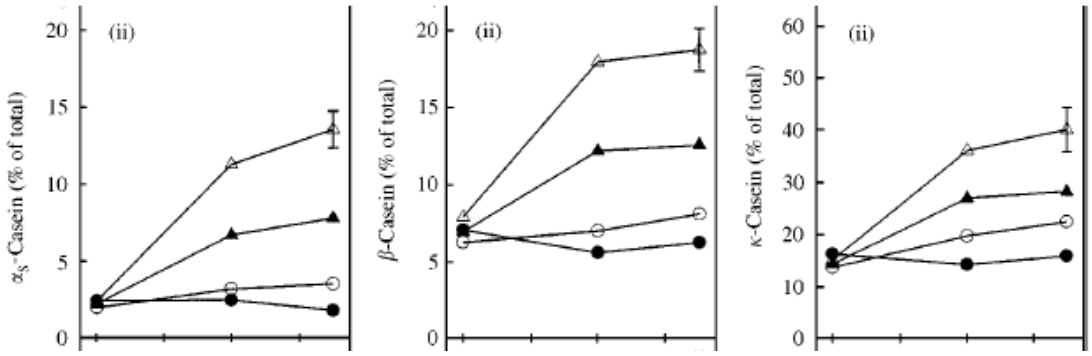

Figure 1.4. Effect of temperature (20°C, 60°C, 90°C) and pH on the level of protein in the supernatants obtained from serum protein-free milk. (a) α_s-casein; (b) β-casein; (c) κ-casein. (●) pH 6.5; (○) pH 6.7; (▲) pH 6.9; (Δ) pH 7.1 (from Anema and Li, 2000).

Previous work by Beliciu et al. (2012) focused on evaluating the effects of two commercial sterilization regimes (continuous-flow UHT treatment and in-container retorting) on the processing stability and physical properties of serum protein- and lactose-depleted MCC, as well as MCC reconstituted from spray-dried MF retentate powder (R-MCC). The study showed that sterilization treatments induced structural changes in MCC and R-MCC, which was most significant during UHT treatment, in which it led to aggregation and coagulation of the samples.

Retorting led to a significantly lower apparent viscosity (η_{100}) of the MCC samples, while the UHT-treated MCC had a much higher apparent viscosity than control samples (Figure 1.5). For the control and retorted samples, dynamic rheological testing revealed a predominantly liquid-like behavior ($G' < G''$), while for the UHT-treated samples a solid-like behavior ($G' > G''$) was observed, which was indicative of structure formation, consistent with the visual observation.

Casein micelle aggregation was demonstrated by an increase in the average effective particle diameter from 180 nm in the untreated MCC to 240 nm in retorted samples. UHT-treatment led to the formation of large aggregates and coagulation, visible with the naked eye. A significantly lower zeta potential was measured after sterilization treatments, indicating a decrease in electrostatic stability of the casein micelles.

Levels of soluble calcium, phosphorus and magnesium decreased significantly, while the total mineral composition was not affected by heat treatment. Lower concentrations of soluble Ca and P were observed after UHT treatment in comparison to retorted MCC, whereas magnesium was not affected by the type of sterilization treatment. The soluble Ca/P ratio was 1.25, 1.11 and 1.02 for control, UHT-treated and retorted MCC, respectively, with similar values for R-MCC.

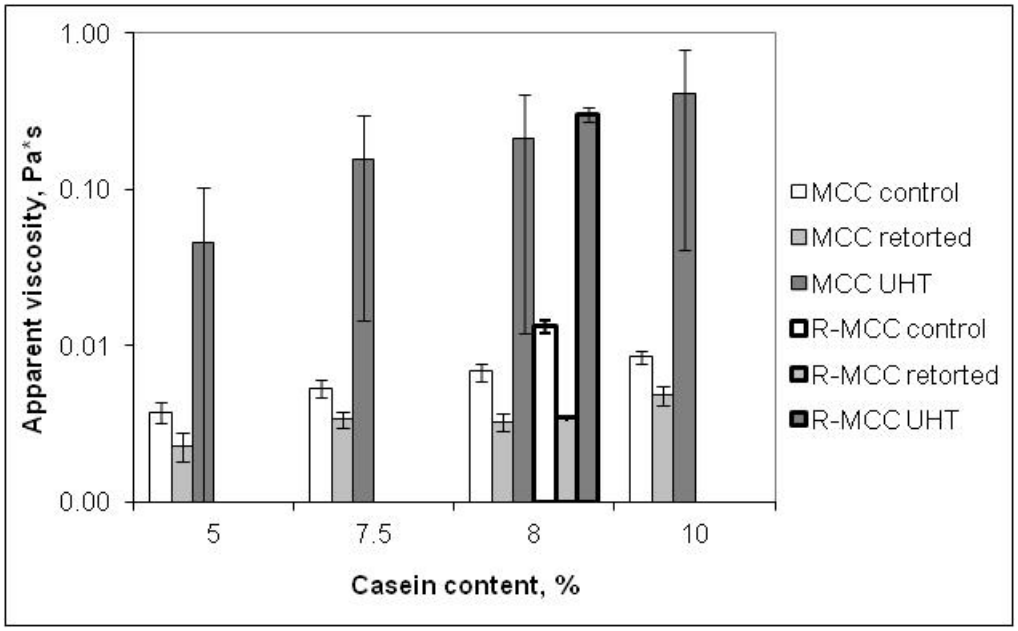

Figure 1.5. Apparent viscosity at 100 s^{-1} for untreated and heat-treated MCC and R-MCC. Data represents the average of three experimental replicates and error bars represent one standard deviation (from Beliciu et al., 2012).

An evaluation of the sterilization behavior of R-MCC obtained by reconstitution of spray dried MF retentate powders showed that drying enhanced the instabilities that occurred during sterilization of MCC, but did not have a significant effect on its own.

The study showed that sterilization treatments led to a partial insolubilization of minerals responsible for micellar stability and a reduction in surface charge of casein micelles, leading to instability and aggregation, specifically in the UHT-treated MCC. Based on the observed difference in stability of MCC to retorting and UHT-treatment, it was suggested that heat-induced changes might be an effect of temperature. Changes in electrostatic stability were attributed to a combination of κ-casein

dissociation as well as the precipitation of calcium phosphate, leading to subsequent micelle aggregation and coagulation.

The effects of heat treatment and pH on the dissociation of caseins from the micelle require further investigation to better understand the observed instabilities during sterilization treatments.

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CHAPTER 2

RESEARCH OBJECTIVES

In recent years, the use of micellar casein preparations obtained by microfiltration has been receiving increased interest from the dairy and food industry. During the microfiltration process, the casein micelle remains close to its native state and serum proteins and lactose are separated, which results in unique functionality and new possibilities for applications. There is a need to understand the functionality and stability of micellar casein concentrates during processing and storage.

The pH-dependent, heat-induced instabilities of the casein micelle have been researched for many decades and considerable progress has been made on explaining the variability and mechanism of heat-induced coagulation. However the phenomenon has not been elucidated on the molecular level yet and only limited information is available on micellar casein systems devoid of serum proteins and lactose.

A systematic evaluation of the rheological and heating behavior of micellar casein concentrates across a range of concentrations, pH, and temperature conditions will contribute to a better understanding of the flow behavior of micellar casein concentrates and the interactions within the casein micelle. The knowledge is necessary in order to provide the dairy and food industry with data necessary for effectively stabilizing micellar casein preparations and developing practical applications for this valuable ingredient. The objectives of this dissertation were:

1. Investigate the steady shear rheological properties of micellar casein concentrates under a range of concentration, temperature and shear conditions and develop a mathematical model able to accurately predict viscosity as a function of these parameters.
2. Evaluate the stability and sedimentation behavior of sterilized micellar casein concentrates during 8 weeks of storage at 25°C.

3. Investigate the effects of pH and temperatures in the sterilization range on the stability of micellar casein concentrates.
    3.1. Quantify the effect of pH, in the range of 6.5-7.3, and temperature, in the range of 110-150°C, on the mineral distribution and dissociation of caseins during heating of micellar casein concentrates.
    3.2. Validate findings by evaluating the dissociation of caseins as a result of commercial high heat treatment regimes to develop solutions for stabilizing micellar casein concentrates during sterilization treatments.

CHAPTER 3

STEADY SHEAR RHEOLOGICAL PROPERTIES OF MICELLAR CASEIN CONCENTRATES (MCC) OBTAINED BY MEMBRANE FILTRATION AS A FUNCTION OF SHEAR RATE, CONCENTRATION AND TEMPERATURE

Abstract

The use of casein preparations obtained by membrane separation is receiving increasing interest from the dairy and food industry. The objective of this work was to generate information about the steady shear rheological properties of micellar casein concentrates (MCC) and the effect of composition, temperature and shear rate on these properties. MCC preparations with two levels of serum proteins (SP) (65% and 95% SP-reduced, respectively), were obtained from skim milk by microfiltration followed by spray-drying. MCC preparations with casein concentrations ranging from 2.5% to 12.5% were obtained by dispersing the MCC powders in ultrapure water. Steady shear rheological analyses at temperatures ranging from 0°C to 80°C were performed using a strain-controlled rheometer. Viscosity vs. shear rate curves were used to evaluate the effect of shear on viscosity, and the apparent viscosity at a shear rate of $100\ s^{-1}$ was used to make direct comparisons between various concentration and temperature conditions.

The 65% SP-reduced MCC had lower viscosity than the 95% SP-reduced MCC at the same casein concentration and temperature. Protein preparations at casein concentrations above 7.5% displayed shear thinning behavior, which was more pronounced as concentration increased. The viscosity of MCC increased exponentially with casein concentration and decreased with temperature. The dependency of viscosity on temperature followed an Arrhenius relationship. A modified Arrhenius model able to

accurately predict rheological properties under desired shear, temperature and concentration conditions was developed and validated. This study provides critical rheological data necessary for developing practical applications of micellar casein preparations.

Introduction

Casein and caseinates are valuable food ingredients because of their nutritive value and physico-chemical and functional properties. Due to their water-binding, emulsifying, whipping, foaming and texturizing properties, casein concentrates are used in a range of commercial applications, including protein fortification of dairy foods, or ingredients for beverages, bakery, or meat products (Mulvihill and Ennis, 2003). Traditionally, caseinates and caseins were prepared by either isoelectric precipitation or rennet coagulation (Fox, 2001). Recently, casein preparations obtained by microfiltration (MF) are receiving increasing interest from the food and dairy industry (Affertsholt, 2009). In this process, casein is separated from serum proteins based on their difference in molecular size (Brans et al., 2004). Additional diafiltration of the retentate can lead to serum protein removal from the casein concentrate of up to 95% (Nelson and Barbano, 2005). In casein concentrates obtained by membrane separation, the casein micelles are closer to their native state than in casein preparations obtained by chemical methods, which is why they are typically called micellar casein concentrates (MCC). MCC have different functionality as compared to traditional casein ingredients, which opens the field for new possible applications (Nelson and Barbano, 2005).

Rheological properties of MCC are very important and have high relevance for any new food application. Such data is necessary for equipment selection and the design of various unit operations and processing steps such as pumping, mixing, heating or cooling. Rheological data can as also provide valuable information about the mouthfeel of the finished product (Hermansson, 1975).

The objective of this study was to evaluate the steady shear rheological properties of MCC under a range of concentration, temperature and shear conditions and to establish a mathematical model able to accurately predict viscosity as a function of these parameters. Such a model will provide the dairy and food industry with critical rheological data necessary for developing commercial applications of micellar casein preparations.

Materials and Methods

Manufacture of micellar casein concentrates

Micellar casein concentrates were obtained by membrane separation in the pilot plant at Cornell University according to the procedure described by Zulewska et al. (2009). Pasteurized skim milk was processed at 50°C using a uniform transmembrane pressure (UTP) microfiltration (MF) system equipped with ceramic Membralox membranes with a 0.1 µm pore size. The MF process was continuous bleed-and-feed at a concentration factor of 3X, which resulted in 65% SP-reduced MCC. In order to obtain 95% SP-reduced MCC, the retentates obtained after the first and second MF stages were diluted with reverse osmosis (RO) water at a 2:1 ratio by weight (2 parts water, 1 part retentate). Both the 65% SP-reduced and 95% SP-reduced retentates were spray dried using a Model 1 Niro Atomizer equipped with a FU11 atomizer rotating at 23,000 rpm (Niro Atomizer Inc., Columbia, MD). The inlet air temperature was 200°C and the outlet air temperature was 95°C. The spray dried powders were stored in the dark at 25°C room temperature until use. For each type of MCC, the processing was replicated 3 times with different batches of milk.

Chemical analysis

Fresh, liquid samples of the 65% and 95% SP-reduced MCC retentates were analyzed for fat, total nitrogen (TN), non-protein nitrogen (NPN), and non-casein nitrogen (NCN) using ether extraction (AOAC,

2005; method 989.05; 33.2.26), Kjeldahl (AOAC, 2005; method 991.20; 33.2.11), Kjeldahl (AOAC, 2005; method 991.21; 33.2.12), and Kjeldahl (AOAC, 2005; method 998.05; 33.2.64), respectively. True protein (TP) was calculated by subtracting NPN from TN and then multiplying by 6.38; casein content (CN) was calculated by subtracting NCN from TN and multiplying by 6.38; and SP content was calculated by subtracting NPN from NCN and multiplying by 6.38. The SP removal was estimated by Kjeldahl analysis (TN, NPN, NCN) of the MF permeates according to Hurt et al. (2010).

After spray-drying, powders were reconstituted to 10% solids and their pH was measured with an Electrolyte 9823 electrode (Mettler Toledo, Columbus, OH). The total solids (TS) content of the powders was measured by forced air oven drying (AOAC, 2005; method 990.20; 33.2.44).

Sample preparation

The protein powders were weighed and slowly added to ultrapure water to obtain suspensions of casein concentrations of 2.5%, 5.0%, 7.5%, 10.0% and 12.5% w/v. The suspensions were stirred for 30 min on a stir plate at moderate speed at 25°C, followed by ultrahigh shear mixing using an Ultra-Turrax Model T25 fitted with a S25N-18G dispersing tool (IKA Works Inc., Wilmington, NC) for 5 min at 21,500 rpm. Following the high shear dispersion the suspensions were kept under continuous stirring at low speed at room temperature for an additional 90 min to allow for full hydration and for the foam to break. A detailed discussion about how rehydration and sample preparation procedure were established is included in the paper by Beliciu and Moraru (2011).

Rheological analysis

The viscosity and flow behavior of the reconstituted 65% and 95% SP-reduced MCC were determined using large deformation rheological analyses. Steady shear rate sweeps were conducted using an Advanced Rheometric Expansion System (ARES) strain-controlled rheometer equipped with a Peltier temperature control system (TA Instruments, New Castle, DE). 2 mL of MCC sample were

carefully loaded onto a parallel plate fixture (diameter: 50 mm, interplaten gap: 1 mm) at 20°C, avoiding the formation of air bubbles. In order to prevent water evaporation during the measurement, a thin mineral oil film was applied around the geometry's circumference. For test temperatures above 50°C, an isothermal chamber was installed around the fixture. The temperature of the Peltier element was set to the desired value (0°C, 20°C, 40°C, 60°C or 80°C) and once the test temperature was reached the samples were allowed a 60 s temperature equilibration and relaxation step before starting the test. Steady-shear rate sweeps were conducted with shear rates ranging from 1 to 631 s^{-1} at a frequency of 6.28 rad/s (1 Hz) in clockwise and counterclockwise direction. For each sample, triplicate measurements were performed.

The data was collected and analyzed using the Orchestrator software (TA Instruments, New Castle, DE). The *Fit Model* component of the software was used to fit the data to rheological models.

Statistical analysis

JMP 8.0 (SAS Institute Inc., Cary, NC) was used for statistical analyses of the data. Analysis of variance was used to determine the effect of serum protein removal, concentration, and temperature. Significant differences among samples were determined by Tukey's HSD test at $p \leq 0.05$.

Results and Discussion

Chemical composition of the powders

A summary of the chemical composition of the 65% and 95% SP-reduced MCC powders is presented in Table 3.1. The casein content in the 65% SP-reduced MCC was 51.10%, which equates to a casein-to-true protein ratio (CN%TP) of 90.23%. For the 95% SP-reduced MCC, the casein content was 80.47% and the CN%TP was 95.64%, due to the additional removal of serum proteins and non-protein nitrogen from the retentate during the 2 diafiltration steps in the production process. The diafiltration

also lead to a significant reduction of lactose to a final concentration of 3.70% in the 95% SP-reduced MCC as compared to 30.34% lactose in the 65% SP-reduced MCC. The fat content was similar in the two types of MCC powders. A detailed discussion about the composition of these retentates is included in the paper by Hurt and Barbano (2010).

Table 3.1. Composition of 65% and 95% SP-reduced micellar casein concentrates (MCC), on dry solids basis (% by weight)

	Composition[1]										
	TN	NPN	NCN	TP	CN	CN% TP	SP	MC	Fat	Lactose	pH
65% SP-reduced MCC	57.92	1.29	6.82	56.63	51.10	90.23	5.53	3.33	2.22	30.34	6.91
95% SP-reduced MCC	84.56	0.42	4.09	84.14	80.47	95.64	3.67	4.55	2.74	3.70	7.20

[1]TN = total nitrogen x 6.38; NPN = non-protein nitrogen x 6.38; NCN = non-casein nitrogen x 6.38; TP = true protein (TN-NPN); CN = casein (TN-NCN); CN%TP = casein as percentage of true protein; SP = serum protein (TP-CN); MC = moisture content. Values are averages of 3 experimental replicates.

Flow behavior

Figure 3.1a shows the flow curves of MCC dispersions with casein concentrations ranging from 2.5-12.5% at 20°C. The MCC with 2.5% concentration had virtually a Newtonian behavior, as the viscosity did not change with shear rate. The MCC with casein concentrations above 5% exhibited a mild shear-thinning behavior, while MCC with casein concentrations ≥ 7.5% exhibited a clear non-Newtonian shear-thinning behavior across the investigated range of shear rates. The flow behavior was also affected by temperature, with more pronounced shear-thinning being observed at low temperatures. Figure 3.1b shows the flow curves for the 12.5% MCC, which exhibited a clear shear-thinning behavior for all of the investigated temperatures (0-80°C). These observations are in agreement with the study by Beliciu and Moraru (2011), who reported that the rheological behavior of dispersions of MCC, soy proteins, and their mixtures show two distinct regimes of flow behavior. In the dilute regime, at protein

concentration ≤ 5%, the samples behaved as Newtonian fluids, while at protein concentration ≥ 7.5% a non-Newtonian, shear-thinning behavior was observed. Shear-thinning behavior is typical for many soft foods, including dairy products, and has implications for both processing (i.e. pumping, pipe flow) and the mouthfeel of food products that contain MCC.

The dependence of viscosity on shear rate for concentrated milk products has been described in the literature using various models, including the power-law model (Vélez-Ruiz and Barbosa-Cánovas, 1998; Solanki and Rizvi, 2001), the Bingham model (Bienvenue et al., 2003a), or the Herschel-Bulkley model (Vélez-Ruiz and Barbosa-Cánovas, 1998).

The Herschel-Bulkley model has the following mathematical form:

$$\sigma = \kappa(\dot{\gamma})^n + \sigma_0 \qquad \text{Eq. 3.1}$$

where σ is the shear stress (Pa), κ is the consistency coefficient (Pa·s^n), $\dot{\gamma}$ is the shear rate (s^{-1}), n is the flow behavior index and σ_0 is the yield stress (Pa).

This model is very general, and it can be used to describe the flow behavior of Newtonian ($\sigma_0 = 0$ and $n = 1$), power law ($0 < n < 1$ for shear-thinning and $1 < n < \infty$ for shear-thickening), and Bingham plastic behavior ($n = 1$) (Steffe, 1996).

The viscosity data for all the MCC samples was fitted to the Herschel-Bulkley model, and the values for κ, n and σ_0 were determined. The fit for all data sets was very good, with $R^2 > 98\%$ in all cases. For all the MCC samples the yield stress was very small ($\sigma_0 < 0.04$ Pa) for the casein concentrations and temperatures evaluated (data not shown).

Flow indices (n) for both the 95% SP-reduced and 65% SP-reduced MCC dispersions are presented in Table 3.2. In Table 3.2, statistically significant ($p \leq 0.05$) differences in n values among samples of varying casein concentrations are indicated by different letters. The shear-thinning behavior of the samples is reflected by the $n < 1$.

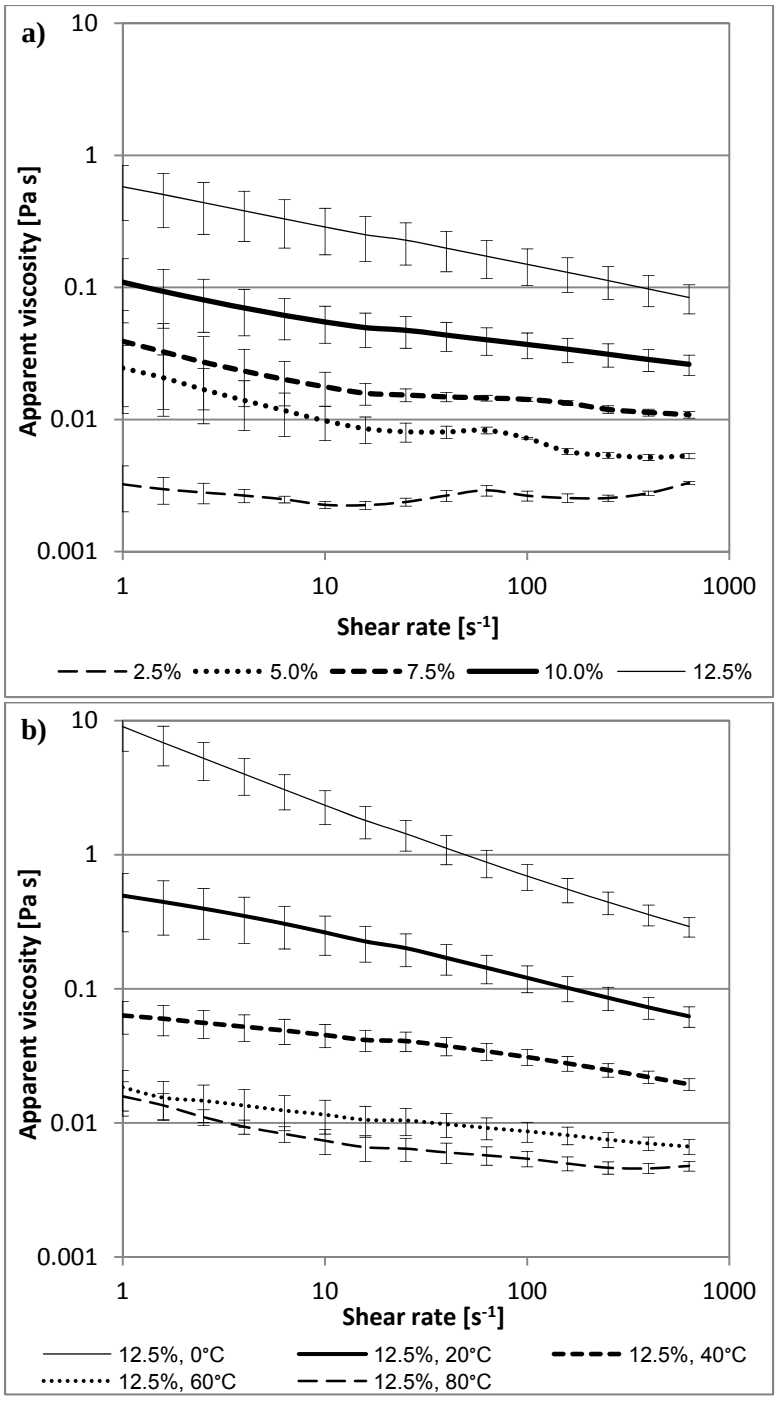

Figure 3.1. Apparent viscosity as a function of shear rate for 95% SP-reduced MCC dispersions of a) 2.5-12.5% casein concentration at 20°C; b) 12.5% casein concentration at temperatures between 0-80°C. Plotted are mean values (n=3) ± 1 SD.

It has been shown before that the flow behavior and the extent of shear-thinning of milk concentrates (Stepp, 1991; Vélez-Ruiz and Barbosa-Cánovas, 1998; Bienvenue et al., 2003a; Bienvenue et al., 2003b) and MF retentates (Solanki and Rizvi, 2001) depend on factors such as composition, concentration, temperature, and storage time. In this work, the shear-thinning behavior of the MCC was more prevalent, i.e. the flow index *n* decreased, with increasing concentration and decreasing temperature. Samples of casein concentrations of 2.5% and 5.0% had flow indices very close to 1, which confirms that these MCC dispersions have a virtually Newtonian behavior, whereas samples of higher concentrations had $n < 1$. The 65% SP-reduced and the 95% SP-reduced MCC showed very similar flow behavior and flow indices across the concentration and temperature ranges used in this study (see Table 3.2).

Table 3.2. Flow indices for 65% and 95% SP-reduced MCC. Shown are mean values (n=3) ± 1 SD

		Flow index *n*				
		Temperature				
MCC type	Concentration	0°C	20°C	40°C	60°C	80°C
95% SP-reduced MCC	2.5%	0.97 ± 0.02[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.98 ± 0.00[a]	0.98 ± 0.00[a]
	5.0%	0.96 ± 0.01[a]	0.97 ± 0.02[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.98 ± 0.00[a]
	7.5%	0.88 ± 0.01[a]	0.95 ± 0.02[a]	0.99 ± 0.01[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a,b]
	10.0%	0.75 ± 0.02[b]	0.85 ± 0.03[b]	0.97 ± 0.01[a]	0.99 ± 0.00[a]	0.99 ± 0.00[b]
	12.5%	0.62 ± 0.07[c]	0.71 ± 0.03[c]	0.86 ± 0.03[b]	0.95 ± 0.01[b]	0.98 ± 0.01[a,b]
65% SP-reduced MCC	2.5%	0.96 ± 0.02[a]	1.00 ± 0.00[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.98 ± 0.00[a]
	5.0%	0.92 ± 0.03[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.98 ± 0.00[a]
	7.5%	0.90 ± 0.08[a]	0.97 ± 0.01[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a]	0.99 ± 0.00[a,b]
	10.0%	0.72 ± 0.03[b]	0.86 ± 0.03[b]	0.97 ± 0.00[a]	0.99 ± 0.00[a]	0.99 ± 0.00[b]
	12.5%	0.57 ± 0.08[c]	0.64 ± 0.03[c]	0.81 ± 0.03[b]	0.95 ± 0.02[b]	0.99 ± 0.01[a,b]

[a–c] Means in the same column not sharing a common superscript are statistically different.

Effect of SP removal on the viscosity of MCC

Since many of the MCC dispersions behaved as non-Newtonian fluids across the investigated range of shear rates, with the viscosity changing as a function of the shear rate, the term 'apparent viscosity' (η_{app}) was used to describe their resistance to flow.

In order to evaluate the effect of composition and temperature on the viscosity of the MCC, comparisons were made using the apparent viscosity at a shear rate of 100 s^{-1} (η_{100}). This shear rate was selected due to its association with stirring, pumping, pipe flow, other processing operations, as well as mastication (Steffe, 1996).

The effect of SP removal on viscosity was evaluated by comparing the apparent viscosity data for the 65% SP-reduced MCC and the 95% SP-reduced MCC. It is important to note that, at the same casein concentration, the 65% SP-removed MCC contained more serum proteins, more lactose and more non-protein nitrogen, and consequently higher total solids (TS) than the 95% SP-removed MCC (Table 3.1). As seen in Figure 3.2a and b, at the same casein concentration and temperature, the 65% SP-reduced MCC dispersions exhibited lower values of η_{100} than the 95% SP-reduced MCC ($p \leq 0.05$). The almost 10-fold higher lactose content in the 65% SP-reduced MCC as compared to the 95% SP-reduced MCC would be expected to increase viscosity. In the study by Morison and Mackay (2001), the addition of varying amounts of lactose to whey protein concentrates (WPC) significantly increased their viscosity. The higher viscosity of the higher purity MCC is consistent with previous reports (Solanki and Rizvi, 2001), and indicates that casein is the main contributor to the viscosity of the MCC. This means that the soluble components (serum proteins, lactose, NPN and minerals) interfered with the casein-casein interactions and thus reduced the viscosity of the 65% SP-reduced MCC samples.

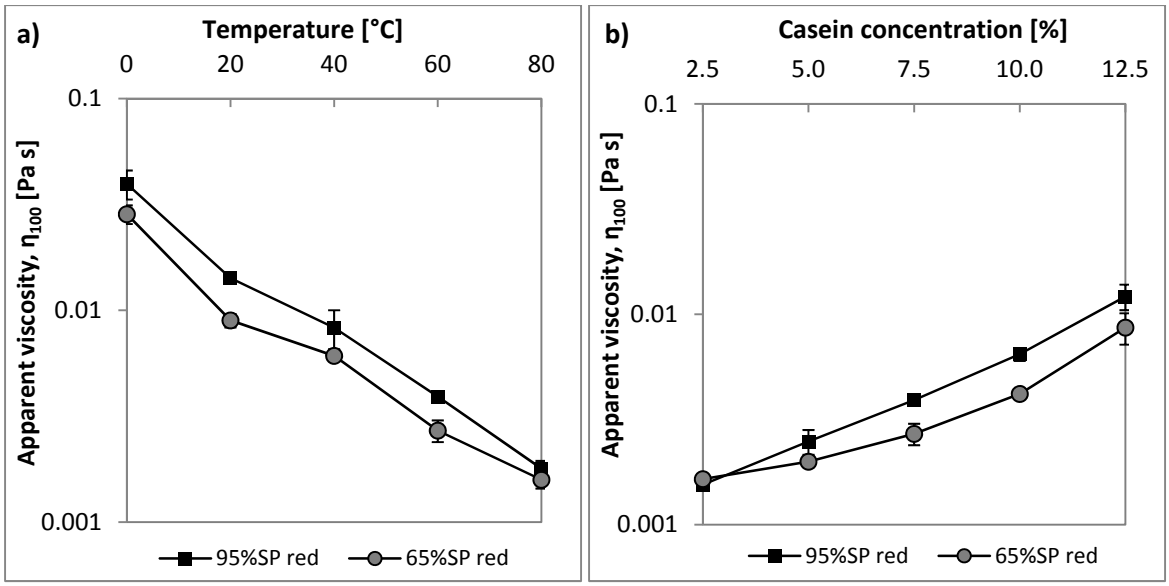

Figure 3.2. Comparison of the viscosity of 65% SP-reduced and 95% SP-reduced MCC: a) 7.5% casein concentration in temperature range 0-80°C; b) range of casein concentrations from 2.5-12.5% at 60°C. Plotted are mean values (n=3) ± 1 SD.

Effect of temperature on the viscosity of MCC

The apparent viscosity at 100 s^{-1} (η_{100}) vs. temperature for the 95% SP-reduced MCC is plotted in Figure 3.3, in which the solid points represent the experimentally measured values. Temperature had a significant effect ($p \leq 0.05$) on the apparent viscosity of MCC, with η_{100} decreasing with increasing temperature, at all casein concentrations.

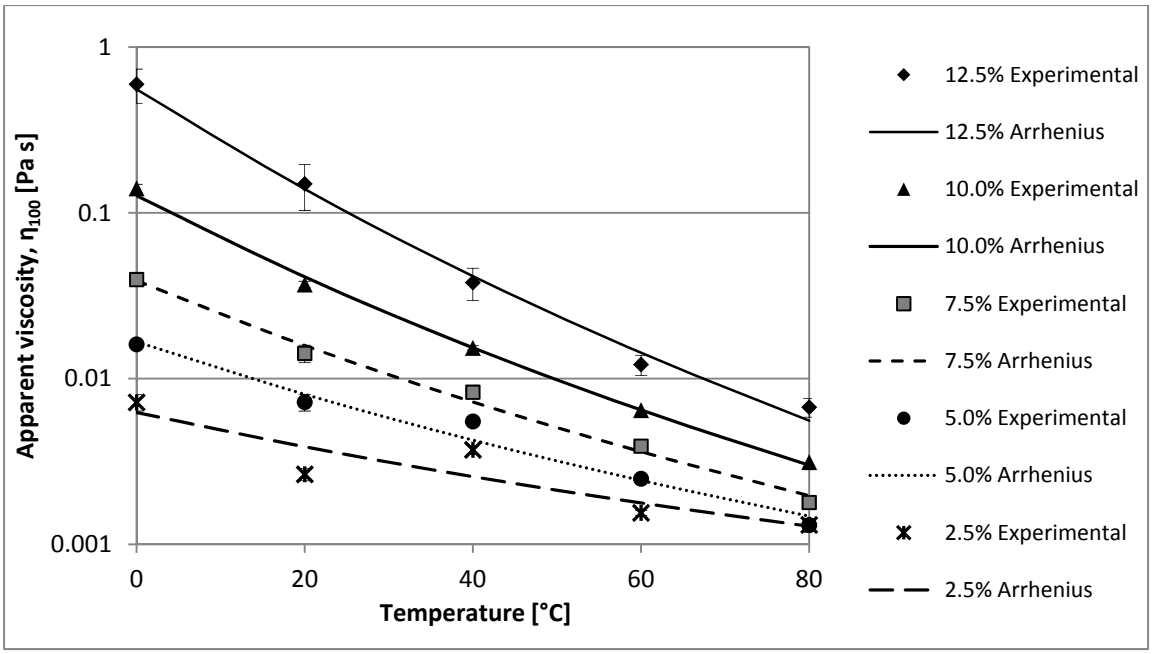

Figure 3.3. Comparison of experimental η_{100} values (points) with values calculated using an Arrhenius relationship (lines), for 95% SP-reduced MCC as function of temperature in the range of 0- 80°C. Plotted are mean values (n=3) ± 1 SD.

The effect of temperature on viscosity of fluids, including concentrated milk products, is commonly described using an Arrhenius-type relationship (Fichtali et al., 1993; Vélez-Ruiz and Barbosa-Cánovas, 1998). In this study, the effect of temperature on the apparent viscosity of the MCC also followed an Arrhenius relationship:

$$\eta_{100} = Ae^{\left(\frac{E_a}{RT}\right)} \qquad \text{Eq. 3.2}$$

where η_{100} is the apparent viscosity at shear rate 100 s^{-1} (Pa·s), A is a pre-exponential constant (Pa·s), E_a is the activation energy of flow (J/mol), R is the universal gas constant (8.314 J/(K·mol)), and T is the absolute temperature (K).

E_a and A were determined by linear regression analysis of 1/T vs. log(η) data for each concentration and replicate. The mean values from the three replicates of A and E_a were used to calculate the apparent viscosity of 95% SP-reduced MCC of different concentrations as a function of temperature, and were plotted as lines in Figure 3.3. As observed in this graphic representation, the

calculated values were very close to the experimental values. A similar analysis was conducted for the 65% SP-reduced MCC.

The activation energy indicates the sensitivity of the viscosity to a change in temperature, with higher values indicating that the viscosity is more sensitive to temperature changes (Steffe, 1996). The values of E_a for the 65% and 95% SP-reduced MCC were in the ranges of 15.1-49.9 kJ/mol and 15.8-46.2 kJ/mol, respectively, and were comparable with values reported in the literature for different types of skim milk concentrates (Solanki and Rizvi, 2001). E_a was found to increase linearly with increasing casein concentration of the 95% SP-reduced MCC dispersions (R^2 > 99%, Figure 3.4a) and 65% SP-reduced MCC dispersions (data not shown). This is in agreement with previous reports that found a linear correlation between activation energy E_a and total solids content (Chang and Hartel, 1997; Solanki and Rizvi, 2001), although other authors reported a non-linear dependence of activation energy with concentration and storage time (Vélez-Ruiz and Barbosa-Cánovas, 1998).

The pre-exponential parameter A indicates the sample's internal resistance to flow that is free from the influence of temperature (Giap, 2010). The values of A for the 65% and 95% SP-reduced MCC were in the ranges of $7.71E^{-6}$-$1.72E^{-10}$ Pa·s and $6.26E^{-6}$-$1.23E^{-9}$ Pa·s, respectively. The values of the pre-exponential parameter A decayed exponentially with casein concentration both for the 95%-SP reduced MCC (Figure 3.4b) and 65%-SP reduced MCC (data not shown).

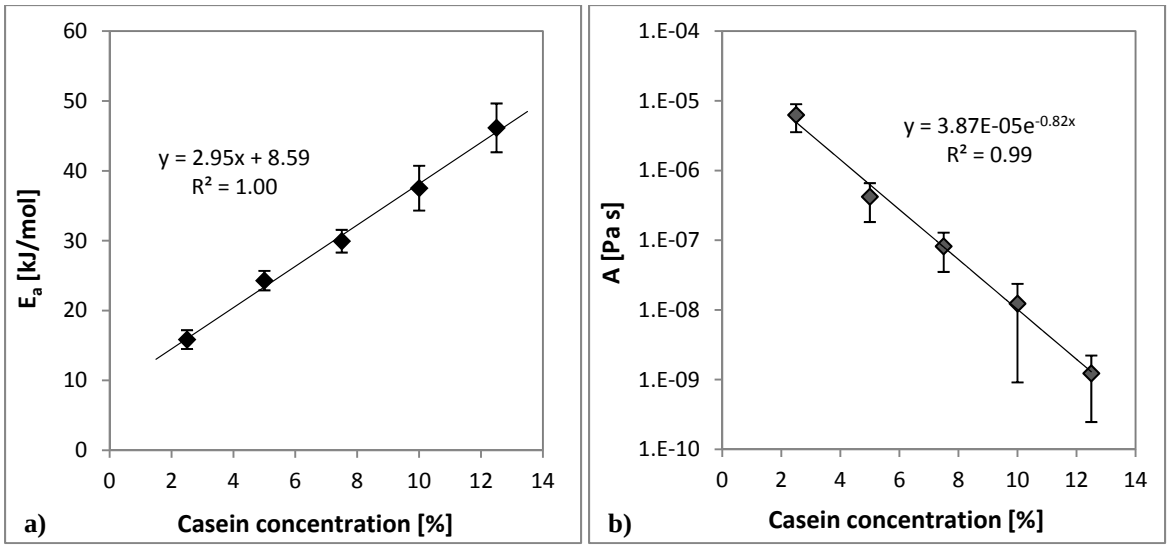

Figure 3.4. Arrhenius constants: a) activation energy E_a; b) pre-exponential parameter A as function of casein concentration for 95% SP-reduced MCC. Plotted are mean values (n=3) ± 1 SD.

Effect of concentration on the viscosity of MCC

The η_{100} of 65% and 95% SP-reduced MCC increased with increasing casein concentration, at all temperatures (see points in Figure 3.5, for the 95% SP-reduced MCC). This increase in viscosity was more pronounced with increasing concentration, as the concentration of total solids (particularly casein) increases, the distance between the casein micelles decreases, leading to a subsequent increase in electrostatic repulsion and resistance to flow. As two particles with the same charge are trying to avoid each other, they are altering their flow path, thus increasing the resistance to flow of the liquid in which they are suspended (İbanoğlu, 2002).

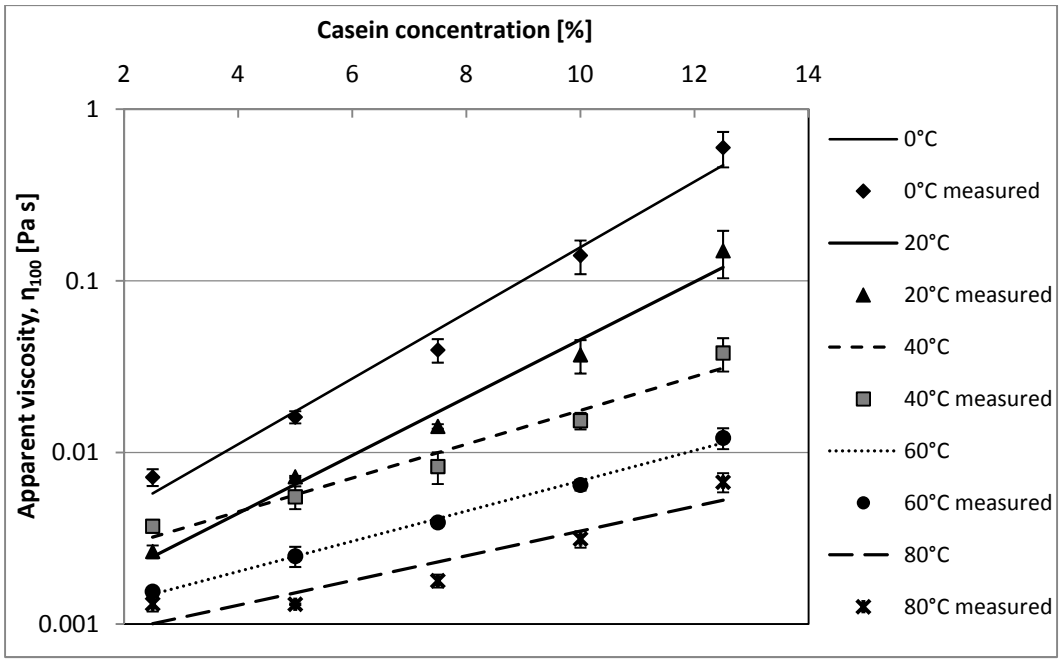

Figure 3.5. Comparison of experimental η_{100} values (points) with values calculated using an exponential relationship (lines) for 95% SP-reduced MCC as function of casein concentration in the range of 2.5-12.5%. Plotted are mean values (n=3) ± 1 SD.

The effect of concentration on viscosity has frequently been described as either a power law (Towler, 1974; Vitali and Rao, 1982) or as an exponential relationship (Rao et al., 1984; Beliciu and Moraru, 2011). For all MCC, the increase in viscosity with concentration followed an exponential relationship, at all tested temperatures:

$$\eta_{100} = a_1 e^{a_2 C} \qquad \text{Eq. 3.3}$$

where a_1 and a_2 are constants and C is the casein concentration (%).

The constants a_1 and a_2 were determined from linear regression analysis of C vs. log(η) data, for all MCC. The values for these coefficients were in agreement with values reported previously by Beliciu and Moraru (2011).

To verify the goodness of the model, the calculated coefficients were then used to calculate the apparent viscosity of the 65% and 95% SP-reduced MCC at different temperatures for the concentration range 2.5-12.5%, and the calculated values are plotted as lines in Figure 3.5. It can be noticed that the

calculated values and experimentally measured values are very close. This was the case for both MCC types, but to avoid redundancy only data for the 95% SP-reduced MCC is shown.

Development of an overall predictive model for MCC viscosity

For many practical applications, it would be very important to have a mathematical model able to accurately predict viscosity at a given shear rate, temperature and concentration.

The determined dependencies of apparent viscosity η_{100} on temperature and concentration of MCC dispersions were used to develop an overall predictive model for both types of MCC. By combining the Arrhenius relationship for the temperature-viscosity dependence and the exponential relationship between concentration and viscosity, the following modified Arrhenius model was developed:

$$\eta_{100} = a_1 e^{(a_2 C + \frac{a_3 + a_4 C}{RT})} \qquad \text{Eq. 3.4}$$

where η_{100} is the apparent viscosity at the shear rate 100 s^{-1} (Pa·s), a_1 (Pa·s), a_2 (dimensionless), a_3 (J/mol), and a_4 (J/mol) are constants, C is the casein concentration (%), R is the universal gas constant (8.314 J/(K·mol)), and T is the absolute temperature (K).

The calculated constants a_1, a_2, a_3, and a_4 for the 65% and 95% SP-reduced MCC dispersions are shown in Table 3.3.

Table 3.3. Determined numerical constants for the predictive model (see Eqs. 3.4, 3.8) for the 65% and 95% SP-reduced MCC. Shown are mean values (n=3) ± 1 SD

	Numerical constants			
MCC type	**a_1** (Pa·s)	**a_2** (Ø)	**a_3** (J/mol)	**a_4** (J/mol)
65% SP-reduced MCC	$2.1\times10^{-4} \pm 8.5\times10^{-5}$	-1.07 ± 0.06	4078 ± 1045	3476 ± 194
95% SP-reduced MCC	$5.0\times10^{-5} \pm 3.1\times10^{-5}$	-0.86 ± 0.15	8591 ± 2137	2955 ± 400

In order to evaluate the fit of the developed model, a regression analysis of the measured vs. predicted values of η_{100} was performed, both for the 95% SP-reduced and for the 65% SP-reduced MCC. The model was found to predict apparent viscosity very well in the range of tested concentrations and temperatures, with $R^2 = 98\%$ for the 95% SP-reduced MCC (Figure 3.6a) and $R^2 = 96\%$ for the 65% SP-reduced MCC (Figure 3.6b). Since slight differences in viscosity can occur due to minor fluctuations in composition of testing conditions, from a practical perspective it is more useful to predict the order of magnitude of viscosity than the actual values of viscosity. For this reason, the fit of the model was evaluated by plotting the logarithm of viscosity instead of the actual viscosity values in Figure 3.6. Nonetheless, the regression parameters were almost the same when comparing the actual viscosity values (data not shown).

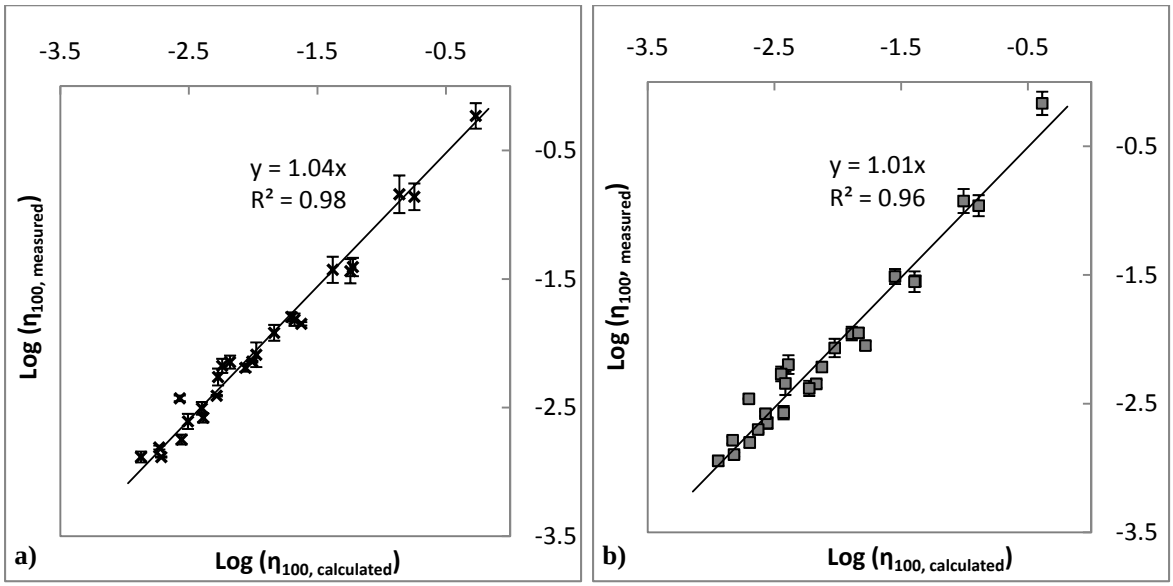

Figure 3.6. Model fit: measured vs. calculated log(η_{100}) values for a) 95% SP-reduced MCC and b) 65% SP-reduced MCC. Plotted are mean values (n=3) ± 1 SD.

Furthermore, the model was validated by generating a set of experimental viscosity data that was not used in the development of the model. For the 95% SP-reduced MCC, the validation was conducted for the following samples: 6% casein concentration at 30°C and 70°C, 9% casein concentration at 0°C, and 13% casein concentration at 0°C, 10°C and 50°C. For the 65% SP-reduced MCC, the validation was

conducted for 4% casein concentration at 30°C and 70°C, and 11% casein concentration at 10°C and 30°C. The predicted vs. measured η values are shown in Figure 3.7. The slope of the regression line of $\log(\eta_{100,\,measured})$ vs. $\log(\eta_{100,\,predicted})$ is basically 1 and the correlation coefficient is very close to 1 for both types of MCC, which demonstrates the strength of the model in predicting the apparent viscosity of MCC dispersions for a wide range of shear rates, casein concentrations and temperatures.

Although η_{100} is the most relevant for a range of practical applications, it may be useful for certain situations to be able to predict viscosity at other shear rates. This can be easily done for each temperature, concentration and MCC type by using the predicted values for η_{100} and the value of the flow index *n*.

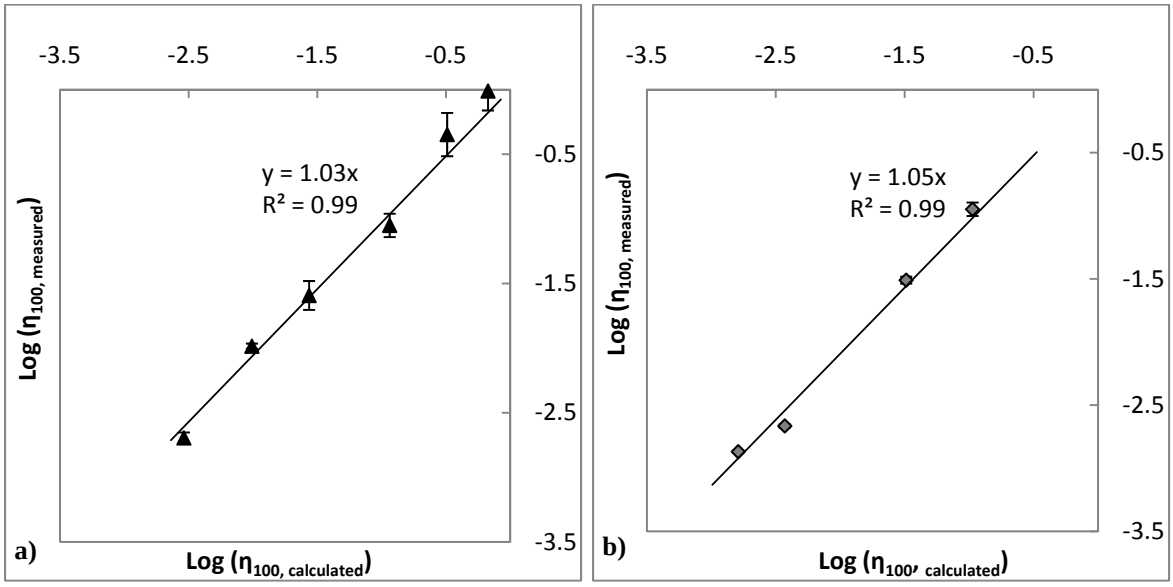

Figure 3.7. Model validation: measured vs. calculated $\log(\eta_{100})$ values for a) 95% SP-reduced MCC and b) 65% SP-reduced MCC. Plotted are mean values (n=3) ± 1 SD.

Since the values for yield stress were very small, as a simplifying assumption it can be considered that the MCC have a power law behavior. After considering $\sigma_0 = 0$ in Eq. 3.1, the equation for the apparent viscosity can be re-written as:

$$\eta_{\dot{\gamma}} = \kappa(\dot{\gamma})^{n-1} \quad \text{Eq. 3.5}$$

where $\eta_{\dot{\gamma}}$ is the apparent viscosity at any shear rate ($\dot{\gamma}$) > 0, n is the flow behavior index and κ is the consistency coefficient (Pa·s^n).

At $\dot{\gamma}$ = 100 s^{-1}, Eq. 3.5 will become:

$$\eta_{100} = \kappa(100)^{n-1} \quad \text{Eq. 3.6}$$

By combining Eq. 3.5 and Eq. 3.6, the following equation will be obtained:

$$\eta_{\dot{\gamma}} = \eta_{100}\left(\frac{\dot{\gamma}}{100}\right)^{n-1} \quad \text{Eq. 3.7}$$

Further, by substituting Eq. 3.4 in Eq. 3.7, an equation that will allow the prediction of apparent viscosity at any shear rate > 0, temperature and concentration will be obtained:

$$\eta_{(\dot{\gamma},T,C)} = a_1 e^{\left(a_2 C + \frac{a_3 + a_4 C}{RT}\right)}\left(\frac{\dot{\gamma}}{100}\right)^{n-1} \quad \text{Eq. 3.8}$$

where $\eta_{(\dot{\gamma},T,C)}$ is the apparent viscosity at any shear rate ($\dot{\gamma}$) > 0 s^{-1}, for the temperature (T) and concentration (C) ranges investigated in this study.

To test the accuracy of this model, values for apparent viscosity for the 95% SP-reduced MCC dispersions of 12.5% casein concentration in the range of shear rates from 1-631 s^{-1} were predicted using eq. 3.8 and plotted against experimentally obtained values (Figure 3.8). The fit for the data across all shear rates and temperatures was very good (R^2 = 99%). The model was able to predict very well the order of magnitude of viscosity, with the slope of the regression line for the measured vs. calculated $\log(\eta_{(\dot{\gamma},T,C)})$ being very close to 1 (see example for 12.5% in Figure 3.8a). The model resulted in a slight underestimation of the actual viscosity values though. The most significant underestimation, of over 20%, occurred for the 12.5% MCC (see example in Figure 3.8b), while for the lower concentrations the underestimation was smaller than 10% (data not shown).

Nonetheless, as mentioned before, this model is extremely powerful and accurate for estimating the order of magnitude of viscosity, which makes it extremely useful for designing application of the types of MCC evaluated in this study.

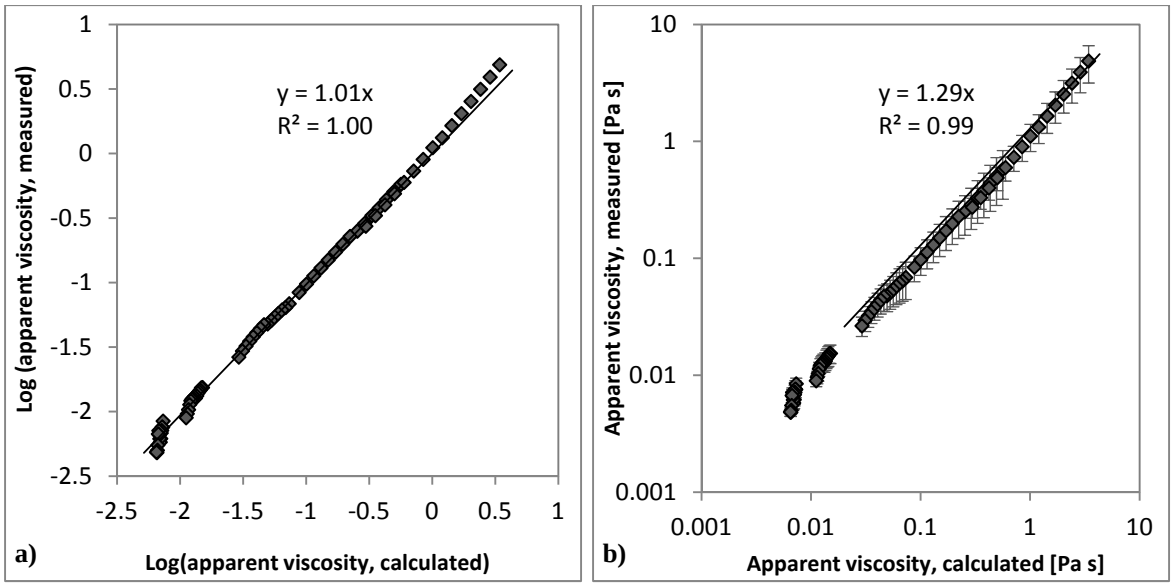

Figure 3.8. General model fit: measured vs. calculated a) log(η_{app}); b) η_{app} values for 95% SP-reduced MCC of casein concentration 12.5%, for shear rates between 1-631 s^{-1} and temperatures between 0-80°C.

Conclusions

The study showed that the apparent viscosity of MCC increased with concentration and decreased with temperature, and at casein concentrations ≥ 7.5% the MCC started showing a clear shear-thinning behavior. The viscosity of the micellar casein preparations was affected by the SP removal, with higher SP-reduction leading to higher viscosity in dispersions with the same casein concentrations. A modified Arrhenius model that incorporates the temperature and concentration dependencies was developed, and is able to predict very accurately MCC apparent viscosity for a wide range of shear rates, temperatures and concentrations of relevance for the practical use of these casein ingredients. The rheological data generated in this study and the predictive model developed will provide the dairy and food industry with critical data necessary for developing applications of micellar casein preparations.

Acknowledgements

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CHAPTER 4

STABILITY OF STERILIZED MICELLAR CASEIN CONCENTRATES DURING STORAGE

Abstract

The use of micellar casein concentrates (MCC) obtained by membrane separation is receiving increasing interest from the dairy industry. One of the most significant potential applications of MCC is its use in shelf-stable beverages. Since protein based beverages tend to show sedimentation during prolonged storage, this work aimed at evaluating the stability, particularly the occurrence of aggregation and sedimentation, in sterilized MCC during storage at 25°C.

MCC with casein concentration ranging from 5-10% were subjected to continuous-flow UHT treatment and in-container retorting, and subsequently stored for 8 weeks at 25°C. As control, non-heat treated MCC with added preservative was used. Sedimentation was evaluated by measuring the protein content in the bottom layer of the storage containers.

Control samples were stable for up to 8 weeks, with no significant sedimentation observed, while retorted samples showed significant sedimentation, with up to 22% increase of protein concentration in the bottom layer of the container. For the retorted samples, sedimentation was the least pronounced in the 10% MCC samples, and most pronounced in the 5% MCC samples. Very strong sedimentation was observed in all UHT-treated samples. In order to estimate sedimentation over prolonged storage, sedimentation kinetics was established for all samples. The rate of sedimentation for 10% MCC ranged from 0.02% protein/day to 0.06% protein/day.

It was concluded that UHT-treated MCC preparations were unstable during storage, and thus may require additional stabilization to increase their storage stability, while the retorted preparations were relatively stable. The results of this study provide valuable information about the storage stability of sterilized MCC obtained by membrane filtration, which can be used for the manufacture of shelf-stable, protein-rich beverages.

Introduction

The use of casein preparations obtained by membrane filtration is receiving increasing interest from the dairy industry, as well as other industries (Affertsholt, 2009). Casein is used as an ingredient because of its nutritive value as well as its physico-chemical and functional properties. It is used in different applications, such as various dairy foods, bakery products, confectionaries and beverages (Mulvihill and Ennis, 2003). Traditionally casein has been obtained by methods like acidification, renneting or co-precipitation which involve a chemical modification or break-down of the casein micelle. In casein concentrates obtained by membrane filtration, the casein micelle is closer to its native state and up to 95% of the serum proteins and lactose have been removed (Nelson and Barbano, 2005). These differences result in different functionality and open further fields for possible applications of the casein concentrates. Regardless of the application however, one important aspect of any food ingredient is its heat stability, as well as its stability over the time of storage. Specifically beverages usually undergo a sterilization step during processing that involves high temperature heat treatments to produce a product that is shelf-stable without refrigeration.

For normal and concentrated milks, heat stability refers to the ability of milk to withstand high processing temperatures without visible coagulation or gelation (Singh, 2004). For shelf-stable beverages, stability must also refer to the effects of sedimentation and aggregation over the extended time of storage.

Casein micelles exhibit great stability against high heat treatments, cooling and drying, which is mainly credited to their electrostatic repulsion, as well as their steric hindrance due to the κ-casein on the surface of the micelles (de Kruif and Holt, 2003). Yet, sedimentation of casein micelles after heat-treatment is still to be expected. Dalgleish (1992) showed that in concentrated milks even unaltered casein micelles would settle during extended time of storage under the force of gravity. The deposition of insoluble calcium phosphate onto the surface of the casein micelle after UHT treatment would increase the micelle weight and thus cause more rapid sedimentation (Wahlgren et al., 1990; Dalgleish, 1992). Additionally the aggregation of casein micelles, leading to a bigger radius as well as higher molecular weight, would speed up sedimentation. Dalgleish et al. (1987) observed an initial increase in micelle size during heating at 130°C, after which it remained constant until just before the onset of coagulation. Guthy et al. (1983) reported aggregation of casein micelles in directly and indirectly UHT-treated skim milks over the time of storage, leading up to age gelation, which they credited to the activity of proteolytic enzymes that survived UHT treatment. Furthermore, heat-induced polymerization of serum protein-free casein micelle dispersions during heat treatment, as well as during storage have been reported (Zin El-Din et al., 1991; Zin El-Din and Aoki, 1993). Lactose, when present, was considered to be the main constituent responsible for polymerization, and cross-linking through Maillard reaction has been established as cause (Andrews, 1975). Without lactose, the formation of thermally generated colloidal calcium phosphate linkages have been suggested in the formation of micelle aggregates (Singh, 1994).

Jelen et al. (1987) examined commercially available whey-based beverages and concluded that partially strong sedimentation might have been one issue in the failed success of the first generation whey drinks. To overcome sedimentation problems, acidified milk beverages are often stabilized with pectins or other food hydrocolloids (Tromp et al., 2004).

The study by Beliciu et al. (2012) focused on evaluating the effects of two commercial sterilization regimes (continuous-flow UHT treatment and in-container retorting) and spray-drying on the stability and physical properties of micellar casein concentrates (MCC) during thermal processing. Retorting resulted in a visually homogeneous product with a lower viscosity and significantly higher particle sizes than the untreated MCC, while UHT treatment caused coagulation and increased viscosity. Spray-drying of MCC further accentuated these instabilities during heat treatment. Beliciu et al. (2012) and Sauer and Moraru (2012) established that the main factors responsible for these instabilities are mineral redistribution and micelle casein dissociation as a result of the heat treatment

In addition to stability during thermal processing, the shelf stability of the sterilized products is also very important. Often times protein based beverages tend to show sedimentation during prolonged storage, which is undesirable for the consumer and consequently will affect the marketability of the products. Therefore, the main objective of this study evaluates the stability and sedimentation behavior of the sterilized MCC during eight weeks of storage at 25°C. The results of this study will provide valuable information about the storage stability of heat-treated MCC obtained by membrane filtration, which will be of interest particularly for the manufacture of shelf-stable, casein-rich beverages.

Materials and Methods

Production and heat treatment of micellar casein concentrates

Micellar casein concentrates were obtained by membrane filtration in the pilot plant at Cornell University. Pasteurized skim milk was subjected to ultrafiltration (UF) at 50°C using a polymeric spiral-wound membrane with a molecular weight cutoff of 10,000 Da, followed by four stages of microfiltration (MF) in order to obtain a low lactose, 98% serum protein-reduced MF retentate with a true protein content above 10%. The MF was performed at 50°C using a graded permeability (GP) ceramic membrane with a nominal pore size of 0.1 µm. The 4^{th} stage MF retentate was diluted with RO

water to micellar casein concentrates (MCC) of four fixed casein concentrations (5%, 7.5%, 8% and 10%). A portion of the MCC was spray dried and reconstituted to a casein concentration of 8% (referred to as R-MCC).

Each of the MCC dilutions and the R-MCC was split into 3 portions: one untreated control, one to be heat treated by continuous-flow UHT, and one to be batch sterilized by retorting.

The UHT treatment was performed on a pilot scale Microthermics heating system (Model 25 HV, Microthermics, Inc., Raleigh, NC), equipped with an Ultra Clean Fill Hood/Sterile Product Outlet, using the following treatment parameters: pre-heating of product to 90°C (194°F) in the first heater within 20 seconds, final heating of the product to 143.3°C (290°F) within 20 seconds in the final heater, holding at 141.7°C (287°F) for 3 seconds, and cooling to 38.3°C (101°F) within 20 seconds at a flow rate of 2 L/min.

A FMC Multipurpose Lab Retort with LogTec Process Management System (Steritort, FMC, San Jose, CA) was used to batch-sterilize the casein concentrates in 8 oz (237 mL) Mason jars (Ball Corporation, Broomfield, CO). The product was treated at 250°F (121.1°C) and a steam pressure of 30 psi (206.8 kPa), in a still cook mode with cascading hot water. The temperature profile included a come-up step (17.5 minutes, product reached 118.9°C (246°F)), a cooking step (9 min, average temperature of 120°C (248°F)) and a cooling step (10 min, final temperature 41.1°C (106°F)).

All casein concentrates were quickly cooled and stored at 4°C overnight for sample preparation.

A more detailed description of the production, preparation and heat treatments of the casein preparations can be found in Beliciu et al. (2012).

Sample preparation

Three liters of each MCC and R-MCC preparation was pooled and 0.02% wt/wt Broad Spectrum Microtabs II (D&F Control System, Inc., Norwood, MA), a combination of Bronopol and Natamycin, was added to prevent the growth of bacteria, yeast and mold. The mixture was placed on a magnetic

stirplate and stirred throughout the process of filling. 60 mL of preparation was filled into sterile AirTite Norm-Ject syringes and the syringes were capped with sterile Luer-Lok caps (both Fisher Scientific, Pittsburgh, PA) and placed vertically into 30 mm test tube racks, which were stored in a temperature-controlled chamber at 25°C for up to 8 weeks. Due to the strong flocculation during the UHT treatments the pooled UHT samples were mixed with an UltraTurrax T25 homogenizer (IKA Works Inc., Wilmington, NC) at 21,500 rpm for 5 min prior to adding the antimicrobial.

Sampling

To gain insight into the sedimentation of micellar casein preparations over time, a total of five layers from the fluid column of each syringe were separated during sampling. For each sample five 130 mL flat bottom glass tubes (Pyrex, from Fisher Scientific, Pittsburgh, PA) were labeled 1 through 5 and placed on a laboratory scale. From each syringe the first 12 g were collected into the first test tube (i.e. layer 1 = bottom layer), the second 12 g were collected into the second test tube (i.e. layer 2) etc. until all 60 mL from one syringe were collected. Five syringes were sampled in total each week to collect 60 g per layer. About 35 mL were used for IR analyses and the rest of the sample was used for particle size measurements (on bottom layer only), brix measurements and pH (on bottom layer only). While data for all five layers (where applicable) are available, the data shown and discussed is data for layer 1 (i.e. the bottom layer), unless noted otherwise, as most of the changes that happened during storage are reflected in that layer.

Chemical and instrumental analyses

IR analysis. Fourier transformation infrared spectrophotometry (FTIR) analysis was used to determine the true protein, fat and lactose content in all samples. The analysis was performed by D.M. Barbano's research group (Northeast Dairy Foods Research Center, Department of Food Science, Cornell University, Ithaca, NY) according to Kaylegian et al. (2006). The MCC samples were preheated to 37°C in

45 mL polypropylene sample vials (Fisher Scientific, Pittsburgh, PA) and subsequently analyzed using a Lactoscope FTIR (Delta Instruments, Drachten, Netherlands). Due to the storage in syringes and the sampling in layers a higher amount of fat was present in the top layers of the product. Therefore the true protein content was reported on the basis of the skim portion. All measurements were done in duplicate.

To verify the FTIR readings from , total nitrogen (TN) measurements were also conducted on one sample set of replicate 3 over the time of storage using Kjeldahl analysis (AOAC, 2005; method 991.20). The non-protein nitrogen (NPN) fraction of the MCC was analyzed in the MCC prior to drying (which represents the 4^{th} stage MF retentate) ((AOAC, 2005); method 991.21). This analysis was conducted for replicate 3 of MCC manufacture. The MCC (4^{th} stage MF retentate) had 11.15% true protein content, and 0.13% NPN. As the NPN fraction was not expected to change during storage, the value was used to calculate the true protein content of the samples as (TN x 6.38 – 0.13) (%).

Refractometric analysis. Due to the presence of sedimentation and high viscosity of some samples, IR analyses could not be performed on all samples. To allow for a quantification of sedimentation a refractometric analysis was run on all samples using an r² mini refractometer (Reichert Analytical Instruments, Depew, NY). The refractometer was calibrated with distilled water every ten measurements and cleaned according to the manufacturer's instructions prior to every measurement. Samples were tempered to 20°C in a water bath and inverted three times until well homogenized, then about 500 µL of sample was placed on the measuring surface of the refractometer. If the sample was too thick to be transferred with a pipette, the vial was stirred and a small amount of sample was placed on the measuring surface using a spatula. The refractometric measurements were performed in duplicate.

Particle size analysis. Dynamic light scattering (DLS) was used to evaluate particle size and particle size distributions in all samples following the procedure described in detail by Beliciu et al. (2012). The

analyses were performed using a 90Plus Nanoparticle Size Analyzer equipped with a Peltier temperature control system (Brookhaven Instruments Corp., Holtsville, NY), at a fixed 90° angle and a wavelength of 658 nm.

Samples were equilibrated to 20°C in a water bath prior to analysis, with proper protection against dehydration. The samples where then diluted in UF permeate to the recommended signal intensity of 700 – 900 kilocounts per second. The UF permeate, obtained during the manufacture of the MCC, was kept frozen at -40°C and placed in a fridge at 4°C four days prior to analysis for thawing. The particle size measurements were performed at a constant temperature of 20°C, following an equilibration step.

Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp.). A dust cutoff filter parameter of 30 was employed to improve the quality of the measurements. Each measurement consisted of 8 subsequent individual runs of 30 s duration and the relative particle size distribution, the intensity weighted effective diameter and the polydispersity index were determined. Measurements were performed in duplicate.

pH measurements

The pH was measured in the bottom layer of the samples, unless otherwise indicated, at 20°C using a Fisher Scientific accumet Excel XL20 pH meter. The pH meter was calibrated at the measurement temperature prior to use (Fisher Scientific, Pittsburgh, PA).

Colorimetric analysis

The color of samples after processing was measured in the Yxy color space using a Chroma Meter CR-400 (Konica Minolta Sensing Americas, Inc., Ramsey, NJ). Samples were poured in 15 mL disposable sampling cups, excess was wiped off and the sampling head was placed vertically above the sample as described in the manufacturer's manual. The measurements were done in triplicate.

To allow for easier comparison of the data the Whiteness Index (WI) was calculated using the following formula, given by the instrument's manufacturer:

$$WI = Y + 800(0.3131 - x) + 1700(0.3191 - y) \quad \text{Eq. 4.1}$$

with Y, x, y being the measurements from the instrument, and 0.3131 and 0.3191 being chromatic coordinates of the used calibration white.

Statistical analysis

Data was analyzed by mixed-model analysis using JMP software (8.0, SAS Institute, Cary, NC). The model included fixed effects (product source, casein concentration, treatment and week) and random effects (replicate, replicate x casein concentration, replicate x casein concentration x treatment, replicate x product source, replicate x product source x treatment), depending on the parameters tested. Similar tests were performed in order to assess the effect of experimental replication; in these cases the randomized variable was casein concentration, which was then crossed with the other variables. Statistical differences between values of different parameters were determined using the Tukey-Kramer honestly significant difference (HSD) test. Differences with $p \leq 0.05$ were considered statistically significant.

Results and Discussion

Correlation of IR data with refractometric measurements

The formation of large aggregates during the UHT treatment of MCC, as well as the sedimentation over time led to a high viscosity in some samples (Beliciu et al., 2012), which prevented the use of IR analysis for testing of some of the MCC samples. To quantify the protein content and thus quantitatively evaluate the rate of sedimentation in those samples, refractometric analysis was performed on all samples in addition to IR analysis.

The value for soluble total solids (in °Brix), measured with a refractometer, was plotted vs. true protein (TP) values obtained using IR analysis, for all MCC samples in week 1, and a linear trendline was fitted (Figure 4.1). The established correlation that 1.47°Brix = 1% TP allowed the estimation of the true protein content of a MCC sample from the refractometric data.

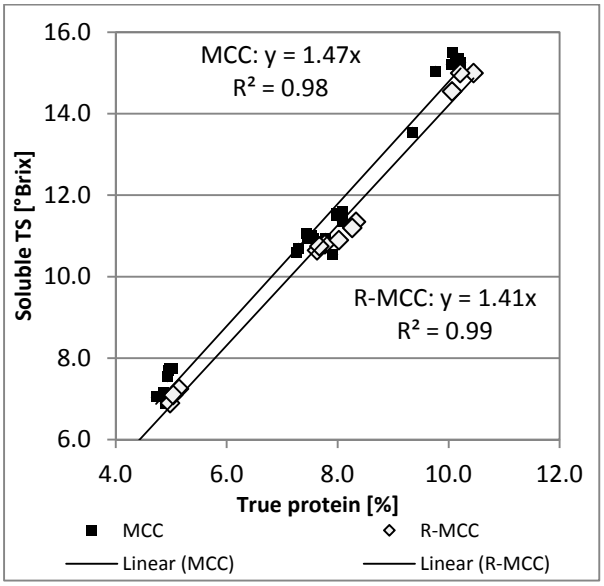

Figure 4.1. Correlation of true protein (IR data) with soluble total solids (refractometric data) for MCC and R-MCC samples in week 1. *Note: Data for MCC samples include control and heat-treated samples. Data for R-MCC samples includes control samples only.*

As establishing a correlation for R-MCC was difficult for cases with only the one concentration used in the study, additional reconstituted samples of concentrations of 2.5%, 5%, 7.5% and 10% were prepared, following the procedure described elsewhere (Sauer et al., 2012). No heat treatments were performed on those samples. The generated data was analyzed as described above, and a correlation of 1.41°Brix = 1% TP was established for R-MCC.

As the correlations of data for TP with soluble TS data was very good (R^2 equal to 98% and 99% for MCC and R-MCC, respectively), the refractometric data was subsequently used to estimate true protein content, whenever IR data was not available. It should be noted however, that the IR milk analyzer used in this study was calibrated for unpreserved standard milks, not for concentrated milk protein

preparations. To obtain accurate results, the IR should ideally be calibrated with calibration samples that resemble the composition of the samples to the tested (Kaylegian et al., 2006; Barbano et al., 2010). As this was not possible, the direct readings from the IR obtained in this study might underestimate the actual protein content of the samples. Nonetheless, since this would lead to a systematic error, the relative values over the time of storage and thus the observed sedimentation kinetics will not be affected.

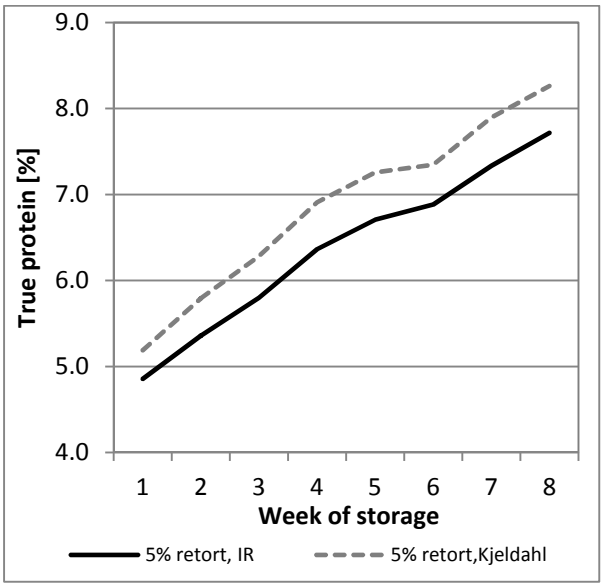

Figure 4.2. Comparison of IR and Kjeldahl data for the measurement of true protein content (%) in the bottom layer of the 5% R-MCC sample of replicate 3 over the time of storage at 25°C.

To evaluate the magnitude of the underestimation of the IR readings, the true protein content of the retorted 5% MCC samples of replicate 3 was additionally analyzed by Kjeldahl analysis over the time of storage (Figure 4.2). The Kjeldahl data was consistently higher than the IR data. The calculated overestimation of the true protein content measured by Kjeldahl in comparison to IR increased over the time of storage, from 0.26% in week 1 to 0.49% in week 7. Kjeldahl analysis itself has been shown to underestimate the casein content in casein concentrates (Hurt and Barbano, 2010), although no information could be found in the literature about the comparison of true protein content. This challenge could not be overcome, due to the fact that both IR and Kjeldahl are standard methods for

normal milks, not concentrated milk protein concentrates, for which standard methods have yet to be developed.

Composition of MCC and R-MCC at the beginning of storage

The composition of the untreated MCC and R-MCC is given in Table 4.1. All samples had a very low lactose content, which was due to the UF step as well as the diafiltration with RO water in between MF stages during the production of the MCC. A higher pH in the lower concentrations of the MCC samples was noted, which was attributed to the pH of the RO water (pH of 7.65 at 20°C), in which the final MF retentate was diluted to achieve the desired casein concentrations. The chemical composition of the final MF retentate prior to dilution can be found in Beliciu et al. (2012). The composition of the R-MCC samples is similar, with a slightly lower pH than the MCC samples of the same casein concentration.

Table 4.1. Composition of untreated MCC and R-MCC at the beginning of storage (week 1). Calculated are averages of three replicates ± one standard deviation.

	Composition			
	protein (%)	lactose (%)	fat (%)	pH
5% MCC	4.98 ± 0.05	0.14 ± 0.02	0.21 ± 0.01	7.16 ± 0.07
7.5% MCC	7.50 ± 0.05	0.14 ± 0.02	0.25 ± 0.01	7.12 ± 0.10
8% MCC	8.05 ± 0.03	0.15 ± 0.02	0.26 ± 0.01	7.10 ± 0.08
8% R-MCC	8.08 ± 0.05	0.14 ± 0.01	0.26 ± 0.01	7.04 ± 0.02
10% MCC	10.15 ± 0.05	0.15 ± 0.02	0.30 ± 0.02	7.09 ± 0.05

The true protein content measured in the retorted MCC was slightly lower than in untreated MCC (Figure 4.3). The measured protein content in the retorted MCC decreased with increasing protein concentration, but this decrease was not proportional with the casein concentration of the MCC. A 1.3% decrease was measured in the 5% samples; in the 10% samples a decrease of 2.3% was observed.

As the coagulation and aggregation that occurred during the UHT treatment did not allow for analysis using IR, the protein content of the samples was estimated using the established correlation with the refractometric data. There was strong variability between replicates for the UHT treatment, as apparent by the large standard deviations in Figure 4.3. In replicate 1 some flocculation occurred and the measured protein content in the samples decreased by about 3% in the 5%, 7.5% and 8% MCC samples and by about 10% in the 10% MCC samples. In replicate 2 the measured true protein content had decreased by about 5%, 30%, 23% and 42% for the 5%, 7.5%, 8% and 10% MCC samples, respectively. Replicate 3 accounted for most of the variability as the samples showed the highest levels of flocculation with a change of protein content of over 50% in all UHT-treated MCC samples.

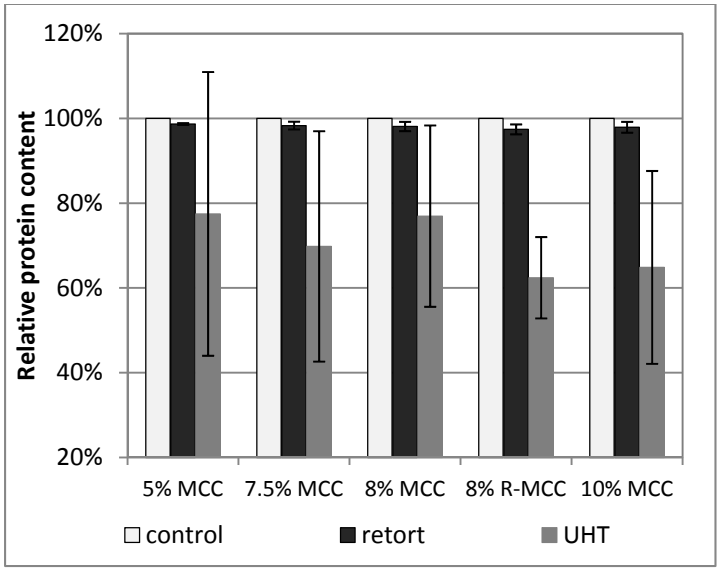

Figure 4.3. Relative change of protein content in MCC and R-MCC samples after heat treatment (at beginning of storage) in comparison to the untreated control samples. Plotted are mean values (n=3), error bars represent one standard deviation.

A similar pattern could be seen in the R-MCC samples. The measured protein content after retorting and UHT treatment decreased by 2.5% and 38%, respectively, but the variability between replicates was lower than in the MCC samples.

Differences in the color of the untreated and treated MCC samples at the beginning of storage were assessed using the Whiteness Index (WI) (Eq. 4.1). WI was chosen for simplicity, as it is easier to compare one value rather than three color scale parameters. The untreated MCC samples showed significantly higher WI values than the retorted and UHT-treated MCC samples (Figure 4.4). Within the untreated control samples the WI was highest for the 5% MCC samples and was decreasing for increasing concentrations. The lower WI in the UHT-treated samples can mostly be credited to the strong flocculation, which made the measuring surface of the samples uneven and therefore might have corrupted the readings. Browning in retort sterilized milk occurs due to Maillard reaction of lactose and proteins (Patton, 1955). Even though the lactose content in the MCC is below 0.2% in all concentrations, browning still occurred in the retorted MCC samples, leading to significantly lower WI than in the untreated MCC. The relatively large standard deviation of the WI for the 7.5%, 8% and 10% retorted MCC is caused by significantly lower WI of those samples in replicate 3 as compared to replicates 1 and 2. This could be explained by the lower pH of the 4^{th} stage MF retentate during the production of micellar casein in replicate 3, as a lower pH has been shown to increase Maillard reaction (Patton, 1955).

The WI of the R-MCC showed a similar trend as described for the MCC samples, but exhibited significantly lower values overall, which was expected as during spray-drying the powder undergoes a strong heat treatment which facilitates Maillard reaction (Patton, 1955).

The significantly lower WI caused by browning of the retorted MCC and R-MCC was surprising, given that the lactose content of the casein concentrates was so low. While data about the lactose content for most of the samples over the time of storage is available, not much information can be inferred from it, as the values are below the reliable detection limit for the IR instrument.

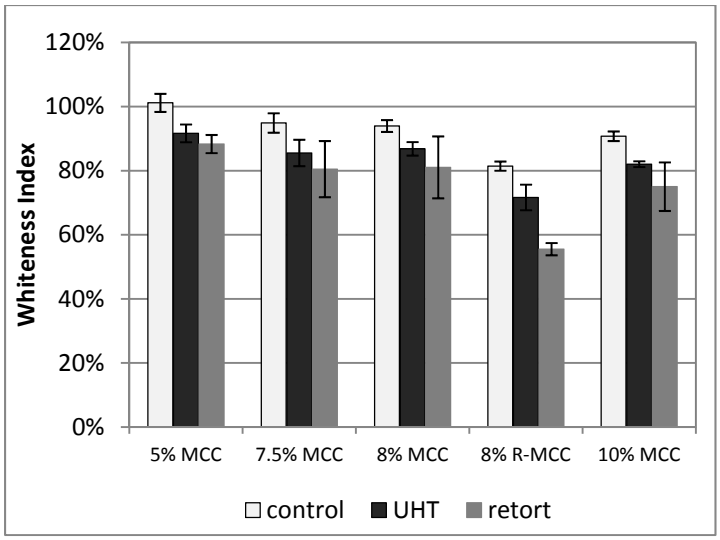

Figure 4.4. Whiteness Index (WI) of control and heat-treated MCC and R-MCC samples. Plotted are mean values (n=3), error bars represent one standard deviation.

Changes in MCC during storage

pH

The pH of the MCC samples over the time of storage at 25°C is plotted in Figure 4.5. These values represent averages of the three experimental replicates, but it is important to note that the pH of all samples from replicate 3 was consistently lower than the pH values of replicates 1 and 2. At the beginning of storage, the pH of the control and UHT-treated MCC samples was comparable, while the pH of the retorted samples was significantly lower by 0.14 pH units. Decreases of pH by 0.2-0.3 units in milk with full lactose content after retorting have been reported in the literature (Berg and Boekel, 1994). Pyne and McHenry (1955) proposed three different causes for decreases in pH during extended high temperature treatments of milk: thermal oxidation of lactose to organic acids, which accounts for 50% of the pH decrease, dephosphorylation of casein, which contributes up to 30% of the decrease in pH, and the precipitation of tertiary calcium phosphate with a concomitant release of protons. Given

that the lactose content of the MCC preparations is very low, most of the pH decrease can be accredited to the last two mechanisms.

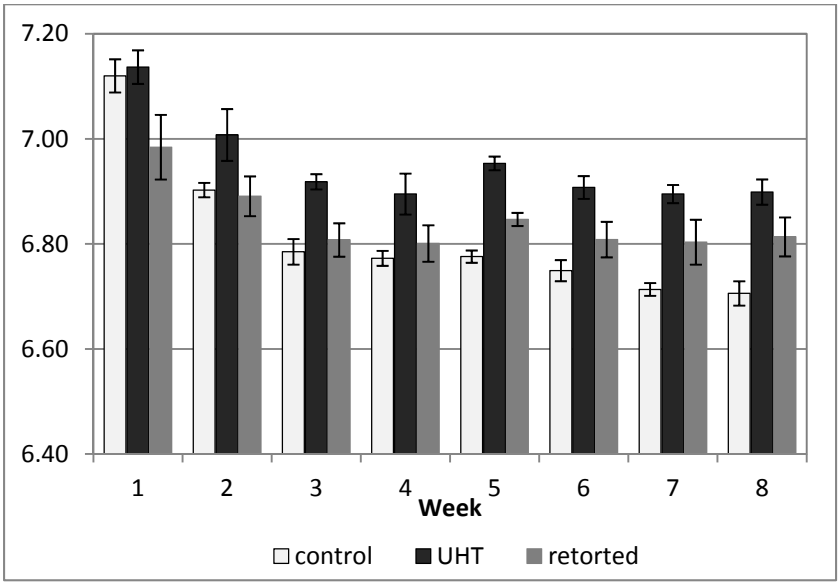

Figure 4.5. pH of MCC over time of storage at 25°C. Plotted values represent averages for all four concentrations and three replicates, error bars represent one standard deviation. *Notes: Measurements for control and retort samples were done on bottom layer, measurements for UHT samples were done on layer 3 beginning in week 2. No measurements could be performed on samples (8%, UHT, rep1, week 8) and (5%, UHT, rep2, week 8) due to UHT age gelation.*

After weeks 1 and 2, significant drops of pH in all MCC samples were observed, which were especially pronounced in the control (untreated) samples. The pH in the control samples decreased by 0.33 pH units to a pH of 6.79 within the first two weeks of storage. From week 2 onwards there were significant differences in the pH of the differently treated MCC samples, with the $pH_{UHT} > pH_{retort} >$ control. Starting in week 3, the pH of the control and retorted samples stayed relatively constant for the rest of the storage time, while the pH of the UHT samples increased up to week 5 and decreased again after that. Over the eight weeks of storage, the pH in the MCC dropped on average by 0.41, 0.24 and 0.17 pH units for the control, UHT and retorted samples, respectively.

Overall the pH of the 5% MCC was higher than the pH of the samples of the other concentrations, which was attributed to the higher amount of RO water used to dilute the samples to this concentration.

The pH of the R-MCC samples over the time of storage was lower than the pH of the MCC samples, but their trend throughout storage was similar. One difference noted was that the UHT-treated R-MCC samples did not show an increase of pH up to week 5, but rather stayed leveled after week 2 for the rest of the storage time (data not shown).

Particle size

Particle aggregation in the MCC preparations over the time of storage was evaluated by determining the effective particle diameter in samples from the bottom layer of each untreated and retorted MCC and R-MCC sample. Accurate particle size data of UHT-treated samples could not be obtained, due to the visibly large aggregates and coagulates formed during the heat treatment that would have caused erroneous readings (Beliciu et al., 2012).

The average diameter of the particles in the untreated MCC for all replicates at the beginning of shelf life was about 165 nm (Figure 4.6), which is about the size of casein micelles, as previously reported (Beliciu and Moraru, 2009). There were no significant differences of particle size between the different concentrations of the untreated MCC samples in week 1 and 2. Beginning in week 3 the effective diameter of the particles in the 10% MCC samples was significantly larger than in the other concentrations. The particle size steadily increased to a maximum size of 403 nm in week 5, after which it decreased again. The 7.5% and 8% MCC samples showed the same trend with a maximum particle size of 240 nm and 257 nm, respectively. The untreated 5% MCC exhibited the overall smallest particle sizes with a maximum particle size of 233 nm in week 7.

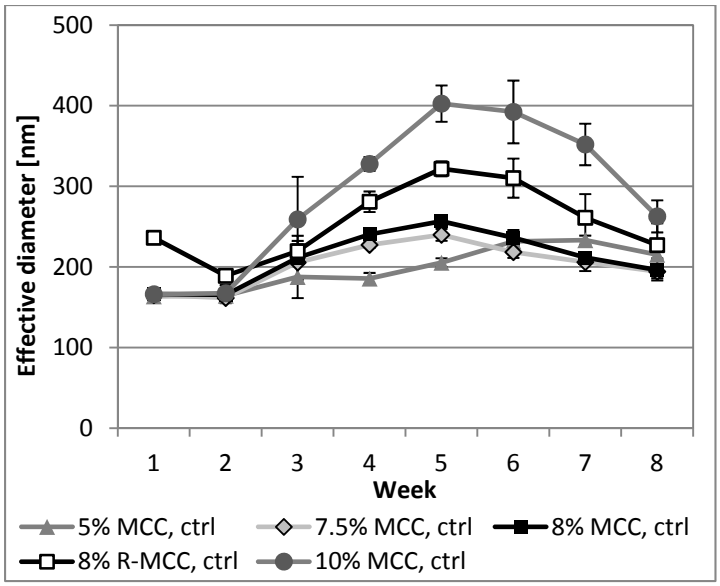

Figure 4.6. Particle size of untreated MCC and R-MCC samples in the bottom layer over time of storage at 25°C. Plotted are mean values (n=3), error bars represent one standard deviation.

In the retorted MCC, there were no significant differences in particle size in samples of the four different concentrations, and no changes were observed over the time of storage (data not shown). However significant differences among replicates were observed. The MCC samples of all concentrations of replicates 1 and 2 were comparable, with an average effective diameter of about 180 nm, which was significantly higher than the initial particle size of the untreated MCC. The average effective diameter of the retorted samples of replicate 3 was 326 nm, which was significantly larger than replicates 1 and 2.

The overall trend in the 8% R-MCC samples was comparable to the 8% MCC samples, which indicated that spray drying did not affect aggregation trends in reconstituted casein concentrates. The untreated R-MCC exhibited a maximum of the particle size in week 5, while the retorted samples showed no significant changes of size throughout storage (data not shown). Yet in the untreated R-MCC samples the behavior during the first two weeks of storage was different compared to the MCC samples. The particle size of the control 8% R-MCC in the first week of storage was about 236 nm, which is significantly higher than the initial size of particles in the 8% MCC. The particle size in the untreated R-MCC dropped to 189 nm in the second week, while there had been no significant change in size within

the first two weeks in the MCC samples. After the second week of storage the particle size in the R-MCC started to increase to a maximum particle size of about 322 nm in week 5, which is significantly higher than the particle size of the untreated 8% MCC samples.

No significant differences between replicates were noted for the retorted R-MCC. The retorted 8% R-MCC samples had a mean particle size of about 215 nm throughout the time of storage, which was significantly bigger than the particle size of the retorted MCC samples of replicates 1 and 2, but significantly smaller than the particle size observed in retorted MCC samples of replicate 3.

Sedimentation

Sedimentation was expressed quantitatively as the change of true protein content in the bottom layer of the stored syringes using IR data where possible. When no IR data was available, due to coagulation or sedimentation of the samples, the estimated protein content calculated based on the refractometric measurements were used instead.

The untreated, preserved MCC exhibited very little sedimentation over the time of storage (Figure 4.7). There was some variation between replicates 1 and 2 in comparison to replicate 3, but on average the increase in protein content in the bottom layer was lower than 5% throughout the time of storage. In most weeks the 5% MCC exhibited the largest degree of sedimentation, while the 10% MCC showed the least. Overall the control samples could be described as stable in terms of protein sedimentation behavior.

The retorted MCC showed sedimentation over the time of storage with strong variation between replicates. Replicate 1 and 2 were comparable with maximum sedimentation occurring in the 5% MCC samples in week 8 (about 22% increase of protein content in the bottom layer). In replicate 3, strong sedimentation in all the retorted MCC samples was observed. The change in protein in the 5% MCC samples increased almost linearly with an almost 10% increase after each week, reaching a maximum of

59% of change of protein in the bottom layer in week 8. The 10% MCC samples increased with a much smaller rate and exhibited the overall lowest sedimentation at the end of storage.

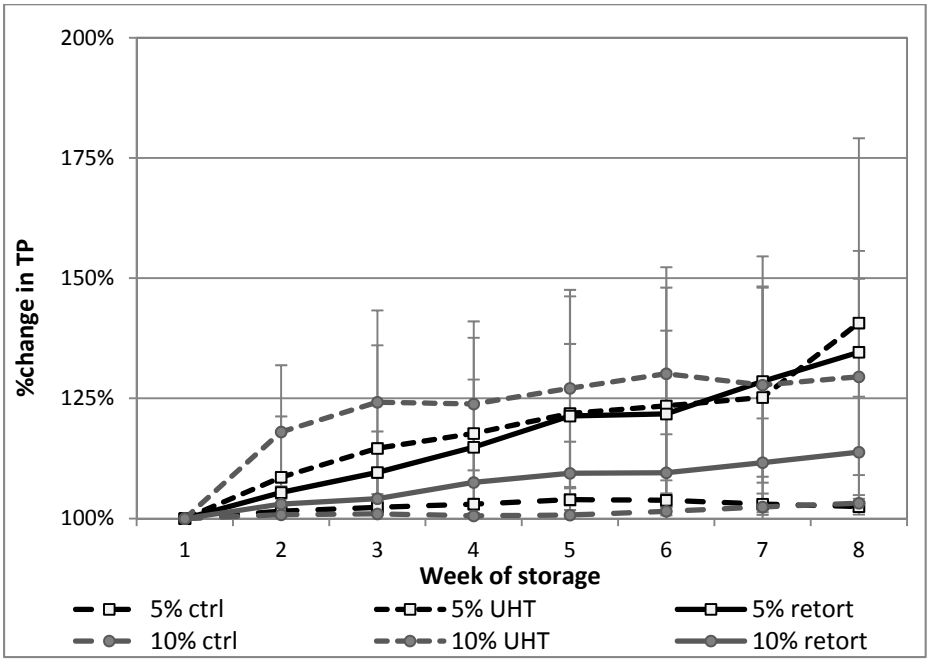

Figure 4.7. Change (%) in true protein content in bottom layer of MCC samples over time of storage at 25°C for control, retorted and UHT-treated 5% and 10% MCC. *Notes: No measurements could be performed on sample 5%, UHT, replicate 2, week 8 due to age gelation. Data for the control samples is based on IR, data for retort samples is a mix of IR data and values estimated using the established correlation of refractometric and IR data. UHT data are estimates from the refractometric data only.*

The UHT-treated samples of the three replicates behaved very differently over the time of storage due to the flocculation that occurred during the heat treatment. It must be noted that the UHT-treated samples were high-shear mixed after the heat treatment as described in the Methods section, in order to even be able to fill the storage containers. This may have disrupted to some extent the aggregates present in these samples prior to storage. Replicate 1 showed moderate sedimentation, with a maximum change of protein content of about 14% in the 5% and 10% MCC samples in week 8. The 7.5% and 8% MCC samples exhibited only little sedimentation. In replicate 2 the maximum sedimentation occurred in the 7.5% sample in week 7 (30% increase of protein content), while the 5% sample showed only little sedimentation. Extensive sedimentation was noted in replicate 3. Within the first week of

storage between 23% and 41% sedimentation occurred in the 5% and 7.5% MCC, respectively. The 10% MCC samples showed the smallest amount of sedimentation with 52% change of protein in the bottom layer in week 8. A maximum of 79% increase of protein content was measured in the 7.5% MCC sample at the end of storage time.

The R-MCC samples showed a somewhat different behavior than the MCC samples. Maximum sedimentation of about 16% occurred in the control samples in the third week of storage, after which sedimentation decreased and leveled off at about 10% (Figure 4.8). The retorted R-MCC samples showed a steady increase of sedimentation over the time of storage and reached about 10% of sedimentation in week 8, which is comparable to the 8% MCC counterparts of replicates 1 and 2. Both the control and the retorted R-MCC exhibited little variability between the three replicates. The UHT-treated R-MCC samples showed strong sedimentation behavior, but also large variation between the three replicates, which is reflected by the large standard deviations (Figure 4.8). After the first week of storage an average of about 24% sedimentation had occurred. At the end of storage the protein content in the bottom layer had increased by about 27%, 45% and 81% for replicates 1, 2 and 3, respectively (data not shown). For all replicates, sedimentation was significantly higher in the UHT-treated R-MCC than their 8% MCC counterparts.

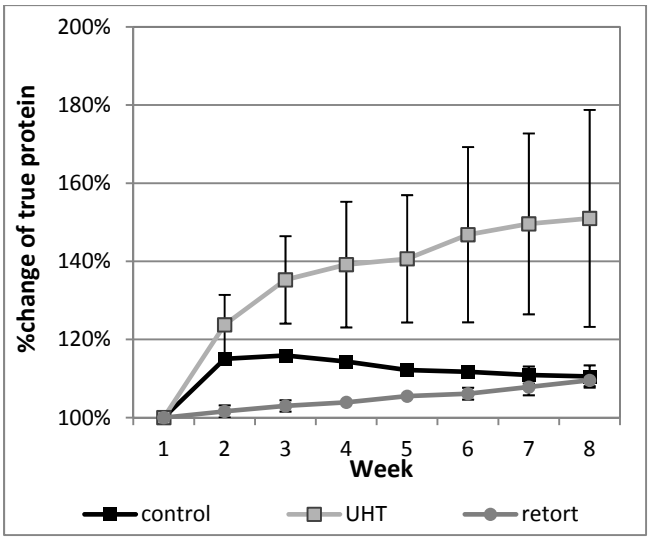

Figure 4.8. Change (%) in true protein content in the bottom layer of R-MCC samples over time of storage at 25°C. Plotted are averages of three replicates, error bars represent one standard deviation.

Based on the change of protein content in the bottom layer over time, sedimentation kinetics was established to predict sedimentation over longer times of storage. A linear regression model was fitted to the data of true protein (%) in the bottom layer vs. storage time (days). Due to the large variability between the replicates, the analysis was done separately for each replicate of the retorted MCC samples. For the retorted R-MCC samples linear regression was fitted to the average of the three replicates.

Using the following formula for the simple linear regression model:

$$y = Ax + y_0 \qquad \text{Eq. 4.2}$$

where y is the true protein content (%) in the bottom layer of the sample, x is the time of storage in days and y_0 is the initial protein content (%) of the MCC preparation in week 1. The constant A reflects the rate of sedimentation, expressed as % protein that sediments per day.

Sedimentation kinetics was established only for retorted samples of the MCC and R-MCC samples. Given the aggregation and coagulation during the treatment and already heavy sedimentation observed in the UHT-treated samples, the prediction of storage behavior over extended periods of time has little

practical value, and the same is true for the untreated control samples stored at 25°C (since these samples are subjected to microbial and enzymatic activity).

It can be seen from the calculated protein data in Table 4.2 that after 180 days of storage at 25°C about 75% of the protein in the 5% MCC samples of replicate 1 and replicate 2 will have sedimented. In replicate 3 after 120 days of storage over 150% of protein would settle in the 5% MCC samples, over 225% would have settled after 180 days of storage. Sedimentation in the R-MCC samples is comparable to the 8% MCC samples of replicate 2, in which after 180 days of storage about 35% of the protein would have settled.

Given that commercially sterilized milk beverages can have an ambient shelf-life of up to 12 month (Aryana, 2007), it can be inferred from the data in Table 4.2 that sedimentation would be an issue and means of stabilization would need to be employed in a potential beverage application of MCC.

Table 4.2. Sedimentation kinetics parameters (Eq. 4.2) and estimation of sedimentation after 120, 180 and 365 days storage at 25°C for retorted MCC and R-MCC samples.

Sedimentation kinetics									
				after 120 days		after 180 days		after 365 days	
	A	y_0	R^2	%protein	%change	%protein	%change	%protein	%change
retorted MCC, replicate 1									
5%	0.02	4.96	0.94	7.46	50.3%	8.71	75.5%	12.55	153.0%
7.5%	0.01	7.47	0.83	8.83	18.1%	9.51	27.2%	11.60	55.2%
8%	0.01	7.99	0.81	9.43	18.0%	10.15	27.1%	12.37	54.9%
10%	0.02	10.08	0.81	11.96	18.7%	12.91	28.0%	15.81	56.9%
retorted MCC, replicate 2									
5%	0.02	4.96	0.85	7.38	48.9%	8.59	73.4%	12.33	148.8%
7.5%	0.01	7.45	0.92	8.93	20.0%	9.68	30.0%	11.97	60.8%
8%	0.02	7.99	0.69	9.83	23.1%	10.76	34.7%	13.61	70.4%
10%	0.02	10.04	0.77	11.89	18.4%	12.82	27.6%	15.66	56.0%
retorted MCC, replicate 3									
5%	0.06	4.86	0.98	12.16	150.5%	15.82	225.8%	27.08	457.8%
7.5%	0.05	7.26	0.87	13.49	85.8%	16.60	128.7%	26.20	260.9%
8%	0.07	7.78	0.95	16.08	106.7%	20.24	160.1%	33.04	324.6%
10%	0.06	9.76	0.95	16.45	68.4%	19.79	102.7%	30.10	208.2%
retorted R-MCC, average of three replicates									
8%	0.01	7.55	0.99	9.30	23.2%	10.18	34.8%	12.88	70.6%

Conclusions

Heat treatment affected the storage stability of micellar casein concentrates (MCC) obtained by membrane separation. UHT-treated concentrates were highly instable. Strong sedimentation was observed over eight weeks of storage at 25°C, which was mainly attributed to the large, visible aggregates that had formed during the heat treatment. Retorted casein preparations showed some browning as well as moderate sedimentation throughout storage time. Significant differences were observed among replicates, which were likely due to compositional and processing differences experienced in the manufacture of the MCC. The effect of spray-drying was limited and the stability of reconstituted casein preparations is primarily determined by the stability of their non-dried counterparts.

Overall, this data shows that additional stabilization or adjusted treatment regimes might be required for the commercial application of heat treated MCC in order to obtain a product that is shelf-stable both from a microbiological and physic-chemical point of view.

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CHAPTER 5

HEAT STABILITY OF MICELLAR CASEIN CONCENTRATE (MCC) AS AFFECTED BY TEMPERATURE AND PH

Abstract

The increasing interest in using MCC obtained by microfiltration in manufacturing shelf-stable high protein products creates a need to understand the effect of sterilization treatments on the stability of this ingredient. The goals of this work were to: 1) elucidate the effects of pH and heat treatment temperatures on the mineral distribution and dissociation of caseins; and 2) use the generated knowledge to develop solutions for stabilizing the MCC during sterilization treatments.

MCC powders were reconstituted and the resulting dispersions were adjusted to pH values of 6.5-7.3. Subsequently, the MCC samples were heated in an oil bath to 110-150°C. The treated samples were evaluated for particle size, soluble minerals and casein dissociation.

At pH < 6.7, all heat treated samples were visibly aggregated or coagulated. At pH 6.9, higher temperatures lead to increased particle size, while at pH > 6.9 little or no changes were observed after heat treatment. Casein dissociation increased with increasing pH for all caseins, at all temperatures, with dissociation of κ-CN and β-CN being most significant. At higher pH, the levels of dissociated α_s-CN decreased after heat treatment, suggesting aggregation of α_s-CN due to the presence of calcium and lost protection by κ-CN. It was concluded that increased stability of MCC requires increasing the pH or lowering the processing temperature. MCC were then submitted to sterilization, utilizing those modifications at equivalent lethality. A significant reduction in particle size was obtained and no coagulation or aggregation occurred after retorting or UHT.

The generated knowledge will allow the effective stabilization of MCC in practical applications, such as the production of novel, shelf-stable protein beverages.

Introduction

The use of micellar casein preparations obtained by microfiltration is receiving increasing interest from the dairy industry and other industries (Affertsholt, 2009). In the microfiltration (MF) process, casein is separated from serum proteins based on their difference in molecular size (Brans et al., 2004), with additional diafiltration of the retentate leading to serum protein removal of up to 95% (Nelson and Barbano, 2005). In casein concentrates obtained by microfiltration, the casein micelles are closer to their native state than in casein preparations obtained by chemical methods. This results in unique functionality and opens the field for possible applications of the casein concentrates, such as premium shelf-stable, high protein nutritional beverages (UBIC Consulting, 2010). Typically the manufacture of such beverages involves a sterilization step, and therefore it is crucial that the casein concentrates are heat stable under a range of pH and temperature conditions.

Casein micelles in milk are remarkably stable systems that can withstand the rigorous conditions applied during commercial processing (Fox, 1982). However under certain conditions of temperature and pH, the colloidal integrity of the casein micelles can be disrupted, resulting in decreased stability, manifested through visible flocculation, gelation or protein separation (Fox, 1982).

The mechanism and the pH-dependence of heat-induced instabilities of the casein micelles have been subject of previous research, most of which has been done on milk and concentrated milk products; only some studies are available on micellar casein systems devoid of serum proteins and lactose.

Previous work investigated the effects of two commercial sterilization regimes (continuous-flow UHT treatment and in-container retorting) and spray-drying on the stability and physical properties of

micellar casein concentrates (MCC) during processing and storage (Sauer and Moraru, 2011; Beliciu et al., 2012). Sterilization affected the colloidal stability, viscosity and flow behavior of MCC. Retorting resulted in a significant increase in particle size, while UHT treatment caused aggregation and coagulation of MCC. The observed effects were in part credited to a loss in solubility of calcium phosphate as a result of the high heat treatment, but it was hypothesized that casein micelle dissociation is also likely to play a role in this instability (Beliciu et al., 2012).

The objectives of this work were to 1) elucidate the effects of pH and heat treatment temperature on the mineral distribution and dissociation of casein micelles; and 2) use the generated knowledge to develop solutions for stabilizing MCC during sterilization treatments.

Materials and Methods

Micellar casein concentrates

Micellar casein concentrate (MCC) was obtained by membrane separation in the pilot plant at Cornell University following the methodology described in detail by Hurt et al. (2010). Pasteurized skim milk was subjected to 3 stages of microfiltration (MF) using a uniform transmembrane pressure system equipped with ceramic Membralox membranes of 0.1 µm pore size. After the first and second MF stages, the resulting retentates were diluted with reverse osmosis (RO) water at a 2:1 ratio by weight (2 parts water, 1 part retentate). The final retentate was spray dried using a Model 1 Niro Atomizer equipped with a FU11 atomizer rotating at 23,000 rpm (Niro Atomizer Inc., Columbia, MD). The inlet air temperature was 200°C and the outlet air temperature was 95°C. The spray dried powder was stored in tightly closed containers in the dark at room temperature (25°C or less) until use.

Experimental design

MCC powder was reconstituted in water and adjusted with 1 M HCl and 1 M NaOH, respectively, to pH values ranging from 6.5 to 7.3. To study the effect of pH and temperature in the sterilization range, samples were heat-treated using a benchtop heating system. In order to minimize the number of experiments, pH-temperature conditions were chosen based on an experimental design derived from the central composite design of response surface method. Specifically, central and corner points of the range of conditions to be investigated were chosen, in order to span a larger range of the effect variables, as shown in Figure 5.1. Non-heat treated samples (controls) were analyzed at all pH conditions.

After investigation of the full range of pH and temperature conditions, commercial sterilization regimes for UHT and retort treatments were designed based on optimal pH-temperature combinations as well as legal sterilization requirements for the dairy industry.

All treatments and analyses were preformed in triplicate.

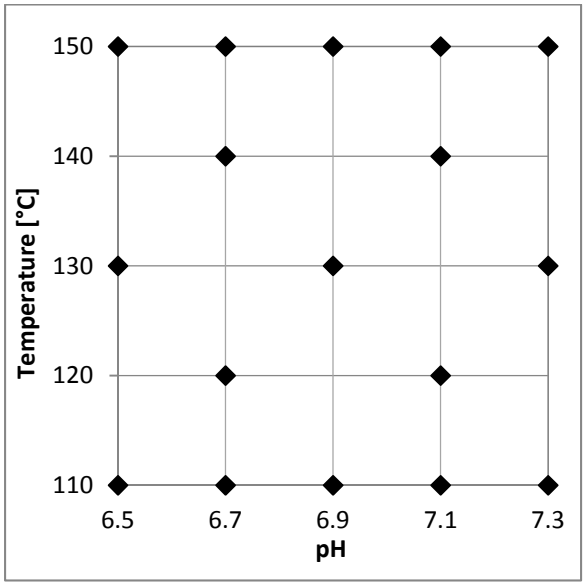

Figure 5.1. Experimental design: pH-temperature conditions chosen for benchtop heat treatments. Non heat-treated samples were analyzed as control samples at all pH conditions.

Chemical analysis

Fresh, liquid MF retentates were analyzed for lactose, fat and true protein (TP) by FTIR using an infrared spectrophotometer (Lactoscope FTIR, Delta Instruments) before spray drying. The powder was reconstituted to 10% solids and was analyzed at Dairy One Inc. (Ithaca, NY) for fat by ether extraction (AOAC, (2005); method 989.05; 33.2.26), and for proteins by Kjeldahl: total nitrogen (TN) (AOAC, 2005; method 991.20; 33.2.11), non-protein nitrogen (NPN) (AOAC, 2005; method 991.21; 33.2.12), and non-casein nitrogen (NCN) (AOAC, 2005; method 998.05; 33.2.64). The total solids (TS) content of the powder was measured by forced air oven drying (AOAC, 2005; method 990.20; 33.2.44).

The chemical composition of the 95% SP-reduced MCC powder is presented in Table 5.1.

Table 5.1. Chemical composition (%) of the 95% SP-reduced MCC powder

	Chemical composition[1]				
	TN	NPN	NCN	CN	Fat
95% SP-red. MCC	86.81	1.15	9.21	77.59	2.51

[1]TN = total nitrogen, NPN = non-protein nitrogen, NCN = non-casein nitrogen, CN = casein = TN - NCN

Sample preparation

MCC powder was reconstituted to casein concentrations of 8% w/v in water. This concentration was chosen based on prior work (Beliciu et al., 2012) and on a typical protein concentration for a high protein beverage. The casein concentration of the MCC was estimated as 98% of the TP values obtained by FTIR measurements (Lactoscope FTIR, Delta Instruments, Drachten, Netherlands), according to Hurt and Barbano (2010). TP of the final MF retentate prior to spray drying was 8.21%, which equates to 8.05% casein.

For the benchtop treatments, ultrapure water was mixed with predetermined amounts of 1 M HCl and 1 M NaOH, respectively, so that the MCC dispersions would reach pH values of 6.5, 6.7, 6.9, and 7.3.

The control samples for the pH study were not adjusted and were at pH 7.1. A 30-min re-suspension step was followed by a 5-min ultrahigh shear mixing step using an Ultra-Turrax Model T25 fitted with a S25N-18G dispersing tool (IKA Works Inc., Wilmington, NC). For the pilot plant treatments, the MCC powder was reconstituted with RO water heated to 40°C, mixed with predetermined amounts of 1 M NaOH, using a 5-min re-suspension step, followed by a 15-min high shear dispersion step. All reconstituted MCC dispersions were stored at 4°C overnight to ensure full hydration and pH equilibration.

Heating treatments

Benchtop treatments. To evaluate the effect of heating temperature in the sterilization range on MCC, samples were heated to 110, 120, 130, 140 and 150°C, respectively. The heat treatments were performed in an insulated oil bath, equipped with a recirculation heater (Thermomix 1480, Braun, Germany). During heating, MCC samples were placed in stainless steel tubes of 3/8 in (0.95 cm) diameter, 0.02 in (0.05 cm) wall thickness and 7.12 in (18.08 cm) length, closed at both ends with self-locking stainless steel cap fittings (Swagelok Company, Solon, OH). Thermocouples (type T) were mounted airtight into the stainless steel cap on one end and connected to a High Accuracy Datalogger/ Thermometer HH506 RA (Omega Engineering Inc., Stamford, CT) for monitoring and acquisition of temperature data.

Five stainless steel tubes per temperature condition were filled with MCC and pre-heated to 40°C, to ensure the same starting temperature for all samples. The tubes were submerged into the hot oil and the timer was started instantly. The oil bath was setup so that samples reached the specified final temperatures in about 52 s, for all heat treatment temperatures. This was done in order to eliminate the effect of the come-up time on the MCC. Immediately after reaching the desired final temperature, the

tubes were removed from the oil bath and placed on ice until the sample temperature reached 20°C. The treated samples were pooled and subjected to chemical analyses.

Sterilization treatments. The temperature-time combinations for the sterilization treatments were designed to ensure an equivalent microbial inactivation effect by calculating the lethality factor, F_0. According to Bylund (2003), minimum F_0 values of 5-6 are typically used to achieve commercially sterile milk.

The UHT treatment was performed on a pilot scale Microthermics heating system (Model 25 HV, Microthermics, Inc., Raleigh, NC), equipped with an Ultra Clean Fill Hood/Sterile Product Outlet. The MCC preparations were preheated from the refrigeration temperature to 40°C (104°F) in a steam kettle and immediately subjected to the sterilization treatment. The following parameters were used for final temperatures and times shown in Table 5.2: pre-heating within 20 s, final heating of the product within 20 s, holding for time at temperature as indicated in Table 5.2, and cooling within 20 s. The flow rate of the product was 2 L/min. The F_0 value for the UHT treatment was calculated based on the average velocity of the product in the UHT unit.

Table 5.2. Sterilization treatment conditions (temperature, holding time, pH) designed to achieve same lethality (F_0 = 6)

Treatment	Holding time	pH	
		7.1 (unmodified pH)	7.3
UHT, 135°C/275°F	15 s	X	X
UHT, 142°C/288°F	3 s	-	X
Retorting, 115.6°C/240°F	21.3 min	X	X
Retorting, 121°C/250°F	6.1 min	X	X

A FMC Multipurpose Lab Retort with LogTec Process Management System (Steritort, FMC, San Jose, CA) was used to batch-sterilize the MCC dispersions in 8 oz (237 mL) Mason jars (Ball Corporation,

Broomfield, CO). The product was treated in a still cook mode with cascading hot water. The temperature-time parameters, shown in Table 5.2, were calculated from the temperature profile recorded in a preliminary trial by thermocouples attached to 4 jars located in the center of the retort. The calculations were performed with a modified Ball's formula, using CALSoft 32, version 1.0 (TechniCAL Inc., Kenner, LA).

pH measurements

The pH of the MCC samples was measured at 20°C using a Fisher Scientific accumet Excel XL20 pH meter, calibrated at the measurement temperature prior to use (Fisher Scientific, Pittsburgh, PA).

Particle size analysis

Dynamic light scattering (DLS) was used to evaluate particle size and particle size distributions in all samples following the procedure described in detail by Beliciu et al. (2012). The analyses were performed using a 90Plus Nanoparticle Size Analyzer equipped with a Peltier temperature control system (Brookhaven Instruments Corp., Holtsville, NY), at a fixed 90° angle and a wavelength of 658 nm.

Samples were equilibrated to 20°C in a waterbath prior to analysis, with proper protection against dehydration. The samples where then diluted with UF permeate to the manufacturer recommended signal intensity of 700-900 kilocounts per second. The UF permeate, obtained during the manufacture of the MCC, was kept frozen at -40°C and placed in a fridge at 4°C four days prior to analysis for thawing. The particle size measurements were performed at a constant temperature of 20°C, following an equilibration step.

Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp.). A dust cutoff filter parameter of 30 was used to ensure the quality of the measurements. Each measurement consisted of 8 subsequent individual runs of 30 s duration. The relative particle size

distribution, the intensity weighted effective diameter and the polydispersity index were determined. Measurements for each experimental condition were performed in duplicate.

Ultracentrifugation procedure

In order to evaluate mineral distribution between the soluble and insoluble phase and the dissociation of casein micelles as a result of the heat treatments, all MCC samples were subjected to an ultracentrifugation step. Samples were ultracentrifuged immediately after the heat treatment by transferring them into polycarbonate centrifuge bottles and centrifuging at 100,000 g for 60 min at 20°C, using a Beckman L8M centrifuge (Beckman Coulter, Inc., Brea, CA) with a standard 70.1 Ti rotor at 38,200 rpm. Special care was taken to not re-suspend the insoluble pellet while handling the ultracentrifuges samples. The resulting clear supernatant was collected using 10 mL syringes (Luer-Lock, Thermo Scientific, Waltham, MA) with 4 in stainless steel needles from 4 bottles per sample and pooled.

Mineral profile characterization

The mineral composition of the ultracentrifugation supernatants was tested at the Dairy One Forage Analysis Laboratory (Ithaca, NY). 5 g of sample were weighed into calibrated CEM Xpress Teflon PFA vessels with fiberglass insulating sleeves. The samples underwent a 30 min digestion step with 8 mL HNO_3 and 2 mL HCl at 1600 W and 190°C, in a CEM Microwave Accelerated Reaction System (CEM, Matthews, NC) equipped with the MarsXpress Temperature Control option. The vessels were then brought to 50 mL volume with 1.5 N HNO_3 + 0.5 N HCl solution to match standards, aspirated and analyzed with a Thermo Jarrell Ash IRIS Advantage HX Inductively Coupled Plasma (ICP) Radial Spectrophotometer (Thermo Scientific, Madison, WI). The mineral analyses for each experimental condition were performed in duplicate.

Quantification of dissociated casein

In order to evaluate dissociation of the casein micelle, individual caseins were identified in the soluble phase after ultracentrifugation using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) by the Proteomics and Mass Spectrometry Core Facility at Cornell University (Ithaca, NY). LC-MS/MS couples the separation capabilities of HPLC, based on hydrophobic interactions between the stationary phase and the solute, with the detection power of mass spectrometry. Mass spectrometers ionize molecules and subsequently sort and identify them according to their mass-to-charge (m/z) ratio. The identification of a protein, using the so called "bottom-up" or "shotgun" proteomics approach, is usually achieved via a four-step process consisting of protein digestion, protein separation, and MS analysis of resulting proteolytic fragments, followed by comparison of the observed peptides to those in a database (O'Donnell et al., 2004).

Prior to the protein digestion, 0.2 pM apomyglobin was added to 3 µL of the ultracentrifugal supernatants as internal standard. Apomyglobin is a protein standard that does not naturally occur in bovine dairy products. The protein mixtures were denatured with 3 M Guanidine HCl, after which disulfide bonds were reduced using 16 mM tris(2 carboxyethyl)phosphine (TCEP) during incubation at 60°C for 1 h. The solution was then treated with 42 mM iodoacetamide (IDA) for 1 h in the dark to alkylate cysteine residues and incubated with 26 mM dithiothreitol (DTT) for 10 min. Prior to the addition of trypsin, samples were diluted with 100 mM Tris buffer for pH adjustment. Sequencing-grade trypsin was added to the solution in the concentration of 40 ng/µg, in order to achieve about a 1:10 ratio of trypsin to protein, and incubated overnight at 37°C. The resulting tryptic-digested peptides were diluted in a 1% formic acid/10% acetonitrile solution.

The samples were desalted and separated on a C18 RP-HPLC column, connected in-line to a triple quadrupole linear ion trap mass spectrometer 4000 Q Trap equipped with a Turbo Ion Spray Head ion source (Applied Biosystems, Foster City, CA). The spray voltage was 2.0 kV and was used in positive ion

mode. Nitrogen was used as the collision gas. In information-dependent acquisition analysis, after each survey scan for m/z 375 to m/z 1600 and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for MS/MS with rolling collision energy applied for detected ions based on different charge states and m/z values.

Statistical analysis

The experimental data was analyzed using JMP 8.0 software (SAS Institute Inc., Cary, NC). Analysis of variance was used to determine the effects of temperature and pH. A general linear model was used to predict values for omitted pH-temperature conditions. Significant differences among samples were determined by Tukey's HSD test at $p \leq 0.05$.

Results and Discussion

Aggregation and coagulation of MCC under different temperature and pH conditions

The occurrence of particle aggregation in the heat treated samples was evaluated by determining the effective particle diameter in the MCC samples (Figure 5.2). Values of the effective diameter at pH-temperature conditions omitted in the experimental design were estimated using a linear regression model based on the statistical analysis of the data, and are indicated by an asterisk in Figure 5.2. Both pH, temperature, and the interaction of the two factors had a significant effect on particle size.

At pH 6.5 and 6.7, MCC samples showed strong aggregation after heating to 110°C and 120°C, and complete coagulation at temperatures of 130°C and above. In all cases aggregation was visible to the naked eye, which rendered dynamic light scattering unusable for these pH conditions.

At pH 6.9, particle size increased significantly with increasing temperature, which points to aggregation of casein micelles in the treated MCC (Figure 5.2). The non-heat treated control samples had an effective diameter of 224.5 ± 35.8 nm, which is slightly higher than what has been reported as

size for casein micelles in raw milk by Beliciu and Moraru (2009). After heat treatment at 150°C, the effective particle diameter was 541.5 ± 36.0 nm, more than twice the size measured in the control.

At pH 7.1, the unadjusted pH of the reconstituted MCC dispersions, the measured particle size measured in the control was 193.9 ± 16.1 nm, similar to the values reported by Beliciu and Moraru (2009). Only slight increases in size were observed after increasing heat treatment temperature (Figure 5.2); after heating to 150°C, an effective diameter of 229.0 ± 25.3 nm was measured.

At pH 7.3 virtually no differences in particle size were observed after heat treatment, for all temperatures. The effective diameter of the non-heat treated control samples was comparable to the control samples at pH 7.1.

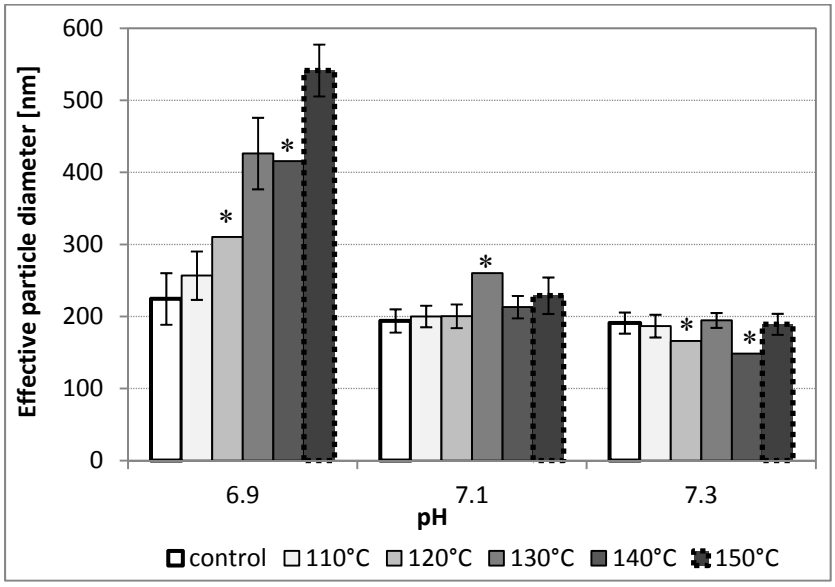

Figure 5.2. Particle size of untreated and heat-treated MCC at different pH values. Plotted are mean values (n=3) ± 1 SD. *Values estimated by general regression model based on statistical analysis.

Zeta potential (ξ-potential), as a measure of the electrical charge of the casein micelle, was determined as a relative indicator of colloidal stability of MCC samples. The ξ-potential values for samples at all pH across the series of temperature treatments were in the range of -15 to -25 mV (data not shown). The ξ-potential became more negative with increasing pH, and less negative with increasing temperature treatment. Although the differences among the samples were not statistically significant,

the observed trend supports the particle size data, showing less propensity for aggregation of the micelles at higher pH.

Changes in mineral solubility as a function of pH and temperature

The total and soluble mineral profile of untreated, unmodified MCC can be found in Table 5.3. The main focus was placed on calcium and phosphorus - the minerals that make up calcium phosphate, which has a critical role in the stability of the casein micelle. The concentration of calcium and phosphorus in the soluble phase of heat-treated MCC samples across a range of pH values is presented in Figure 5.3a and b. Temperature had a significant effect on minerals in the serum phase, with the levels of Ca and P decreasing with increasing heat treatment temperature across the investigated pH range. Levels of soluble calcium at pH 6.5 were significantly higher compared to those at higher pH (Figure 5.3a). There were no differences in the soluble calcium concentration in the MCC samples treated at 140°C and 150°C for pH 6.9 or higher.

Table 5.3. Total and soluble mineral composition (mM) of the pH-unadjusted 8% MCC. Shown are mean values (n=3) ± 1 SD, unless otherwise noted.

	Mineral composition					
	Ca		P		Na	
	Total	Soluble	Total	Soluble	Total	Soluble
8% MCC	69.5±5.35	5.1±0.3	62.7±11.7	4.9±0.6	6.8*	6.3*

* Only two replicates available

The effect of temperature on soluble phosphorus was similar to soluble calcium (Figure 5.3b). Soluble phosphorus levels did not change when pH was lowered, but increased when pH was increased; the highest levels of soluble P were detected in samples of pH 7.3.

To better understand the effect of pH and thermal treatments on colloidal calcium phosphate, the Ca/P ratios for all MCC samples were calculated. Calcium phosphate salts in milk can exist in different

forms, which are characterized by different Ca/P ratios (Gaucheron, 2005). The major form of colloidal calcium phosphate (CCP) is tricalcium phosphate ($Ca_3(PO_4)_2$), which corresponds to a Ca/P ratio of 1.5 (Cross et al., 2005; Lucey and Horne, 2009).

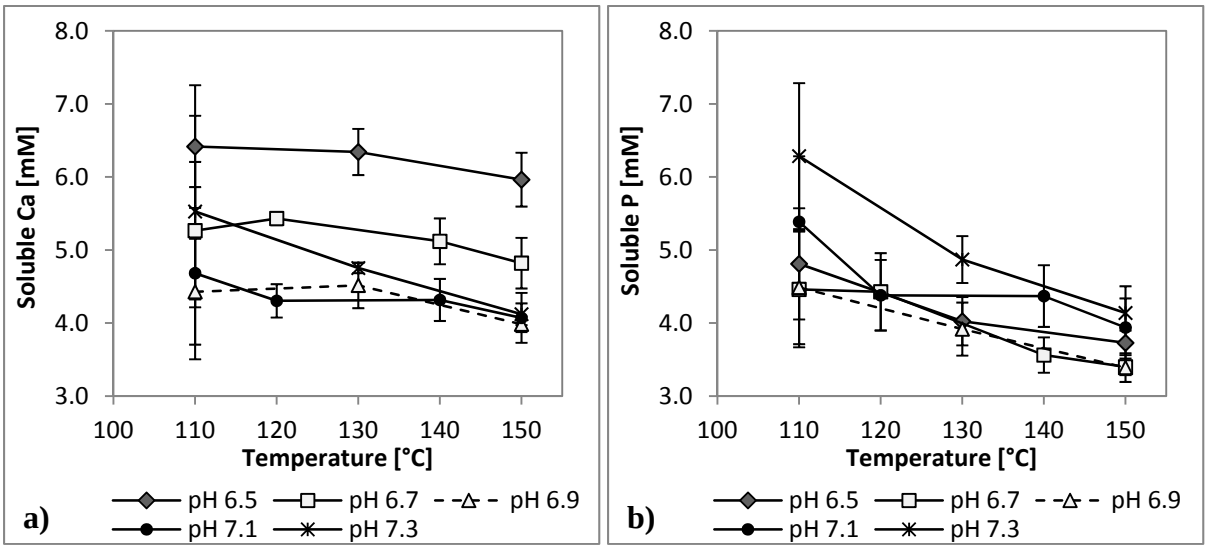

Figure 5.3. Mineral concentration in the soluble phase of heat treated MCC: a) calcium; b) phosphorus. Plotted are mean values (n=3) ± 1 SD.

The Ca/P ratios in the soluble phase for all the MCC samples are plotted in Figure 5.4. For all heat treatments and the controls, Ca/P ratios decreased from about 1.5 at pH 6.5 to about 1 at pH 7.3. Since there were no statistically significant differences in the Ca/P ratio between the controls and the samples treated at different temperatures for any of the pH levels, the average Ca/P ratio across samples of a given pH and subjected to different temperatures was calculated and is shown as a fitted trendline in Figure 5.4. The average Ca/P ratio at pH 7.1, the unadjusted pH of the reconstituted MCC dispersions, was 0.98 ± 0.07; no difference in Ca/P ratio between pH 7.1 and 7.3 was found. A Ca/P ratio of 1 is comparable to the ratio found in the native serum phase of milk (Gaucheron, 2005).

Upon acidification, the Ca/P increased, reaching a value of 1.52 ± 0.12 at pH 6.5. This indicates that as pH decreased CCP dissolved from the casein micelle and migrated into the serum phase. This is

an expected and known effect, and has a significant contribution to the destabilization of casein micelles at low pH and their subsequent aggregation during the heat treatment.

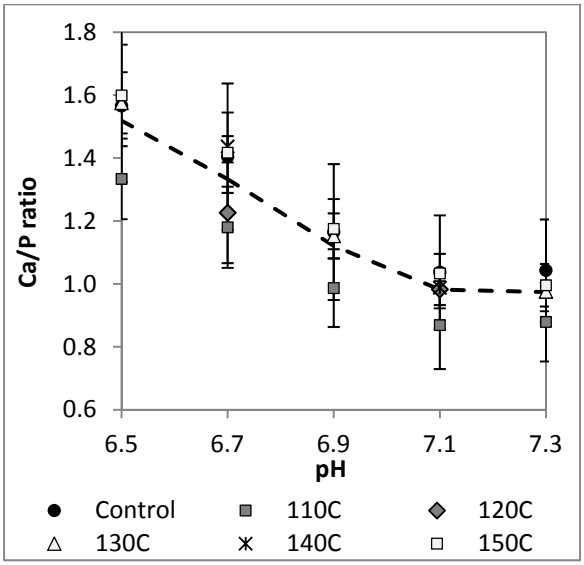

Figure 5.4. Ca/P ratio for untreated and heat treated MCC across the full range of pH. Plotted are mean values (n=3) ± 1 SD.

Evaluation of casein dissociation

Dissociation of casein micelles was evaluated by relative quantification of individual caseins in the soluble phase after ultracentrifugation of heat treated samples. A total of 23 peptides were identified as digest products of α_{s1}-, α_{s2}-, β-, and κ-casein for the control and heat-treated MCC samples, in addition to 6 peptides for the internal standard apomyoglobin. The nomenclature, peptide sequences, mass-over-charge-ratios (m/z), and retentions times for these peptides are shown in Table 5.4. Generally, responses of the peptides from all caseins followed the same trends, but showed different intensity from peptide to peptide. For each type of casein, the peptide with the strongest intensity response was selected as representative for the peptides of that casein and used as an indicator for dissociation in the MCC samples.

The non-heat treated, pH-unadjusted (pH 7.1) MCC control samples showed a significant amount of soluble α_{s1}-, α_{s2}-, β-, and κ-casein (Figure 5.5a). This was to be expected, as not all caseins are bound as casein micelles (Fox, 2003). pH had a strong effect on casein dissociation, regardless of temperature treatment. At lower pH (in the acidified samples), there were significantly less individual caseins in the soluble phase, which points toward casein aggregation and subsequent precipitation into the pellet upon ultracentrifugation. At pH 7.3 significantly higher amounts of individual caseins were found in the supernatant, suggesting increased dissociation of caseins from the casein micelle. Figure 5.5b and Figure 5.5c show the data for samples heated at 110°C and 150°C, but similar trends were obtained for the other heat treatment temperatures. The heat treated samples showed the same trends observed for the controls, with increasing concentration of soluble caseins with increasing pH (Figure 5.5b, c). Across the pH range, soluble β- and κ-casein were detected in much higher concentration as compared to α_{s1}- and α_{s2}-casein.

These observations are in agreement with previous reports, in which increasing amounts of dissociated α_s-, β-, and κ-casein were observed with increasing pH from 6.5-7.1 upon heating skim milk from 80-120°C (Anema and Klostermeyer, 1997a) and whey protein-free milk at 60°C and 90°C, respectively (Anema and Li, 2000).

Table 5.4. Identified peptide names, peptide sequences, m/z values for first and second mass spectrometer Q1 and Q3 and retention times (RT)

Protein	MultiQuant Component	Peptide Sequence (charge (z))	Q1 Mass (m/z)	Q3 Mass (m/z)	RT (min)
κ-casein	k-CN (1y8)	46-YIPIQYVLSR-55 (2+)	626.4	975.6	18.8
	k-CN (1y6)		626.4	765.5	18.8
	k-CN (2y12)	90-SPAQILQWQVLSNTVPAK-107 (2+)	990.6	1370.7	20
	k-CN (2y11)		990.6	1242.7	20
	k-CN (2y7)	90-SPAQILQWQVLSNTVPAK-107 (3+)	660.8	716.5	20
	k-CN (2b7)		660.8	738.5	20
	k-CN (2y8)		660.8	829.6	20
α_{s1}-casein	as1-CN (1y8)	106-YLGYLEQLLR-115 (2+)	634.4	991.7	21.2
	as1-CN (1y6)		634.4	771.6	21.2
	as1-CN (2y8)	38-FFVAPFPEVFGK-49 (2+)	692.9	920.6	21.8
	as1-CN (2y9)		692.9	991.8	21.8
α_{s2}-casein	as2-CN (1y5)	48-ENLCSTFCK-56 (2+)	579.8	642.4	12.1
	as2-CN (1y6)		579.8	802.5	12.1
	as2-CN (2y6)	96-ALNEINQFYQK-106 (2+)	684.4	827.5	14.8
	as2-CN (2y7)		684.4	940.7	14.8
β-casein	b-CN (1y5)	192-AVPYPQR-198 (2+)	415.7	660.4	9.2
	b-CN (1y4)		415.7	563.3	9.2
	b-CN (3y7)	199-DMPIQAFLLYQEPVLGPVR-217 (3+)	729.5	737.5	23.8
	b-CN (3y10)		729.5	1157.6	23.8
	b-CN (3y8)		729.5	866.5	23.8
	b-CN (2y11)	199-DMPIQAFLLYQEPVLGPVRGPFPIIV-224 (3+)	970.6	1151.9	27.5
	b-CN (2y10)		970.6	1094.7	27.5
	b-CN (2b9)		970.6	1029.6	27.5
Apomyoglobin (internal standard)	Apo (1y5)	120-HPGDFGADAQGAMTK-134 (3+)	501.7	507.2	11
	Apo (1b6)		501.7	611.3	11
	Apo (1y6)		501.7	635.4	11
	Apo (2b5)	65-HGTVVLTALGGILK-78 (3+)	460.4	494.3	19.2
	Apo (2y6)		460.4	600.4	19.2
	Apo (2y5)		460.4	487.3	19.2

Figure 5.6 illustrates the effect of heating temperature on dissociation in samples of different pH values. Due to the different concentration of the individual caseins in the supernatant, in this analysis a relative change in dissociation was determined, by comparing the concentration of each type of soluble casein in the heat treated samples to the concentration of that casein in the control. This analysis does not include the samples for which visual aggregation or coagulation occurred (pH 6.5 and 6.7). At pH 6.9 and 7.1, larger amounts of soluble β- and κ-casein were observed with increasing heat treatment temperature (Figure 5.6a, b). At pH 7.3, levels of β- and κ-casein were comparable across the treatment temperature range (Figure 5.6c). Levels of soluble α_{s1}- and α_{s2}-casein, however, decreased with increasing temperature at all pH levels, but the changes were most significant at pH 7.3. Anema and Klostermeyer (1997b) previously reported increasing dissociation of κ-casein and decreasing levels of α_s-casein when heating skim milk at different pH at temperatures from 70-90°C.

This data shows that increasing the pH will lead to dissociation of the casein micelles in the non-heat treated controls. The subsequent decrease in levels of α_{s1}- and α_{s2}-casein upon heating of MCC at pH 7.3 suggests that the α_s-caseins may form insoluble aggregates. This is possible when considering the fact that dissociated α_{s1}- and α_{s2}-casein will be present in the serum phase with a concentration of calcium of about 6 mM (see Table 5.3). It is known that α_{s1}- casein can precipitate at calcium concentrations of 3-8 mM, and α_{s2}-casein at about 2mM (Aoki et al., 1985). These aggregates are probably very small, which leads to a smaller average particle size in the heat treated MCC samples of pH 7.3 as compared to pH 6.9 and 7.1 (Figure 5.2). Therefore, although an increase in pH will still lead to changes of the “native” casein micelles, it will prevent a visible aggregation and coagulation of MCC during high heat treatment.

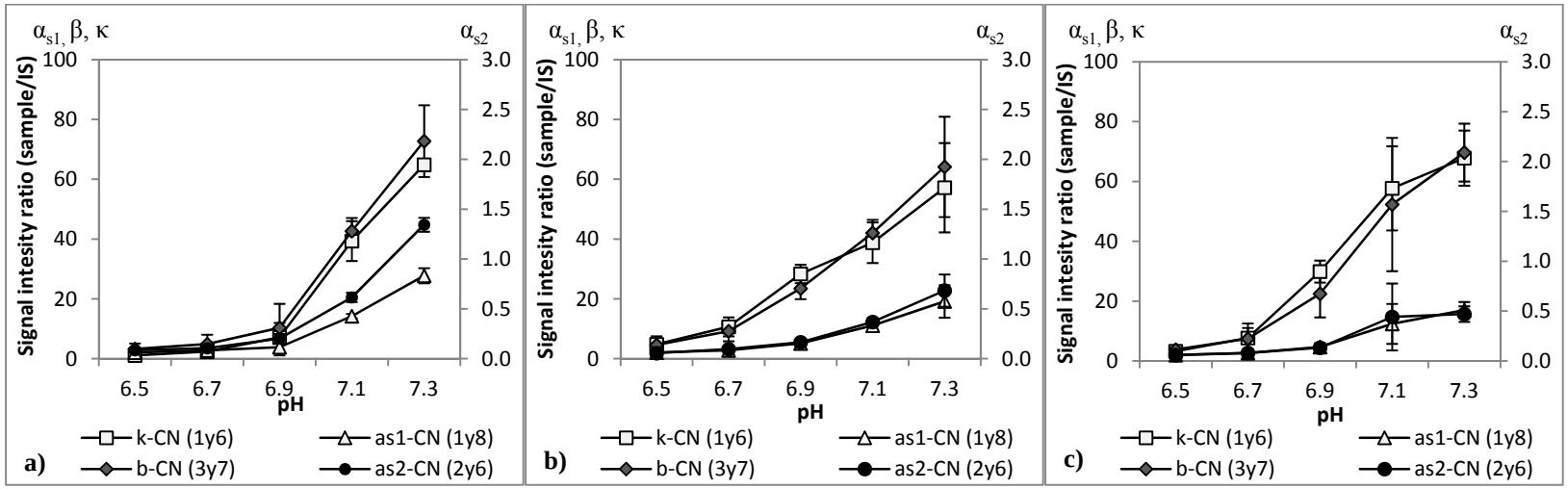

Figure 5.5. Casein dissociation, measured as ratio of signal intensity of sample divided by signal intensity of internal standard (IS) as function of pH in a) control sample; b) samples heated to 110°C; c) samples heated to 150°C. Plotted are mean values (n=3) ± 1 SD.

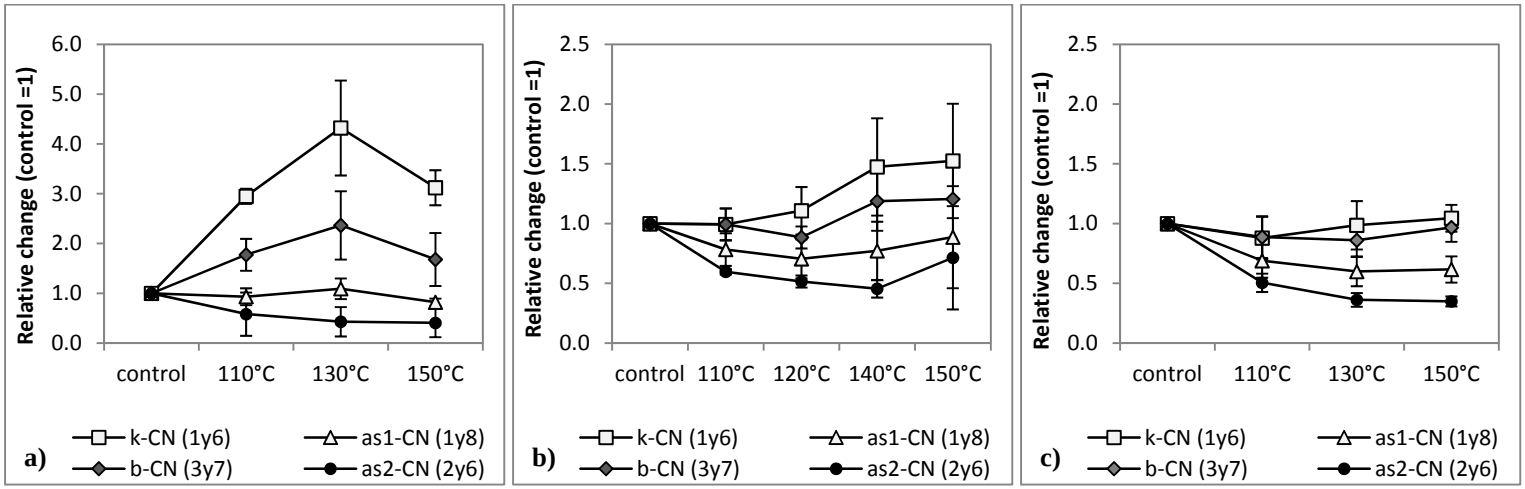

Figure 5.6. Relative change of casein dissociation with increasing temperature at a) pH 6.9; b) pH 7.1; c) pH 7.3. Dissociation level at control treatment equals 1. Plotted are mean values (n=3) ± 1 SD.

It has been found that the addition of 20-100 mM NaCl enhances heat stability of milk and shifts the HCT-pH curve to more alkaline values, which was suggested to be due to the reduction of the negative micellar charge by Na^+ (Morrissey, 1969; Grufferty and Fox, 1985). The effects of sodium in this study, however, were expected to be negligible, as only a small concentration of Na^+ in the form of NaOH was added (1.83 mM of NaOH for adjustment from pH 7.1 to 7.3).

Evaluation of commercial sterilization treatments with modified conditions

The knowledge gained about the effect of pH and temperature on the stability of MCC in the benchtop heating treatments was used to modify sterilization treatment conditions (both continuous-flow UHT treatment and batch retorting) such that the heat stability of MCC would be increased. Based on the results reported above, the pH of the MCC was adjusted to 7.3, 0.2 pH units above the pH of the reconstituted MCC, and sterilization treatment temperatures were decreased. The modified temperature-time combinations for the sterilization treatments were designed to ensure an equivalent microbial inactivation effect as the original treatments, by calculating the lethality factor F_0 according to Bylund (2003). The calculated F_0 value for all four treatments was 6, which is in the range of values typically used to achieve commercially sterile milk (Bylund, 2003). The pH, time and temperature conditions used in these treatments are shown in Table 5.2.

A significant reduction in particle size was achieved in the MCC samples treated at lower sterilization temperatures as compared to the original sterilization treatments, both for the adjusted and unadjusted pH (Figure 5.7). The smallest particle size was observed for MCC with adjusted pH, retorted at 115.6°C and 121°C, with effective diameters of 211.2 ± 3.7 nm and 211.9 ± 0.8 nm, respectively. Visual observation of the samples after sterilization treatment showed that samples retorted at the lower temperature, 115.6°C for 21.3 min, showed significant browning as compared to the other heat treated samples, due to the long duration of the treatment.

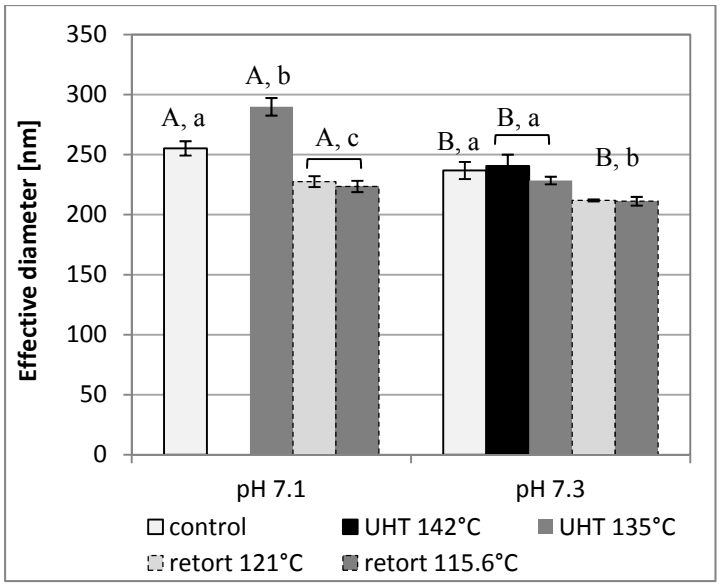

Figure 5.7. Particle size of MCC, untreated and heat-treated by sterilization treatments. Treatments not connected by the same capital letter (A, B) are different across the two pH categories. Treatments not connected by the same small letter (a, b, c) are different within the same pH category. Plotted are mean values (n=3) ± 1 SD.

The increase in pH also resulted in a decreased effective diameter for the control, UHT-treated and retorted samples. The control sample of pH = 7.1 had an effective diameter of 255.3 ± 6.0 nm, as compared to 236.8 ± 7.1 nm in the pH-adjusted control sample. These reductions in particle size were significant; statistically significant differences between the two levels of pH are indicated by capital letters in Figure 5.7, and differences among heat treatments within one pH category are indicated by small letters.

It must also be noted that the particle size for the control MCC in this part of the study was larger as compared to the benchtop heat treatments. This difference can be attributed to the different reconstitution methods used in the two situations, caused by the very different amounts of samples that were handled in the two situations.

Evaluation of calcium and phosphorus content in the serum phase showed significantly lower levels of soluble minerals in the heat-treated MCC as compared to the control samples; both soluble

calcium and phosphorus concentrations were about 2 mM lower in the heat-treated MCC than in the untreated control samples. However, there were no differences in soluble Ca and P between the four different sterilization treatments, both within the same pH group as well as between the two different pH levels (data not shown). Calculation of the Ca/P ratio found no effect of pH on this ratio, for all heat treatments; however, there we significant differences in Ca/P amongst the different heat treatments. The average Ca/P for the control samples at both pH levels was 1.06 ± 0.06, which decreased to 0.98 ± 0.06 in the samples sterilized by UHT treatment at 135°C. A similar Ca/P ratio for the product treated by UHT treatment at 142°C was observed. The average Ca/P ratios for retorted MCC at 115.6°C and 121°C was 0.88 ± 0.05 and 0.91 ± 0.05, respectively. Overall, there seems to be an effect of the duration of heating on the Ca/P ratio rather than the treatment temperature, as the lowest Ca/P ratio was observed for the MCC treated at the lowest sterilization temperature, with the longest hold time.

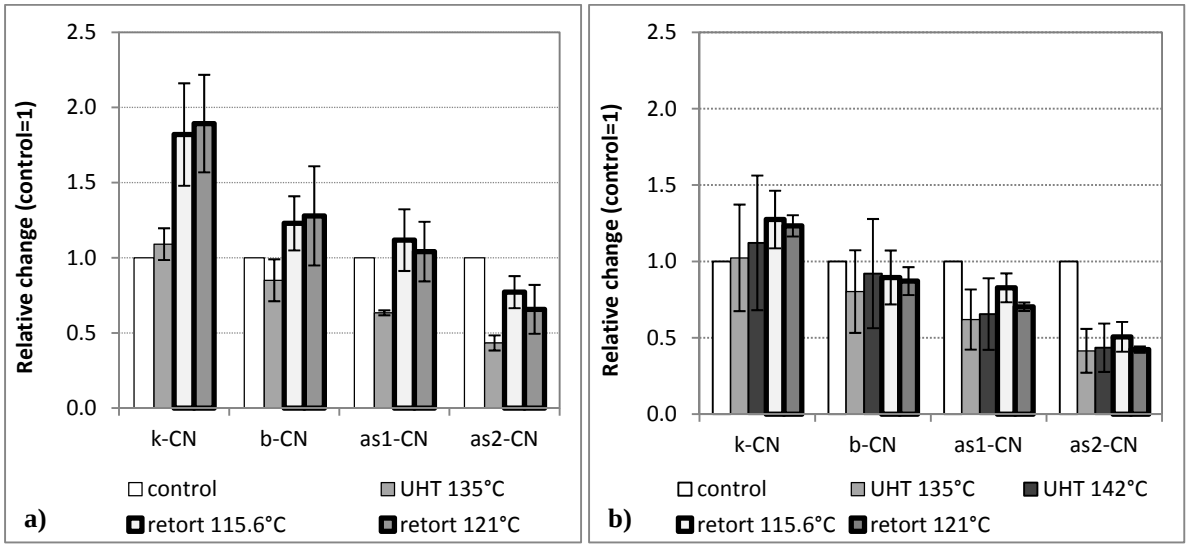

Figure 5.8. Relative change of casein dissociation after commercial sterilization treatments for a) unadjusted pH (pH = 7.1); b) adjusted pH (pH = 7.3). Plotted are mean values (n=3) ± 1 SD.

The effect of sterilization treatments on the dissociation of caseins is presented as relative change in the amount of casein in the soluble phase (Figure 5.8). Distinct differences were observed in the dissociation of casein among the different sterilization treatments at either natural pH or pH-adjusted to

higher value. At the un-adjusted pH, strong dissociation of κ-casein was detected after retorting at both temperatures, while UHT treatment showed no difference in dissociation compared to the control. The dissociation behavior of β-casein was comparable to that of κ-casein but less pronounced for the retorted samples. Significantly less α_{s1}- and α_{s2}-caseins were found in the soluble phase after UHT treatment, while retorting had only a small effect on the dissociation of these two caseins. At the adjusted pH, κ- and β-casein showed no distinct differences in dissociation between different sterilization treatments, however the levels of soluble α_{s1}- and α_{s2}-casein were both significantly lower after all heat treatments. These findings confirm the hypothesis formulated after the benchtop study: as κ-casein is dissociating from the micelle, protection for calcium sensitive caseins against soluble calcium is lost, and α_{s1}- and α_{s2}-casein will participate in formation of aggregates. The aggregates present in the pH- adjusted, heat-treated MCC will most likely have a different composition and will be slightly smaller than the original casein micelles. They will be however small enough to stay in dispersion and not form large aggregates, thus allowing their use in protein based formulations.

Conclusions

The main conclusion of this study is that MCC is unstable to heat temperature treatments in the sterilization range, and this instability increases with treatment temperature. At high temperatures, changes in the mineral equilibrium and partial disintegration of the casein micelle occur, which lead to aggregation and even coagulation. Aggregation and coagulation of MCC can be prevented by increasing the pH and/or decreasing the temperature of the heat treatment. These findings will help processors design appropriate sterilization conditions for products with a high MCC content, and identify new opportunities for product development.

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CHAPTER 6

CONCLUSIONS

The increased interest from the food and dairy industry for the use of casein concentrates obtained by membrane filtration created the need to understand the functionality and stability of micellar casein concentrates during heat treatment and storage.

The systematic evaluation of the steady shear rheological properties of micellar casein concentrates (MCC) showed that the apparent viscosity of MCC increased with concentration and decreased with temperature. The viscosity of the micellar casein preparations was affected by the serum protein (SP) removal, with higher SP reduction leading to higher viscosity in dispersions with the same casein concentrations. A modified Arrhenius model that incorporates the temperature and concentration dependencies was developed, and was able to predict very accurately MCC apparent viscosity for a wide range of shear rates, temperatures and concentrations of relevance for the practical use of these casein ingredients.

The investigation of the behavior of MCC during heat treatment and storage showed that MCC was unstable to temperature treatments in the sterilization range, and this instability increased with treatment temperature and decreasing pH. At high temperatures, changes in the mineral equilibrium and partial disintegration of the casein micelle occur, which led to aggregation and coagulation and in turn affected the storage stability of micellar casein concentrates. UHT-treated concentrates were highly instable and strong sedimentation over the time of storage at 25°C was observed, which was mainly credited to the large, visible aggregates that had formed during the heat treatment. Retorted casein preparations showed some browning as well as moderate sedimentation throughout storage time.

It was found that aggregation and coagulation of MCC can be prevented by increasing the pH and/or decreasing the temperature of the heat treatment.

The generated high quality data of casein dissociation will contribute to a better understanding of the interactions in the casein micelle and the effects of pH and temperature on its stability. Furthermore, the findings from these studies and the developed predictive rheological model will provide the dairy and food industry with critical data, necessary for developing applications of micellar casein preparations.