THE CASEIN MICELLE: STABILITY AND HEAT INDUCED INTERACTIONS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Cosmin M. Beliciu

January 2011

THE CASEIN MICELLE: STABILITY AND HEAT INDUCED INTERACTIONS

Cosmin M. Beliciu, Ph. D.

Cornell University 2011

The increasing availability of micellar casein obtained by membrane separation creates a need to better understand the effect of processing conditions, on the stability and properties of this ingredient.

This work focused on studying the heat-induced interactions in micellar casein concentrates, and in micellar casein (MCN)-soy protein (SP) blends. These changes were investigated using a range of physical measurements (zeta potential and particle size measurements, rheological analyses) and chemical analyses (compositional, differential solubility and Native-PAGE analyses).

First, heat induced effects on the properties of micellar casein (MCN)-soy protein (SP) blends (1:1 ratio) were evaluated. Individual and mixed protein systems of 2.5% - 15% concentration were subjected to temperatures between 40ºC - 95ºC. Heat treatments at T≥80ºC induced soy protein (glycinin) denaturation, which resulted in protein aggregation and network formation by disulfide bonding, when soy protein content >6.3%. There was no evidence of cross-linking between soy proteins and casein micelles, as disulfide bonding seemed to occur exclusively between soy protein molecules. In MCN-SP mixtures with soy protein content <6.3%, thermal denaturation of soy proteins induced local phase separation between the casein micelles and the soy protein molecules. This resulted in protein mixtures with low shear viscosity, Newtonian flow behavior and very good stability to sedimentation.

In the second part, the effect of commercial sterilization (UHT - ultra high

temperature - treatment and retorting) on micellar casein concentrates (MCC) of 5% - 10% casein concentration was evaluated. Sterilization affected the stability, viscosity and flow behavior of MCCs, mainly due to loss in solubility of calcium phosphate at the micelle level. Retorting resulted in slight aggregation of casein micelles, while UHT caused the formation of visible aggregates. The UHT treated MCCs had higher viscosity than retorted MCCs, and displayed a solid-like behavior, indicative of structure formation. Drying of MCCs affected their sterilization behavior, as reconstituted micellar casein concentrates (R-MCCs) were more unstable to UHT sterilization than MCCs. The calcium concentration per gram of casein was identified as critical parameter for the heating stability of MCCs.

This work provides critical information for developing commercial applications of micellar casein, as well as micellar casein-soy mixtures.

BIOGRAPHICAL SKETCH

Cosmin Marius Beliciu was born in the beautiful city of Braila, Romania. He grew up discovering this old port on the Danube, learning about its rich cultural heritage and admiring its old architecture. Cosmin graduated from the Gheorghe Munteanu Murgoci High School and went on to the Lower Danube University of Galati. Aspiring to follow in his father's footsteps, Cosmin was admitted in the Food Science and Engineering Department. He strived for academic excellence, got involved in research activities but also sought exposure to the Food Industry through a number of summer internships. Cosmin proudly served as a student member of the Executive Board of the Food Science and Engineering Department and as a student representative in the Lower Danube University Senate. After receiving his Bachelor of Science degree in Food Science and Engineering as valedictorian, Cosmin got married and spent a year working in the brewing industry, for InBev Romania. There he quickly realized that acquiring a new set of skills in Graduate School would provide a boost to his career. Cosmin was admitted in the Cornell University's MS/PhD program, so he and his wife Carmela moved to beautiful Ithaca, NY. In the spring of 2005, Cosmin joined the research group of Dr. Carmen I. Moraru, in the Department of Food Science. The next 6 years were full of educational and fulfilling experiences and quite a bit of hard work. The birth of their daughter Caitlin Dalia brought Cosmin and Carmela the joys and responsibilities of parenthood. With their love and support, Cosmin was able to achieve his research goals. Upon completing his dissertation, Cosmin will remain in Ithaca where he will begin a career in product development as a Food Scientist at International Food Network, Inc.

To my wife Carmela and our daughter Dalia for their love, support, patience and encouragement

ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. Carmen I. Moraru, for her academic guidance and support during my pursuit of the PhD degree, as well as her thoughtful advice in choosing a career path. I would like to thank her for the patience and understanding she displayed whenever my life outside the lab made demands on my time. I would also like to acknowledge Dr. Syed S.H. Rizvi for being my Food Engineering minor committee member, for his guidance, assistance with strategic planning and unique perspective. I would also like to acknowledge Prof. David J. BenDaniel for serving as my Business Administration minor advisor, and for helping me expand my understanding of entrepreneurship and business management. I would also like to acknowledge Dr. D. M. Barbano for serving on my committee, for the extensive logistical help he provided me with and for the engaging discussions.

I would like to acknowledge the past and present members of the Moraru Lab for all their help and lasting memories. Over the last 6 years we have forged strong relationships within these soon-to-be rebuilt Food Science halls. Thank you to Aaron Uesugi, Anne Sauer, Julia Fritsch, Lillian Hsu, Michelle Wang, Mila Wihodo, Teng Ju Tan, and our summer scholars Cynthia L. Lopez-Pena and Isabel Doehner. I would like to also acknowledge the help of Mark Newbold, Michael Adams, Emily Hurt and Tom Gibson for the long processing work days we spent together.

I would also like to acknowledge the Food Science administrative staff of Jannette Robbins, Tom Burke, Sean Schell, Cindy Uhrovcik, Marva Francis, Shelly Green and Matt Stratton for all their help.

Most of all, I would like to thank my wife Carmela for supporting me during the frantic times, for her patience and for everything she had to give up in the past 6 years. I want to thank the rest of my family for their support and encouragement.

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LIST OF ABBREVIATIONS

CCP = colloidal calcium phosphate

EDTA = Ethylenediaminetetraacetic acid

UHT = ultra high temperature

UF = ultrafiltration

PSD = particle size distribution

MSD = multimodal size distribution

DLS = Dynamic Light Scattering

SMUF = simulated milk ultrafiltrate

ANOVA = analysis of variance

kcps = kilo-counts per second

SDS = sodium dodecyl sulfate

HSD = honestly significant difference

CN = casein

MCN = micellar casein

DSC = differential scanning calorimetry

DDSC = first derivative of the DSC curve

LVR = linear viscoelastic region

RC-DC = reduced agent compatible, detergent compatible

PAGE = polyacrylamide gel electrophoresis

APS = additional protein solubilization

MCC = micellar casein concentrate

RO = reverse osmosis

MF = microfiltration

DF = diafiltration

CIP = cleaning in place

RI = refractive index

R-MCC = reconstituted micellar casein concentrate

LIST OF SYMBOLS

ζ = zeta potential, mV

$|\zeta|$ = absolute value of zeta potential, mV

$\overline{d_{eff}}$ = average weighted diameter (effective diameter)

η_{100} = apparent viscosity measured at a shear rate of 100 s^{-1}, Pa*s

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

n = flow behavior index

σ_0 = yield stress, Pa

F_0 = lethality factor

z = the increase in temperature (°C) needed to obtain the same lethal effect in 1/10 of the time. For dairy products, z = 10ºC.

CHAPTER 1

INTRODUCTION: THE CASEIN MICELLE STRUCTURE AND PROPERTIES

Casein ingredients are widely used in foods due to their availability, high nutritive value, blandness and physico-chemical and functional properties (Southward, 1985; Chandan, 1997). The number of new products containing casein/caseinates launched in the US has grown on average by about 22% per year between 2000 and 2008 (Affertsholt, 2009). Due to its exceptional water binding capacity (Walstra, 1990), emulsifying and foaming properties (Phillips et al., 1994; Damodaran, 1997), and viscosity (Konstance and Strange, 1991), casein can be used in a variety of applications. In dairy products, casein ingredients have been used to adjust the protein content and enhance the sensory properties of low-fat dairy products (Mulvihill and Ennis, 2003). Their whipping and foaming properties make them a good candidate for applications in beverages or dessert-type products. The emulsification and water-binding properties of casein and caseinates are useful for confectionery applications and, due to their lysine-rich aminoacid profile, they make a great supplement in bakery products and pasta (Crowley et al., 2002). The majority of the casein preparations traditionally used as ingredients in the food industry have been obtained through methods that involve the destabilization or chemical modification of casein micelles at some point in the production process by acidification, renneting or co-precipitation.

Recent developments in membrane filtration technology have allowed the large scale production of micellar casein concentrates obtained by microfiltration (MF) (Le Berre and Daufin, 1996; Nelson and Barbano, 2005). In these preparations, micellar casein is closer to its native state than in casein ingredients obtained by chemical methods. The differences in the manufacturing process also result in different functionality of the casein preparations obtained by membrane separation as compared

to the traditional casein ingredients. As the availability and affordability of these ingredients increase, there is a need to understand their functionality, stability and the way they interact with other proteins.

Casein micelles: properties and models

Caseins are defined as phosphoproteins that precipitate at an isoelectric pH of 4.6, and make up 82% of the true protein in milk. About 95% of the casein of milk is organized in heterogeneous, roughly spherical aggregates known as casein micelles; these are composed of four individual casein proteins - α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein - in the weight ratio 3:0.8:3:1 (Schmidt, 1982). The major function of caseins in milk is to efficiently transport calcium, phosphate, and protein from the mammary gland to the newborn (Holt, 1992). Their association in micelles allows the total concentration of calcium phosphate in milk to exceed the threshold solubility level, without causing its uncontrolled precipitation in the mammary gland (Holt and Sawyer, 1988; van Kemenade and de Bruyn, 1989). In the absence of these salts, the casein components would have very high viscosity as a result of their open structures (Farrell et al., 2006).

Casein micelles are polydisperse particles with diameters ranging between 100 and 500 nm and a weighted average diameter of 200 nm (de Kruif, 1998; Beliciu and Moraru, 2009). Approximately one third of the micellar volume in solution is casein while two thirds represent hydration of the porous structure, which explains the generally high viscosity of casein suspensions (Dalgleish, 1997).

The structure of the casein micelles is still a matter of debate and different models are discussed in literature (McMahon and McManus, 1998; de Kruif and Holt, 2003; Dalgleish et al., 2004; Horne, 2006). However, a few structural elements are widely agreed upon and their inclusion is critical for successful models. Casein

micelles contain ~6% colloidal calcium phosphate (CCP) on a dry matter basis, which is regarded as the cement holding the micelle together; its removal by EDTA sequestration of calcium (Griffin et al., 1988) or by dialysis against a calcium phosphate free buffer (Holt et al., 1986) results in dissolution of the micelles. The α_{s1}-, α_{s2}- and β-caseins are precipitated by calcium, which binds to their phosphoserine residues at the concentrations of protein and calcium found in most milks. However, κ-casein is not only soluble in calcium, but also interacts with and inhibits the precipitation of the calcium-sensitive group by calcium, initiating the formation of the stable colloidal state (Fox, 2003).

The other critical micelle feature is the location of κ-casein which, according to Fox (2003), must be located so as to be able to: (1) stabilize the calcium-sensitive caseins; (2) allow rapid and specific hydrolysis by chymosin and similar proteases; and (3) permit complex formation with whey proteins when heated in normal milk. All these conditions are satisfied if κ-casein is present in micelles as a surface layer, which is in accordance with the observation that κ-casein content is inversely proportional to micelle size across the micelle size distribution in bovine milk (Donnelly et al., 1984; Dalgleish et al., 1989). Strong proof that κ-casein is essential for the assembly of casein micelles has been brought by Shekar et al. (2006), who showed that by "knocking off" the κ-casein expression gene in mice they are rendered unable to lactate.

For many years the most widely accepted representation of casein micelles was the subunit model (Figure 1.1). It envisioned casein molecules aggregating via hydrophobic interaction into subunits of 15–20 molecules each and creating a well-defined substructure on a scale of 20 nm (Slattery and Evard, 1973; Schmidt, 1982; Walstra, 1999). The pattern of interaction was believed to involve a variation in the κ-casein content of these submicelles, so that the submicelles which were rich in κ-

casein would congregate on the micelle surface, while those that were poor or totally deficient in κ-casein would be located in the interior of the micelle. These sub-units were assumed to be linked by small calcium phosphate clusters.

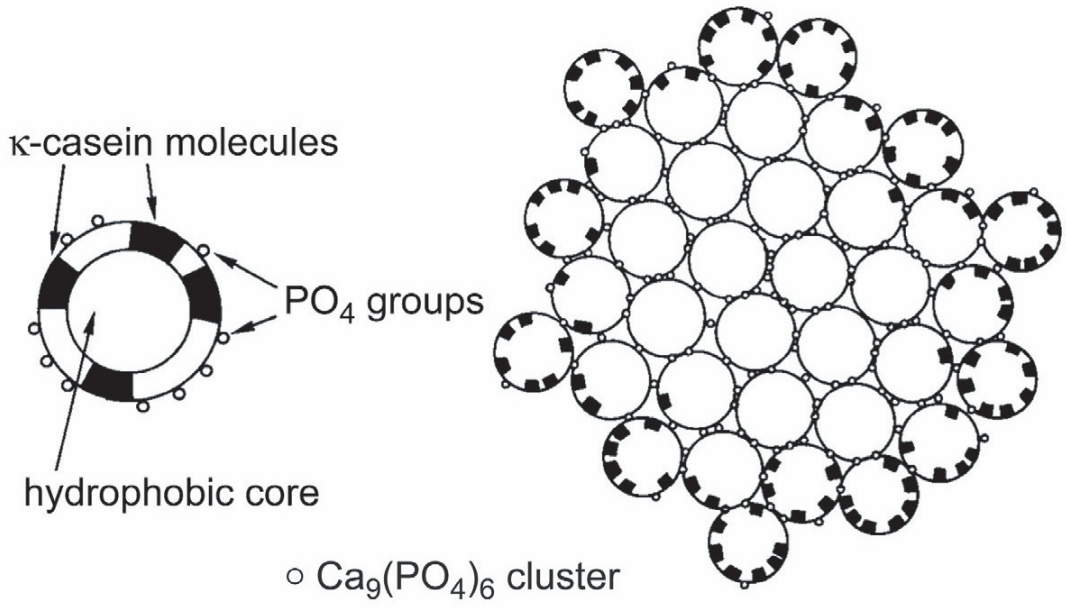

Figure 1.1 The subunit model of casein micelles, showing the surface arrangement of κ-casein (Schmidt, 1982).

The model is not clear in a few regards: it does not explain what drives the division of micelles into κ-casein rich micelles or κ-casein poor micelles, or why κ-casein molecules sometimes favor association with other κ-casein molecules (to form the separated patches in κ-casein rich submicelles), but also associate with other caseins (to complete the building of the submicelle). Another criticism was that in this model calcium phosphate was pictured as a component that enters the casein micelle assembly process after the association of casein chains into submicelles, while both calcium and phosphate are involved in the phosphorylation of the protein chain that occurs immediately post-translation (Horne, 2006).

Newer models question the existence of casein submicelles and adopt the

rheomorphic concept of casein micelle structure (Holt and Sawyer, 1993), which considers that the unstructured proteins form around the amorphous inorganic species, and their ability to bind to the calcium phosphate gives rise to their structure. No specific protein secondary structures or protein–protein interactions are invoked by this concept, however it proposes that κ-casein is located at the surface of the casein micelle.

Based on data showing that the phosphopeptide fraction of β-casein could bind to and stabilize calcium phosphate aggregates (Holt et al., 1998), de Kruif and Holt proposed that the nanoclusters were the centerpiece of casein micelle structure (Figure 1.2). The formation of nanoclusters would drive micelle formation by randomly binding phosphoproteins and causing the formation of an inverted micelle; this new hydrophobic surface can be coated by even more proteins which, in turn, bind more calcium phosphate until a size limited colloid is formed (de Kruif and Holt, 2003).

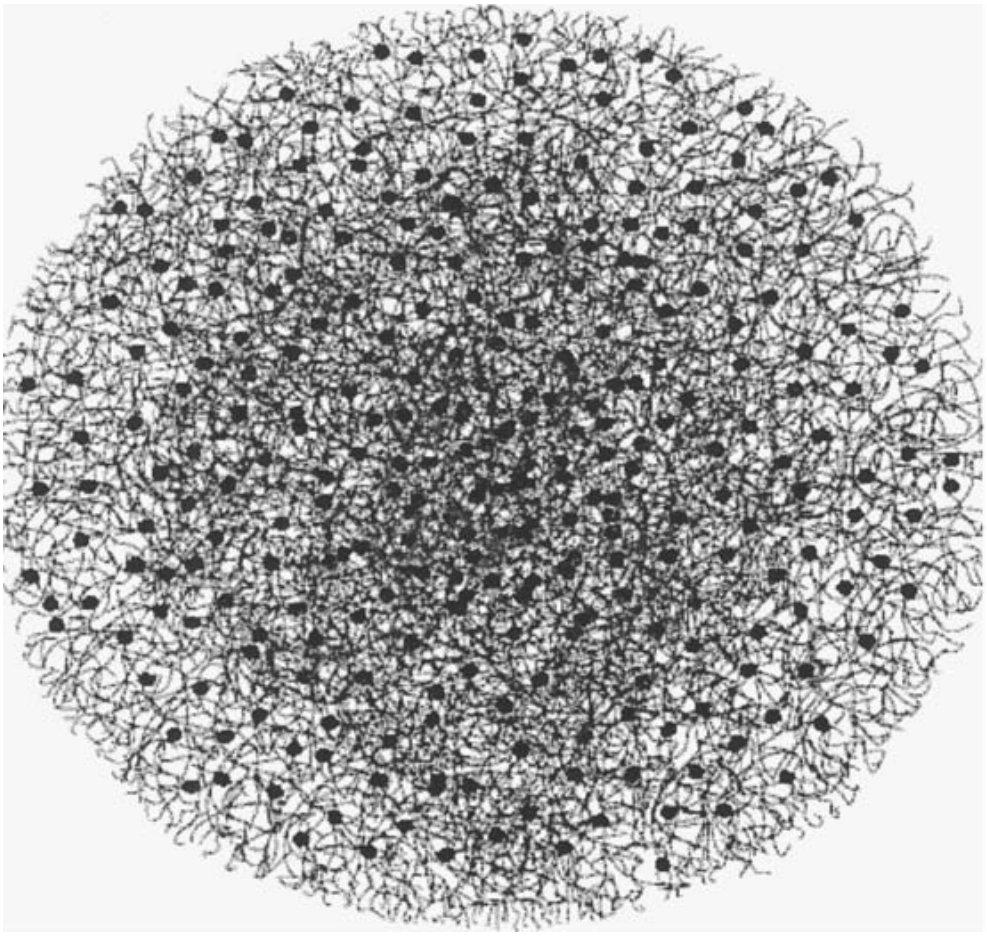

Figure 1.2 The rheomorphic internal model proposed by Holt (2003). Casein monomers are thread-like, while the dark circles represent the calcium phosphate nanoclusters.

The rheomorphic concept and casein micelle model are taken one step further in the dual binding model (Horne, 1998), which accounts for distinct hydrophilic and hydrophobic regions of the casein molecules. Analogous to diblock copolymers, the hydrophobic regions associate, stabilizing the core of the micelle, while the colloidal calcium phosphate particles are attached at the hydrophilic regions.

In this view the amphiphilic nature of the caseins causes them to act more as block copolymers of alternating charge and hydrophobicity - for example, a charged phosphopeptide loop and a hydrophobic train for β-casein or an N-terminal hydrophobic train, followed by a charged loop and a final C-terminal hydrophobic train for α_{s1}-casein (Figure 1.3). Horne proposed that the growth of the calcium phosphate nanoclusters begins the process of micelle formation by binding to the phosphopeptide loop regions, and further protein–protein interactions occur between to the hydrophobic blocks. Micelle formation leads to an internal gel-like structure with embedded nanoclusters of calcium phosphate, and the reaction of κ-casein, which contains only one phosphoserine residue, limits micellar growth by acting as a capping unit, in analogy with the growth of synthetic polymers.

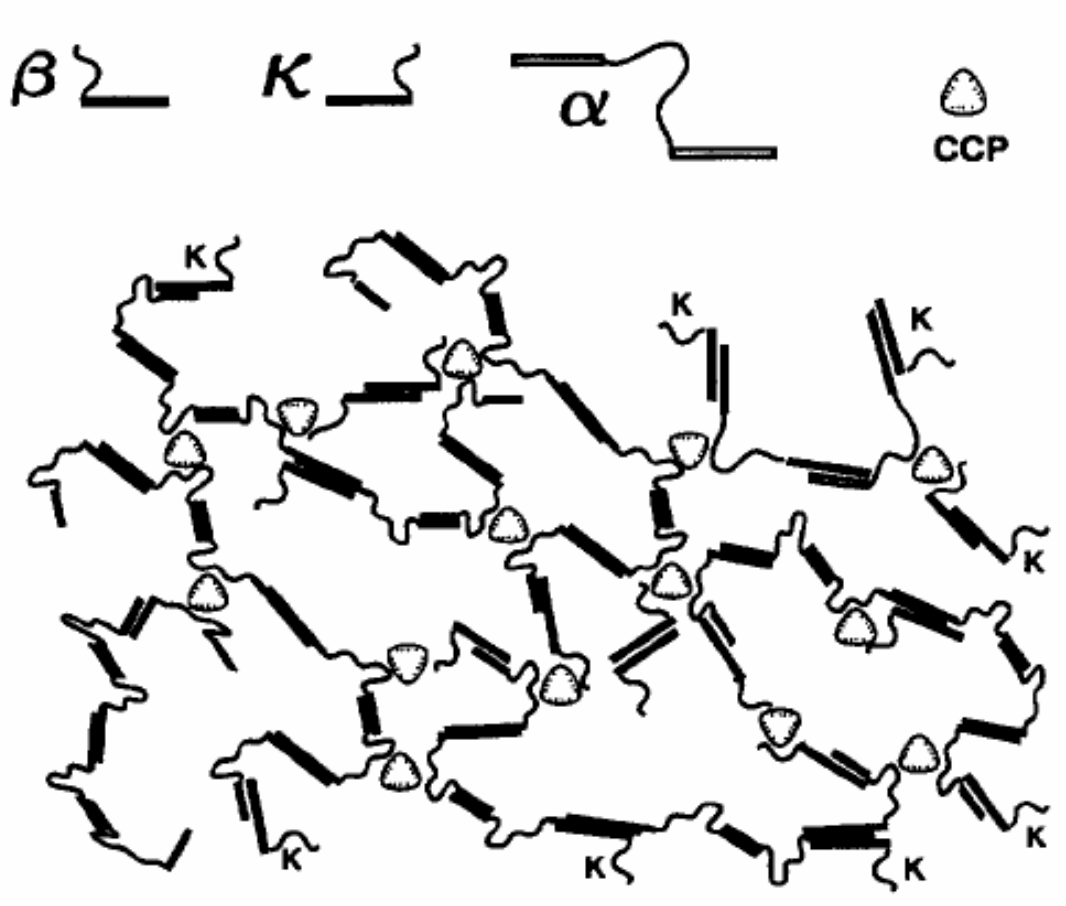

Figure 1.3 The dual binding model of Horne (1998), which considered the casein molecules as diblock polymers, with hydrophobic regions represented as rectangular bars and hydrophilic regions as loops.

The rheomorphic concept of casein micelle structure was reinforced by McMahon and Oomen's (2008) high-resolution transmission electron microscopy stereoscopic images of casein micelles' interior structure. The interlocked lattice model (Figure 1.4) proposes that the caseins form linear and branched chains (2 to 5 proteins long), interlocked by the casein-stabilized calcium phosphate nanoclusters. The "building blocks" of the model are similar to those proposed by Horne (1998): chain extenders (β-casein or α_{s1}-casein), chain branch points (α_{s1}-casein or α_{s2}-casein), chain terminators (κ-casein), and interlocking points (calcium phosphate nanoclusters).

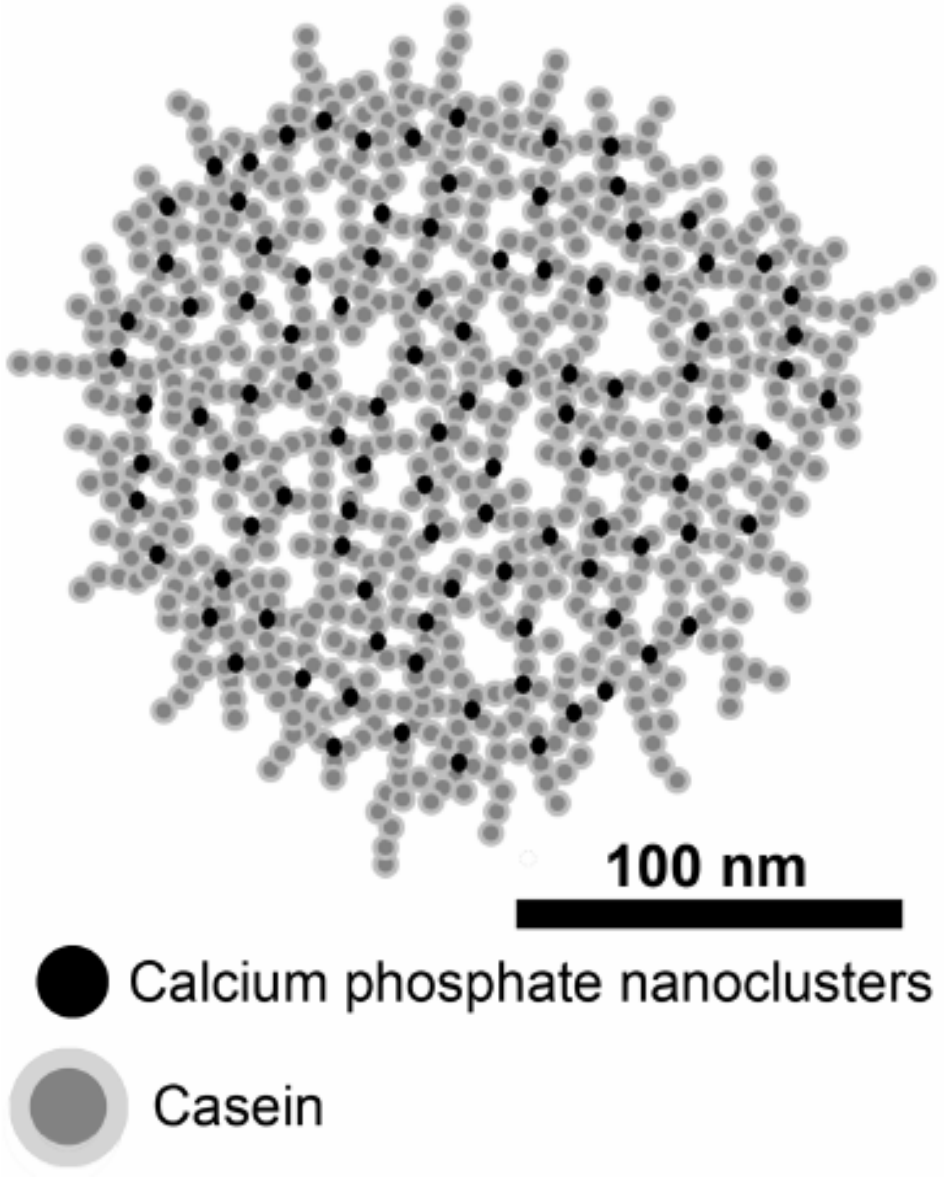

Figure 1.4 The interlocking lattice model of McMahon and Oomen (2008). Casein-calcium phosphate aggregates are present throughout the entire casein micelle and chains of proteins are extended between them.

This model suggests that stabilization of calcium phosphate nanoclusters by phosphoserine domains of α_{s1}-, α_{s2}-, or β-casein would orient their hydrophobic domains outward, allowing interaction and binding to other casein molecules. Hydrophobic interactions as well as calcium bridging are considered the predominant interactions between the caseins; they are involved in chain formation and occur between caseins bound to the calcium phosphate nanoclusters. Hydrogen bonding and other electrostatic interactions are thought to play a role in maintaining the casein micelle's integrity. The combination of the interlocked lattice structure and multiple interactions results in an open, sponge-like colloidal supramolecule that is resistant to spatial changes and disintegration.

Factors that affect the stability of the casein micelle

It is widely agreed that the integrity of casein micelles depends on strong linkages to calcium phosphate, together with some less clearly defined factors such as hydrophobic bonding, hydrogen bonding, salt bridging and entropic forces such as chain entanglements. Because of the multitude of forces responsible for their association, micelles are capable of responding to environmental changes in a variety of ways and at different rates (de Kruif and Holt, 2003). A schematic representation of the effect of some environmental factors on the casein micelles in milk is presented in Figure 1.5 (Gaucheron, 2005).

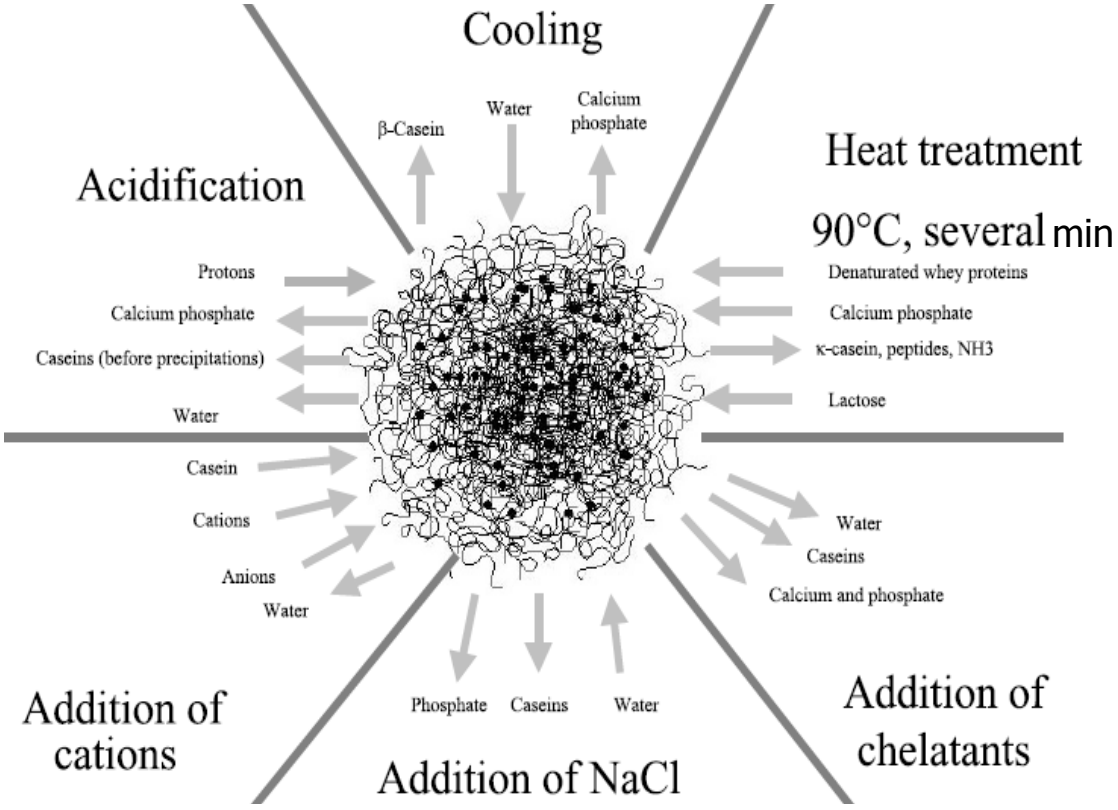

Figure 1.5 The effect of some environmental factors on the casein micelle (Gaucheron, 2005).

When the pH of milk is decreased, the different acido-basic groups of milk's constituents (organic and inorganic phosphate, citrate, carboxylic residues) become increasingly more protonated. As a consequence, micellar calcium phosphate is dissolved and the caseins are released into the diffusible fraction of milk. The extent of dissociation depends on both pH and temperature (Visser et al., 1986; Dalgleish and Law, 1989). It has been shown that pH does not affect the size of casein micelles until a pH of about 5.5 (Dalgleish and Law, 1988), and that the micelles do not dissociate due to their reduced charge when calcium phosphate is dissolved by acidification. In fact, even after the pH is reduced to the isoelectric point (4.6) and the caseins aggregate, the particles retain the appearance of casein micelles, but the separate particles merge over the course of a few hours, indicating more subtle changes in structure following the loss of calcium phosphate (Rollema, 1992).

Casein micelles are remarkably stable against heat treatments. However, several changes were documented, depending on the intensity of the heat treatments. Among these changes, it has been described that calcium phosphate present in the diffusible fraction becomes less soluble during heat treatment (Holt, 1995), but this does not correspond to the simple precipitation of diffusible calcium phosphate. The new, more insoluble phases formed are not clearly identified although some authors reported the formation of crystalline β-tricalcium phosphate (Nelson et al., 1989) or hydroxyapatite (Visser et al., 1986).

As opposed to the heat treatments, the solubility of micellar calcium phosphate increases when the temperature is decreased, and consequently the cooling of milk induces dissolution of micellar calcium phosphate (Pierre and Brulé, 1981). These changes are reversible and the original distribution may be restored upon warming. Low milk temperature was also shown to have an impact on the native casein micelles – Creamer et al. (1977) showed that 26% of the β-casein exits the micelles in 24 hrs at

4°C, due to decreased intensity of hydrophobic bonds. The upper limit of the fraction of β-casein that migrates from the micelle was established at 40% (Davies and Law, 1983), which suggests that this might be the portion of total micellar β-casein that is not bound to the calcium phosphate in the micelles.

The addition of NaCl to milk leads to a slight decrease in pH and an increase in Ca^{2+} concentration in the diffusible phase. These changes were proposed to correspond to exchanges of Na^{+} with divalent cations or protons which were attached directly to phosphoseryl residues of casein molecules (Le Ray et al., 1998). Philippe et al. (2005) investigated the effect of calcium chloride addition to milk (8 mM/kg), and after separating the aqueous phase via ultracentrifugation (100,000g/1hr, 20°C) it was observed that about 80% of the added ion was associated to casein micelles, while inorganic phosphate and citrate ions tended to be displaced from the diffusible fraction towards the micelles. As a result, casein micelles were modified - more specifically, their zeta potentials became less negative, hydration as well as their heat stability decreased.

Addition of κ-casein to milk was thought to induce micelle dissociation, but a study by Holt (1998) showed only a small reduction over 2 days in the proportion of larger casein micelles and an increase in the proportion of smaller micelles; even at the end of the holding time there were large micelles of similar size to those found in the original milk, which implies considerable strength of the cross-links with calcium phosphate in the micelles.

Heat-induced changes to casein micelles in milk

Heat induced changes in milk occur as a function of temperature, varying with respect to rate. Up to 90°C, reactions occur at a relatively slow rate and are largely reversible - with the exception of serum protein denaturation. At higher temperatures,

reactions occur rapidly and are mostly irreversible (O'Connell and Fox, 2003). Heating milk at temperatures >90°C was reported to lead to an increase in casein micelle size (Mohammad and Fox, 1987), which was confirmed in UHT-sterilized milk and UF-concentrated milk (McMahon, 1996). Electron micrographs of casein micelles in direct UHT treated milk showed a large number of small particles, presumably consisting of aggregated serum proteins and/or dissociated caseins (Rollema et al., 1987). The amount of casein that could not be sedimented via ultracentrifugation increased when serum protein free casein dispersions were heat treated at temperatures >110°C, and it was demonstrated that ~40% of the total dissociated protein is κ-casein (Aoki et al., 1974).

Fox et al. (1967) suggested that when heating milk, the calcium that has been previously associated with citrate precipitates as calcium phosphate, thus rendering the citrate free. It was proposed that the citrate solubilizes colloidal calcium phosphate, thus compromising the integrity of casein micelles and leading to dissociation of κ-casein. It has also been suggested that, on heating, the net negative charge on the casein micelles increases to such an extent that hydrophobic bonds [much weaker at temperatures >100°C, according to Poland and Scheraga (1965)] can no longer maintain micellar integrity (Singh and Fox, 1987).

Another change that can occur due to high heat treatments in skim milk is dephosphorylation of casein (hydrolysis of organic phosphate from phosphoserine); Belec and Jenness (1962) have shown that after 20 minutes at 120°C, about 10% of micellar casein has undergone dephosphorylation. This reaction reduces the heat stability of the system and has the potential to disrupt the native micellar structure, which is dependant on colloidal calcium phosphate links (Dalgleish et al., 1987). Some proteolysis also occurs on heating, as it has been shown that heating milk to sterilization conditions (120°C for 30 min) increases the non protein nitrogen content

by 35% as compared to untreated milk (Saidi and Warthensen, 1993). Heat-induced polymerization has been reported in UHT-treated serum protein-free micellar casein dispersions (Zin El-Din and Aoki, 1993), which is thought to result due to different types of crosslinks such as disulphide bonds, crosslinks arising from the Maillard reactions (when lactose is present) or through the formation of thermally generated colloidal calcium phosphate links (Singh, 1994).

It is widely accepted that the pH of milk decreases during heating, due to a number of reactions: thermal oxidation of lactose to organic acids (responsible for 50% of heat-induced acidification) such as formic acid (80% of the total acids that form), lactic acid, acetic and pyruvic acid (van Boekel et al., 1989). Primary calcium phosphate - $Ca(H_2PO_4)_2$ - and secondary calcium phosphate - $CaHPO_4$ - can precipitate during heat treatments as tertiary calcium phosphate - $Ca_3(PO_4)_2$, concomitantly releasing H^+, which is thought to contribute 30% of heat-induced acidification (O'Connell and Fox, 2003). As a point of reference, for milk the pH decreases slightly during UHT sterilization and decreases by 0.2-0.3 units during batch sterilization (Berg and van Boekel, 1994). Decreases in pH may lead to destabilization of the micelle due to dissolution of micellar calcium phosphate.

Milk is supersaturated with respect to calcium, magnesium, phosphate and citrate, but their salts are kept in dispersion because a large part of the calcium and phosphate are incorporated into the casein micelles (Nieuwenhuijse and van Boekel, 2003). Holt's model, referenced in the previous section, implies that micellar calcium phosphate behaves as if in thermodynamic equilibrium with the serum phase, which is in accordance with work by Geerts et al. (1983), who established that many heat induced changes in salts are largely reversible on cooling.

Zeta potential (ζ) can be considered a relative indicator for the colloidal stability of the casein micelles. Experiments performed by Anema and Klostermeyer

(1997) on reconstituted skim milk showed that, when samples were heated at 140°C for a short time (5 min), the absolute value of zeta potential |ζ| increased; if the heat treatment was continued over an extra 15 minutes, the |ζ| linearly decreased with treatment time (Figure 1.6). It was proposed that initial changes in the zeta potential are due to association of whey proteins with casein micelles while subsequent changes in the zeta potential are caused by precipitation of calcium phosphate onto the micelles and κ-casein dissociation. If the pH was increased to 7.1, micellar κ-casein was more susceptible to dissociate and formed complexes with the serum proteins in the continuous phase; the increase in |ζ| was no longer observed.

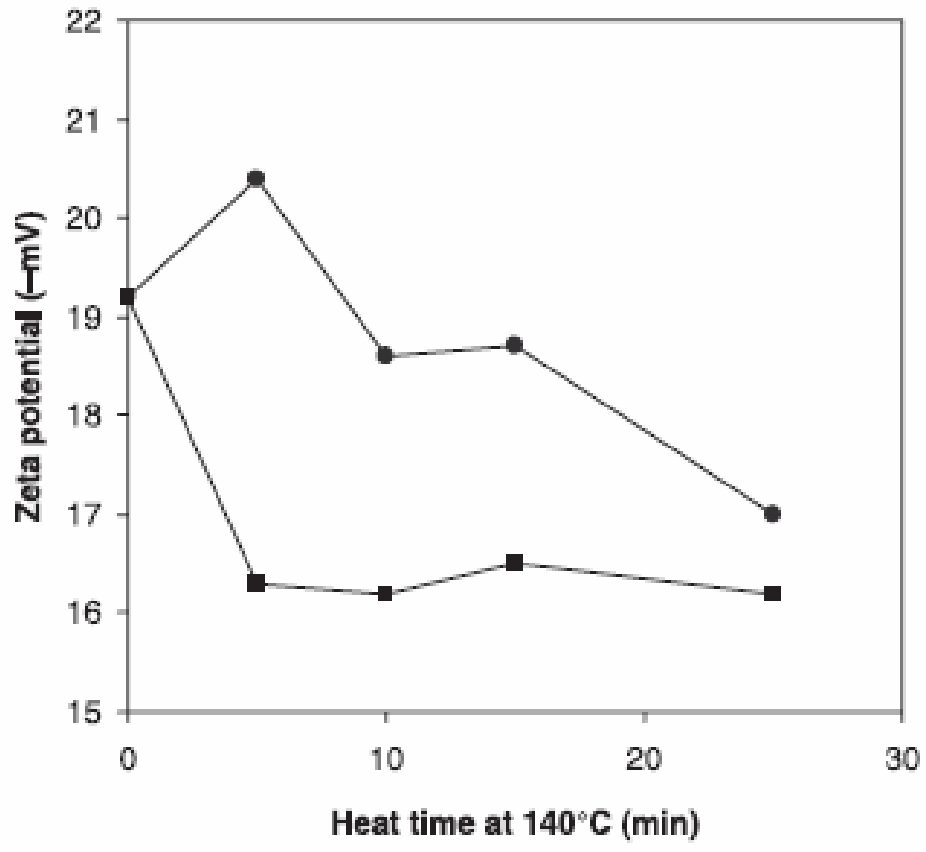

Figure 1.6 Effect of heating time at 140ºC on the zeta potential of casein micelles in skim milk that was adjusted to pH 6.5 (●) or 7.1 (■) previously to heating (Anema and Klostermeyer, 1997).

Dalgleish (1992) identified two major mechanisms that could induce a tendency of casein micelles to sediment in stored UHT treated milks and UF concentrated milks that were UHT treated. One of the mechanisms involves serum protein denaturation. He proposed that the native structure of casein micelles in high heat-treated milk products could be modified due to heat effects, which can cause the serum proteins to denature and react with the micellar κ-casein via disulfide bonds (Dannenberg and Kessler, 1988; Dalgleish, 1990; Beaulieu et al., 1999), forming an altered surface layer and possibly inducing some limited aggregation. The second possible mechanism involves deposition of calcium phosphate onto the micelles (Wahlgren et al., 1990). This process may also lead to increased micelle weight and a tendency to sediment over long timescales.

The majority of the experimental work reviewed in this chapter has been conducted in skim milk or reconstituted skim milk. However, with the increased popularity and accessibility of micellar casein preparations there is a need to understand the properties of casein micelles under different conditions than in milk. Micellar casein is obtained through membrane separation, an exclusively physical process in which components such as the casein micelles in the feed material are concentrated, while serum proteins, lactose and salts pass through the membranes' pores and are removed in the permeate fraction. The advantages of membrane filtration technology are that there is no chemical modification of the casein micelles and limited heat treatment is involved in processing – which leaves the micelle intact and ready to be studied.

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CHAPTER 2

RESEARCH OBJECTIVES

Objective I

Investigate the effect of environmental conditions (dispersing medium and temperature) on the casein micelle size.

Objective II

Evaluate the heat induced interactions between micellar casein and soy proteins.

a. Effect of protein concentration and heat treatment temperature on the physical properties of micellar casein – soy protein mixtures
b. Evaluation of intermolecular interactions in heat treated micellar casein – soy protein mixtures

Objective III

Evaluate the effect of commercial sterilization regimes on micellar casein concentrates and investigate the mechanisms responsible for heat induced changes.

CHAPTER 3

THE EFFECT OF ENVIRONMENTAL CONDITIONS (DISPERSING MEDIUM AND TEMPERATURE) ON THE CASEIN MICELLE SIZE

Abstract

The objectives of this study were to investigate the effect of the solvent on the accuracy of casein micelle particle size determination by DLS at different temperatures and to establish a clear protocol for these measurements. DLS analyses were performed at 6°C, 20°C and 50°C using a 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments, NY). Raw and pasteurized skim milk were used as sources of casein micelles. Simulated milk ultrafiltrate (SMUF), ultrafiltered (UF) water, and permeate obtained by UF of skim milk using a 10KDa cutoff membrane were used as solvents. The pH, ionic concentration, refractive index and viscosity of all solvents were determined. The solvents were evaluated by DLS to ensure that they do not have a significant influence on the results of the particle size measurements. Experimental protocols were developed for the accurate measurement of particle sizes in all solvents and experimental conditions. All measurements had good reproducibility, with coefficients of variance below 5%. Both the solvent and the temperature had a significant effect on the measured effective diameter of the casein micelles. When UF permeate was used as a solvent, the particle size and polydispersity of casein micelles decreased as temperature increased. The effective diameter of casein micelles from raw skim milk diluted with UF permeate was 176.4 ± 5.3 nm at 6°C, 177.4 ± 1.9 nm at 20°C and 137.3 ± 2.7 nm at 50°C. This trend is justified by the increased strength of hydrophobic bonds with increasing temperature. Dilution with water led to micelle hydration, which significantly affected the particle size measurements, especially at 6°C and 20°C. Overall, the results of this study suggest that the most suitable solvent

for the DLS analyses of casein micelles is casein-depleted UF permeate. SMUF seemed to give accurate results only at 20°C, while water should not be used as a solvent.

Introduction

Particle size and particle size distribution (PSD) of dispersed systems affect key properties such as surface area, reactivity, opacity, packing density and rheological properties. Quantifiable changes in particle size or PSD give valuable indications about aggregation or dissociation phenomena, which can help predict the stability and a range of macroscopic properties of colloidal systems.

The particle size and structure of casein micelles are of great interest and have been the focus of numerous investigations. Holt (1985) critically compared different methods used for measuring the average size and size distribution of casein micelles, including electron microscopy, light scattering and controlled pore glass chromatography. He concluded that natural variations in average micelle size, deficiencies in electron microscopy methodology, as well as overestimation of micelle effective diameter by light scattering methods have led to discrepancies in reported casein micelle size and particle distribution data. In recent years, Dynamic Light Scattering (DLS), which is both fast and non invasive, has become the method of choice for evaluating particle size and PSD in a range of colloidal systems. Since casein micelles scatter light very well, the development of DLS had brought a lot of promise for the accurate determination of casein micelle size, as well as the investigation of its interactions with other molecules or its response to environmental factors. The main advantages of light scattering are that it avoids drastic changes in the environment of micelles and that the measurements are performed on a large number of particles (Holt, 1975).

Dalgleish and Pouliot (1987) studied the effect of high heat treatment of milk on the casein micelles size, and were able to effectively show that micelle size increased as a result of aggregation of κ-casein and serum proteins. Walstra et al. (1981) used DLS to show that the hydrodynamic diameter of casein micelles decreased by about 10 nm when rennet was used to cleave the κ-casein and remove the stabilizing macropeptide. Dalgleish and Horne (1989) confirmed that the surface of the casein micelle is covered predominantly by κ-casein by analyzing the protein composition of micellar fractions whose sizes had been established by DLS. In another study, Griffin (1988) used DLS to investigate the effect of the partial removal of micellar calcium phosphate on the structure and size of the casein micelle. Later, de Kruif (1997) successfully used DLS to study and characterize the kinetics of casein micelle aggregation as a result of acidification.

The DLS method correlates the fluctuations of the average intensity of scattered light over time with the size of particles in suspension. The main quantity measured is the translational diffusion coefficient *D*, which can be used to determine the apparent particle diameter *d*, using the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi\eta(t)d} \qquad (1)$$

where:

k_B = Boltzmann constant (1.38×10^{-23} m^2 kg s^{-2} K^{-1})

T = temperature (K)

η(t) = viscosity (Pa*s)

t = time (s)

In biological systems, in which a wide distribution of particle sizes is typically present, the measured effective diameter ($\overline{d_{eff}}$) is an average diameter weighted by the

intensity of light scattered by each particle.

One of the main challenges associated with the particle size measurement by DLS of food colloids, including casein micelles, is the fact that measurements need to be performed in clear solutions (Alexander and Dalgleish, 2006). Moreover, since the autocorrelation function of the DLS software compares the signal with a time delayed version of itself, the accuracy of the measurement is susceptible to secondary scattering. To avoid this, protein systems must be diluted to very low concentrations in order for the incident photons to be scattered only once by the sample, typically in the range of 10^{-3} μg/mL. As most food systems, including milk, are both opaque and concentrated, significant dilution is required (Dalgleish and Hallett, 1995). When doing this, much consideration must be given to ensuring that the analyzed particles maintain their native structure during the DLS analyses. This is rather challenging, because the use of the wrong diluting agent can lead to a change of the native pH and ionic equilibrium, which in turn can result in dissociation or aggregation of the studied colloidal particles, thus affecting the results of the particle size measurement.

Although DLS has been used for some time for measuring the size of casein micelles, the protocols used for performing these measurements have not been consistent from one study to another. Different solvents have been used for diluting the milk. Some studies used lactose-free simulated milk ultrafiltrate, also known as SMUF (Walstra, 1981; Roesch et al., 2004). SMUF was originally designed by Jenness and Koops (1962) as a lactose-free buffer for the dispersion of caseins or serum proteins in studies of physico-chemical properties such as heat stability, electrophoresis or ultracentrifugation. SMUF has since become a very popular solvent or model system for various investigations, including studying the effect of milk salt concentration on the structure of acidified micellar casein systems (Auty et al., 2005), or the influence of whey protein heat-denaturation on the acid induced gelation of

casein (Schorch et. al., 2001). de Kruif (1997) used ultrafiltered (UF) water as a solvent in measurements of casein micelle sizes, while Ono et al. (1983, 1990) used both lactose-free SMUF and permeate from skim milk UF. In the latter case, the size of casein micelles was calculated based on a wavelength vs. turbidity relationship. Tuinier et al. (1999) and Maroziene et al. (2000) used milk UF permeate as a solvent in their investigations of casein micelles interactions with exocellular polysaccharides and pectin. Karlsson et al. (2005) also used UF permeate as a solvent in their DLS investigation, while Anema and Li (2003) used a Ca-immidazole buffer. Strawbridge et al. (1995) and Spagnuolo et al. (2005) also used a buffer containing 5 mM $CaCl_2$ and 20 mM imidazole as a solvent for DLS analysis.

The choice of solvent and the different protocols used for DLS analyses could be the reason why the micelle sizes reported in literature are not always consistent from one study to another. Therefore, the objectives of this study were to investigate the effect of the diluting medium on the casein micelle particle size measurement using DLS and to establish a clear methodology for these measurements. The effect of temperature on the casein micelle particle size was also investigated.

Materials and methods

Materials

Raw skim milk and HTST pasteurized skim milk (Cornell Dairy, Ithaca, NY) with a residual fat content of about 0.1% were used as sources of casein micelles in this study. Three different solvents were used as diluting media for particle size measurements: ultrafiltered water ("water"), lactose-free simulated milk ultrafiltrate ("SMUF") and casein-depleted fresh permeate from milk ultrafiltration ("UF permeate"). SMUF was prepared using analytical grade mineral salts according to the method reported by Jenness and Koops (1962). The composition of SMUF is shown in

Table 3.1. The UF permeate was obtained in the pilot plant at Cornell University (Ithaca, NY) from pasteurized skim milk, using a polyethersulphone spiral wound membrane with a nominal cut-off of 10 kDa (GEA NIRO Inc., Hudson, WI). The total residual protein concentration in the UF permeate was 0.06% w/w. In order to remove any impurities, all solvents were filtered immediately before use in the particle size analyses using a 0.2 μm nylon syringe filter (Fisher Scientific, Pittsburgh, USA).

Table 3.1 Composition of the lactose-free simulated milk ultrafiltrate (SMUF) (adapted from Jenness and Koops, 1962)

Ingredient	**Amount (g/L)**
KH_2PO_4	1.58
K_3 citrate·H_2O	1.20
Na_3 citrate·$2H_2O$	1.79
K_2SO_4	0.18
$CaCl_2·2H_2O$	1.32
$MgCl_2·6H_2O$	0.65
K_2CO_3	0.30
KCl	0.60
KOH	Add to pH 6.6

Particle size analyses

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

To maximize the accuracy of the measurement, skim milk was diluted prior to the DLS analysis using the three solvents mentioned above. As a measure of an acceptable sample concentration, the DLS equipment manufacturer's recommendation that the signal intensity measured by the instrument be between 700 and 900 kilocounts per second (kcps) was followed. The milk: solvent ratio required to achieve the recommended signal intensity was 150µL milk per 1L solvent.

The particle size measurements were performed at constant temperatures of 6°C, 20°C and 50°C, respectively. The lower temperature was chosen to represent refrigeration conditions, 20°C was chosen since this is a temperature commonly used for DLS analysis, while the higher temperature was selected to be just below the denaturation temperature of serum proteins. The skim milk samples were equilibrated either in the refrigerator (at 6°C) or in a water bath (for 20°C and 50°C) for at least an hour before the measurements. The solvents were also tempered at the measurement temperature and were filtered immediately before dilution. The exception was the SMUF used in the 50°C measurements. In this particular case, SMUF was kept under moderate stirring at room temperature, then filtered using the 0.2 µm syringe filter, and heated on a water bath immediately before the dilution step. For all solvents and temperatures, the disposable cuvettes used for the measurements were equilibrated in the temperature controlled chamber of the particle size analyzer for 5 minutes. After that the diluted test samples were pipetted in the cuvette and allowed a 2.5 min temperature equilibration, after which the DLS measurement was promptly started.

Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp., Holtsville, NY), which converted the experimental data into size distributions. The software contains a Dust Filter algorithm that improves the quality of the measurements by rejecting data that was corrupted by scattering from random particles such as air bubbles or dust. The dust filter cut-off

parameter was set at 30, which is the optimal value suggested by the manufacturer when the expected particle size is in the range of hundreds of nm. Seven replicate measurements were performed for each experimental condition. Each measurement consisted of 7 subsequent individual runs of 30 s duration. For each measurement, the relative particle size distribution, the intensity weighted effective diameter ($\overline{d_{eff}}$) and the polydispersity index (p) were determined.

Identification of milk protein classes and residual fat globules

In order to positively identify each class of milk proteins by DLS, a native solution of serum proteins was obtained by precipitating casein out of skim milk at isoelectric pH (4.6) according to the IDF sample preparation procedure for quantifying the non-casein nitrogen in milk (IDF, 1964). 10 mL of milk were diluted with 75 mL of 40°C distilled water in a volumetric flask that was then placed in a water bath at 40°C. 1 mL of 10% acetic acid was added to the flask, which was gently mixed for 10 min before the addition of 1 mL 1N sodium acetate solution. The flask was cooled in an ice bath to 20°C, diluted to 100 mL with 20°C distilled water and filtered through dry Whatman No. 42 filter paper (Whatman Int. Ltd., Maidstone, UK). The serum protein solution obtained using this procedure was very clear and had a protein concentration that did not require further dilution prior to particle size analyses.

The residual fat globules in the skim milk were isolated and their size distribution evaluated by DLS using the method of Michalski et al. (2001). Skim milk (0.1% residual fat) was diluted (1:1) with 35 mM/L EDTA/NaOH, pH 7.0 buffer in order to dissociate casein micelles and aggregates. This mixture was dispersed in a 0.1% SDS solution and analyzed by DLS at 20°C. Both the 35mM/L EDTA/NaOH, ph 7.0 buffer and the 0.1% SDS solution were filtered using a 0.2 μm nylon syringe filter, immediately before the dilution step. In this experiment, the dilution ratio was

adjusted to 150 μL skim milk per 1L solvent.

Viscosity and refractive index measurements

In order to account for the solvent properties in the calculation of particle sizes, the viscosity and refractive index for each solvent and experimental temperature were determined and their respective values were introduced manually in the BIC software. The viscosity of each solvent at the three experimental temperatures was determined using an Advanced Rheometric Expansion System (ARES) strain-controlled rheometer, in conjunction with the Orchestrator data collection and analysis software (TA Instruments, New Castle, DE). Measurements were performed using the double wall couette geometry. Temperature control was achieved using a Julabo FS18-MW heating and refrigerated circulator (Julabo USA, Inc., Allentown, PA). The viscosity was measured using a steady mode, single point test set-up at a shear rate of $1s^{-1}$. The refractive index for each solvent was determined using a digital fiber optic refractometer, after the filtration step (Misco Products Division, Cleveland, OH). The viscosity and refractive index values of the solvents under the test conditions are shown in Table 3.2.

Table 3.2 Viscosity and refractive index of the solvents studied at the three test temperatures.

Solvent	Temperature, °C	Average viscosity ± SD, cP	Refractive index
UF permeate	6	1.809 ± 0.022	1.341
	20	1.154 ± 0.019	1.342
	50	0.804 ± 0.041	1.341
SMUF	6	1.756 ± 0.037	1.335
	20	0.996 ± 0.021	1.334
	50	0.521 ± 0.008	1.334
Water	6	1.652 ± 0.077	1.333
	20	1.023 ± 0.015	1.333
	50	0.467 ± 0.029	1.333

Mineral composition analysis

The ionic concentration in the solvents was tested at the Dairy One Forage Analysis Laboratory (Ithaca, NY, 14850). The following ions were quantified: calcium, phosphorous, magnesium, potassium, sodium and chloride. With the exception of chloride, all other ions were determined using a Thermo Jarrell Ash IRIS Advantage HX Inductively Coupled Plasma (ICP) Radial Spectrophotometer (Thermo Scientific, WI 53711). Samples were acidified to match standards (5mL sample + 545µL 1.5N HNO_3 + 250µL 0.5N HCl), then aspirated by ICP. Results from ICP were multiplied by a correction factor of 1.159 to account for the dilution during acidification. The chloride content was determined by acidifying 5 mL of sample with 45ml 0.2N HNO_3, followed by potentiometric titration with $AgNO_3$ using Brinkman Metrohm 716 Titrino Titration Unit (Brinkmann Instruments Inc., NY 11590)

equipped with a silver electrode. All ionic concentrations were reported in milligrams per liter (Table 3.3).

Table 3.3 Ionic concentrations for the solvents used to dilute the milk samples (measured values) and milk serum (values from literature).

Ion	UF permeate	SMUF	UF water	Milk serum [a]
	Ion concentration (mg/L)			Ion concentration (mg/kg)
1.Calcium	253	351	2.10	390
2.Phosphorus	407	352	0.05	360 [b]
3.Magnesium	69	78	4.10	70
4.Potassium	1423	1429	< 0.01	1500
5.Sodium	268	368	0.70	450
6.Chloride	1026	1140	1.10	1100
Total 1 to 6	3446	3718	< 8.06	3870

[a] Average values. Source: Walstra et al. (1999), p. 8

[b] Estimated from total phosphate

Statistical analysis of data

One way analysis of variance (ANOVA) and two-way ANOVA with an interaction effect were performed in order to determine significant differences between the values of effective diameter and polydispersity ($p < 0.05$). Differences between means were compared using the Tukey-Kramer HSD test and the statistical software package JMP version 7.0 (SAS Institute Inc., Cary, NC).

Results and Discussion

Before the results of the study are presented, a brief discussion about the representation of DLS results is necessary. The results of PSD measurements can be displayed either as a lognormal distribution or as a multimodal size distribution (Figure 3.1). The lognormal distribution has been widely used before software packages associated with the PSD instrumentation had the capacity to create multimodal size distributions. This distribution offers a simplified representation of PSD, which allows relatively easy comparisons between different samples or replicate measurements of the same sample, as well as the calculation of a single effective diameter for the analyzed sample. The multimodal size distribution on the other hand offers more information regarding the presence of groups of particles or molecules of different sizes, but the accuracy of such representations depends greatly on the algorithms used by the specific software. When determining PSD in systems known to have multiple classes of particles, it is very important that both the lognormal and multimodal representations are used when analyzing the data, which was the approach used in the current study.

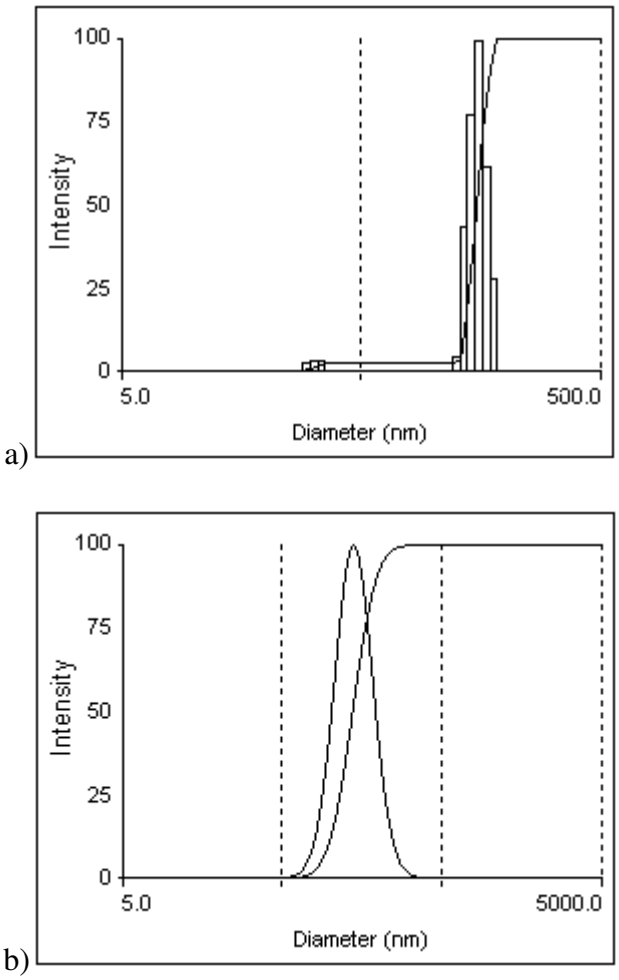


Figure 3.1 Different representations of particle size distributions: a) multimodal size distribution (MSD); b) lognormal distribution. Sample: raw skim milk in UF permeate at 50°C.

Evaluation of solvents

Before determining the PSD of casein micelles, PSD measurements were performed for all three solvents at 6°C, 20°C and 50°C, and the results of these measurements were defined as the "solvent baselines". This was an important step in ensuring that the solvents themselves did not bring any significant contributions to the particle size distribution of the samples.

As expected, water was virtually free of particles, as indicated by negligible signal intensity (1.5 to 2 kcps). For SMUF, the baselines at 6°C and 20°C were also free of particles and showed very low signal intensity (1.5-2.5 kcps). When determining the baseline of fresh SMUF at 50°C, a pronounced and rapid increase in particle count and size during the measurement was observed (Figure 3.2). This phenomenon has also been noted by Jenness and Koops (1962) when heating SMUF to 100°C, and was attributed to the dissolution and precipitation of amorphous calcium phosphate (Andritsos et al., 2002; Spanos et al., 2007). Although Jenness and Koops (1962) reported that the process was reversible upon cooling to 3°C, in the current study the presence of particles was noticed even after cooling, which indicated that the precipitation process was not fully reversible. It is also worth noting that precipitation was observed when a sample of SMUF was kept under stirring overnight at room temperature (T ~ 23°C) which indicated that, given sufficient time, precipitation can take place at lower temperatures than previously reported. In order to avoid this problem, the SMUF was filtered using a 0.2 μm syringe filter immediately before using it to dilute the skim milk samples. Baselines of filtered SMUF at 50°C had signal intensities that never increased above 9 kcps, as precipitation occurred at a much slower rate when the "seed" particles were removed.

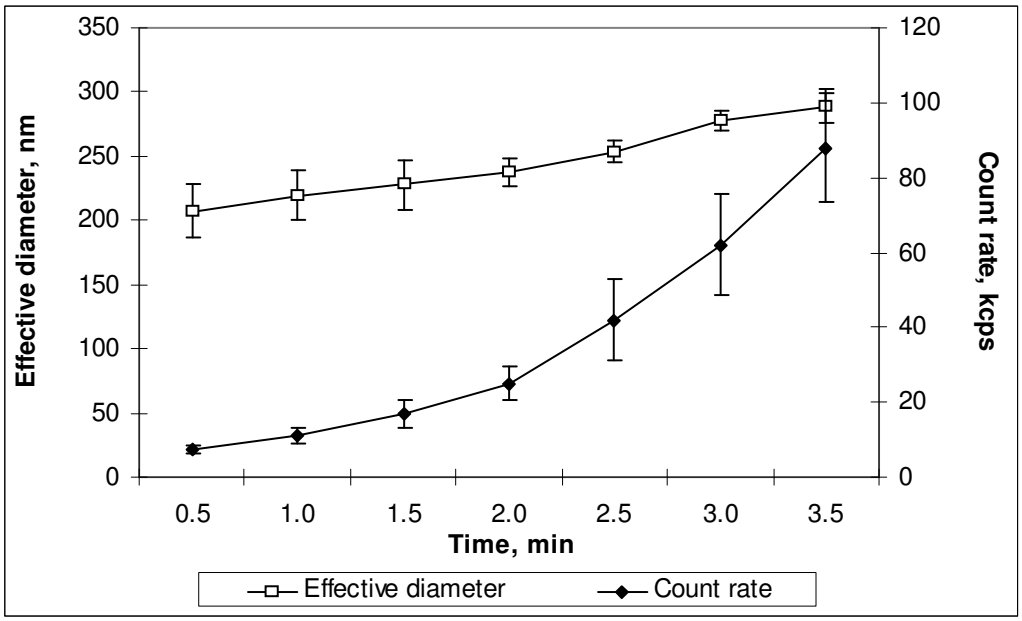
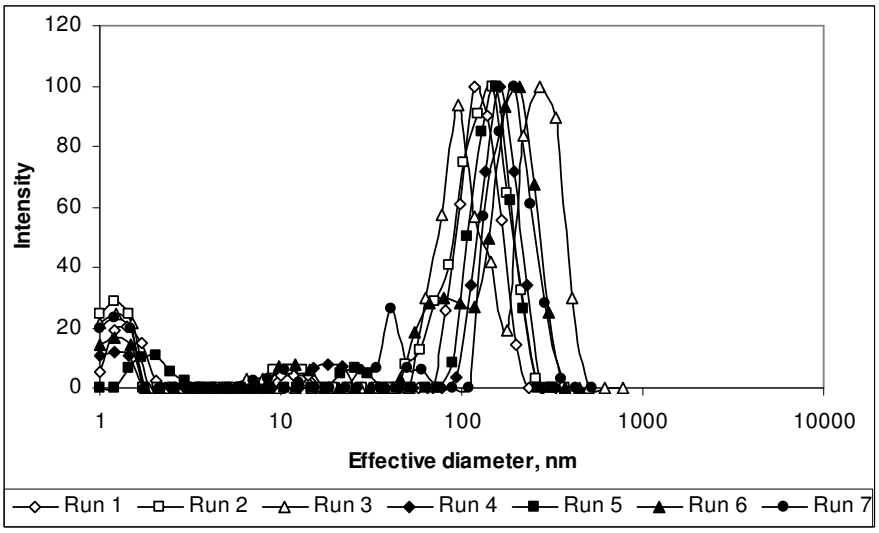


Figure 3.2 Dynamics of signal intensity (count rate) and effective diameter of unfiltered SMUF at 50°C. kcps = kilocounts per second.

The UF permeate was the solvent closest to milk serum, the natural environment of casein micelles. Baseline determinations performed at 6°C, 20°C and 50°C were characterized by a low signal intensity (<23 kcps). DLS analyses indicated the presence of two classes of particles: one in the nanometer to tens of nanometers range, and a second one in the hundreds of nanometers range. These particles were presumed to represent serum proteins and casein micelles, respectively. This hypothesis was verified by performing DLS measurements on a solution of serum proteins obtained from skim milk using a quantitative casein precipitation procedure. According to the multimodal size distributions in Figure 3.3a and 3.3b, which show the results obtained at 20°C, the particles in the smaller range represent serum proteins, while the larger particle size group represents casein micelles. The presence of CN micelles in permeate obtained by UF with a 10 kDa cutoff was due to the membrane having a minor leak. However, because the classes of particles present in

permeate were the same as in skim milk, it is reasonable to assume that they would not affect the trends observed in this study.

a)



b)

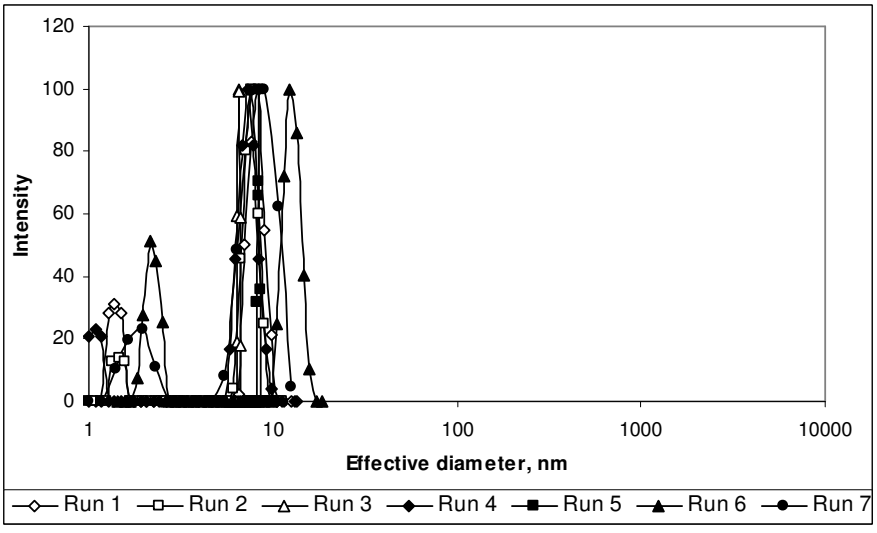


Figure 3.3 Multimodal size distributions: a) UF permeate, and b) serum protein solution at 20°C.

Size distribution data from multiple runs was shown in order to clearly define the particle size ranges, as distribution curves varied slightly among different replicate runs. When evaluating the multimodal particle size distributions in Figure 3.3, it is

important to keep in mind that they offer information about the size range of particles, not quantitative information about the size of individual particles. The calculated effective diameter of the serum proteins at 6°C, 20°C and 50°C is shown in Table 3.4. A one way ANOVA test indicated that these values were not statistically different. The important conclusion was that, due to the low signal intensity (7 to 23 kcps), the UF permeate was not considered to influence the results of the CN micelle size measurement significantly.

Table 3.4 Effective diameters ± standard deviations of particles in a serum protein solution separated using the International Dairy Federation casein precipitation procedure**.** Different letters indicate statistically significant differences ($p<0.05$).

Temperature, °C	**Average effective diameter, nm**
6°C	8.1 ± 1.0 (a)
20°C	8.2 ± 0.7 (a)
50°C	8.4 ± 0.7 (a)

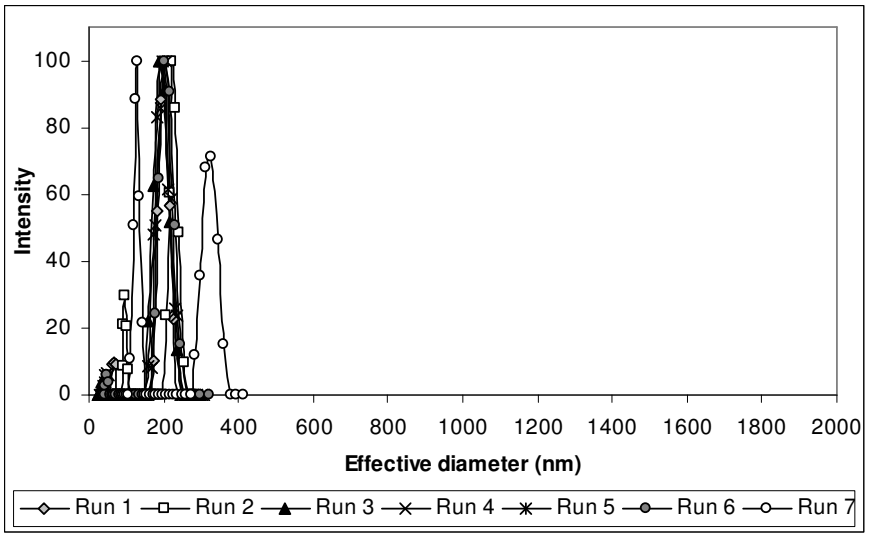
Effect of fat globules and serum proteins on the particle size distribution in skim milk

Particle size measurements were performed both in raw skim milk and HTST pasteurized skim milk. For all solvents and temperatures, the particles observed by DLS ranged between several tens of nm to several hundreds of nm.

In addition to the influence of the solvent, which was discussed above, it was considered that the presence of residual fat globules in the skim milk might also affect the accurate evaluation of casein particle size distribution. Although the fat content of the skim milk samples was very low (~ 0.1%), the slightly larger size of the fat globules as compared to the casein micelles may affect the accuracy of PSD analyses,

as it has been previously reported (Holt, 1985). In order to assess this influence, casein micelles were dissociated by treating the skim milk with a mixture of EDTA and SDS, as previously reported by several researchers (Holt, 1985; Michalski et al., 2001; Ye et al., 2004a,b). Particle size measurements were then performed at 20°C. The particles observed after this treatment ranged in diameter from 50 nm to 1400 nm (Figure 3.4b), and the average effective diameter was 299.1 ± 21.4 nm. Two relatively distinct populations of particles were observed: one in the 100-180 nm range and a second one in the 400-1100 nm range. It is possible that the smaller particles represented casein micelles that were not dissociated as a result of the EDTA/SDS treatment, while the second class was most likely predominantly composed of fat globules. It is interesting to note that the size of the latter class of particles was not observed during the DLS analyses of skim milk, in which the largest particles were in the 300-400 nm range (Figure 3.4a). The signal intensity obtained in the measurement of the residual fat globules was very weak (36.7 ± 9.8 kcps) as compared to the skim milk analyses (700 to 900 kcps). However, since the fat globules are larger than the casein micelles, it is possible that, even if they are present in trace amounts, they distort slightly the size distribution of the casein micelles, shifting it towards larger sizes. This effect of the fat globules needs to be acknowledged, but it is very difficult to accurately and quantitatively account for it when determining the size of the casein micelles. As it will be discussed later in this manuscript, the effect of fat globules can become significant if an unsuitable solvent is used for dilution prior to DLS analyses.

a)



b)

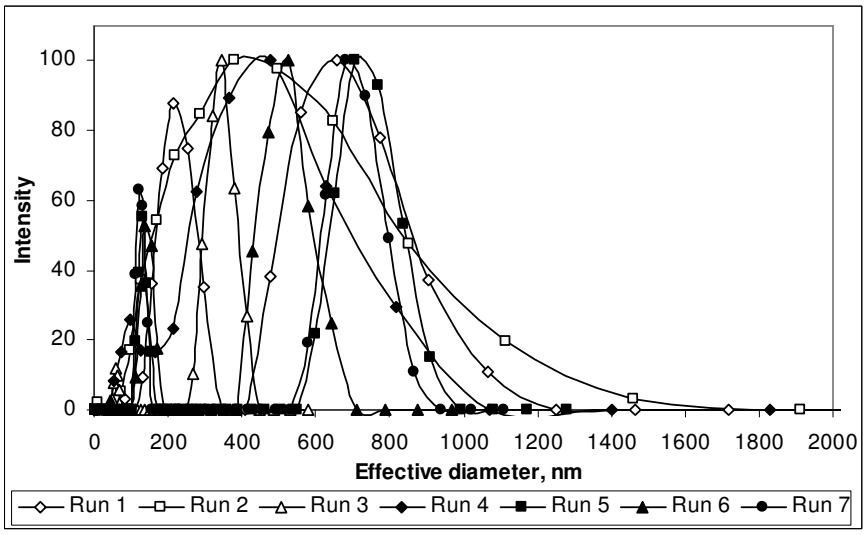


Figure 3.4 Comparative particle size distributions: (a) skim milk in UF permeate, (b) skim milk after dissociation of casein micelles in EDTA: SDS. Measurements were performed at 20°C.

Serum proteins are also present in the skim milk samples. However, due to the small amount and their small size (see Figure 3.3), their effect on the effective diameter, which is an intensity weighted value, can be considered insignificant.

Particle size measurements in skim milk: temperature and solvent effects

Based on the discussion above, the quantitative results of the particle size analyses performed on skim milk were considered to be due predominantly to the casein micelles, and from this point on the discussion will focus on the casein micelles alone.

Table 3.5 contains the values of the effective diameters calculated after the PSD analyses of raw skim milk (Table 3.5a) and pasteurized skim milk (Table 3.5b). All measurements had good reproducibility, with standard deviations in the range of 1.9 to 8.8 nm, corresponding to coefficients of variance of 1.1 to 4.9%.

Table 3.5 Calculated average effective diameter ± standard deviations for casein micelles in skim milk measured by dynamic light scattering under different experimental conditions. Different letters indicate statistically significant differences ($p<0.05$).

a) Raw skim milk

Temperature / Solvent	Average effective diameter, nm		
	6°C	**20°C**	**50°C**
UF permeate	176.4 ± 5.3 (a)	177.4 ± 1.9 (a)	137.3 ± 2.7 (d)
SMUF	153.3 ± 2.7 (b)	176.3 ± 2.5 (a)	194.9 ± 6.0 (c)
Water	180.5 ± 8.8 (a)	180.0 ± 2.7 (a)	200.6 ± 2.5 (c)

b) HTST pasteurized skim milk

Temperature / Solvent	Average effective diameter, nm		
	6°C	**20°C**	**50°C**
UF permeate	190.5 ± 4.3 (b,c)	176.9 ± 4.1 (a)	141.0 ± 2.1 (e)
SMUF	176.6 ± 4.8 (a)	198.8 ± 4.1 (b,d)	205.9 ± 7.6 (d)
Water	178.8 ± 5.5 (a)	189.6 ± 5.3 (c)	198.7 ± 6.3 (b,d)

A two-way ANOVA analysis indicated that the nature of the solvent had a significant effect on the measured effective diameter of the casein micelles, both in raw skim milk ($F(2,18)=128.4$, $p<0.0001$) and pasteurized skim milk ($F(2,18) = 132.52$, $p<0.0001$). Temperature had also a significant influence on particle size ($F(2,18) = 20.5$, $p<0.0001$ for raw skim milk and $F(2,18) = 11.23$, $p<0.0001$ for pasteurized skim milk), and a very strong interaction between solvent and temperature was also observed ($F(4,54)=187.8$, $p<0.0001$ for raw skim milk and $F(4,54) = 125.6$, $p<0.0001$ for pasteurized milk). A Tukey-Kramer HSD analysis was then performed to identify statistically significant differences between the means.

When UF permeate was used as a solvent, the effective diameter of casein micelles decreased as temperature increased, with the effective diameter being significantly lower at 50°C as compared to 6°C and 20°C (see Table 3.5). The decrease in micelle size at 50°C as compared to the other two temperatures is in agreement with previous reports (Davies and Law, 1983; Ono et al., 1990; Gaucheron, 2005), and it can be explained by the increased strength of hydrophobic interactions at that temperature. At low temperatures the hydrophobic bonds become weaker, which causes the micelle structure to become looser and more porous and the micelle diameter to become larger. Additionally, the solubility of calcium phosphate increases, leading to a partial dissolution of micellar calcium phosphate, which also causes the voluminosity of the casein micelle to increase (Gaucheron, 2005). While particle sizes were larger at the lower temperatures for both types of milk, a significantly higher particle size at 6°C as compared to 20°C was only observed in the pasteurized skim milk samples. The “shrinking” of casein micelles at 50°C and their “loosening” at 6°C can also be observed when analyzing the polydispersity (p) data. The interpretation of p values is as follows: $0 \leq p \leq 0.02$ indicates monodisperse or nearly monodisperse systems, $0.02 < p \leq 0.08$ indicates narrow particle size distributions, while $p > 0.08$ is

characteristic for broader size distributions (Brookhaven Instruments, 1995). According to the polydispersity data in Table 3.6, micelles in UF permeate and SMUF displayed narrow distributions at 50°C ($p \leq 0.08$) and broader size distributions at the lower temperatures.

Table 3.6 Polydispersity values ± standard deviations for casein micelles in skim milk measured by dynamic light scattering under different experimental conditions. Different letters indicate statistically significant differences ($p<0.05$).

a) Raw skim milk

Temperature / Solvent	Average polydispersity		
	6°C	**20°C**	**50°C**
UF permeate	0.16 ± 0.02 (a)	0.09 ± 0.02 (b)	0.05 ± 0.01 (c)
SMUF	0.15 ± 0.01 (a)	0.09 ± 0.01 (b)	0.05 ± 0.01 (c)
Water	0.14 ± 0.03 (a)	0.10 ± 0.01 (b)	0.09 ± 0.03 (b)

b) HTST pasteurized skim milk

Temperature / Solvent	Average polydispersity		
	6°C	**20°C**	**50°C**
UF permeate	0.21 ± 0.01 (b)	0.12 ± 0.01 (a,c)	0.07 ± 0.01 (d)
SMUF	0.20 ± 0.03 (b)	0.12 ± 0.01 (a,c)	0.08 ± 0.02 (c,d)
Water	0.13 ± 0.02 (a)	0.07 ± 0.02 (d)	0.12 ± 0.05 (a,c)

When SMUF and water were used as solvents, the effect of temperature on particle size was different than the one observed in UF permeate, with particle sizes becoming larger as the temperature increased. In order to understand the reason for this behavior, a more detailed analysis of the PSD data was performed. Specifically,

the evolution of the effective diameter and of the signal intensity during the PSD measurement was examined. As mentioned in the Materials and Methods section, each measurement consisted of 7 subsequent individual runs of 30 s duration, whose results were combined by the software into one final effective diameter calculation. The data for the individual runs was manually de-convoluted and analyzed, and the dynamics of the effective diameter over the duration of the measurement (3.5 min) was represented for each solvent and measurement condition. The data for the effective diameter measured in raw skim milk is shown in Figure 3.5 and that for signal intensity is shown in Figure 3.6. The trends observed for pasteurized skim milk were virtually identical with those for raw skim milk (data not shown).

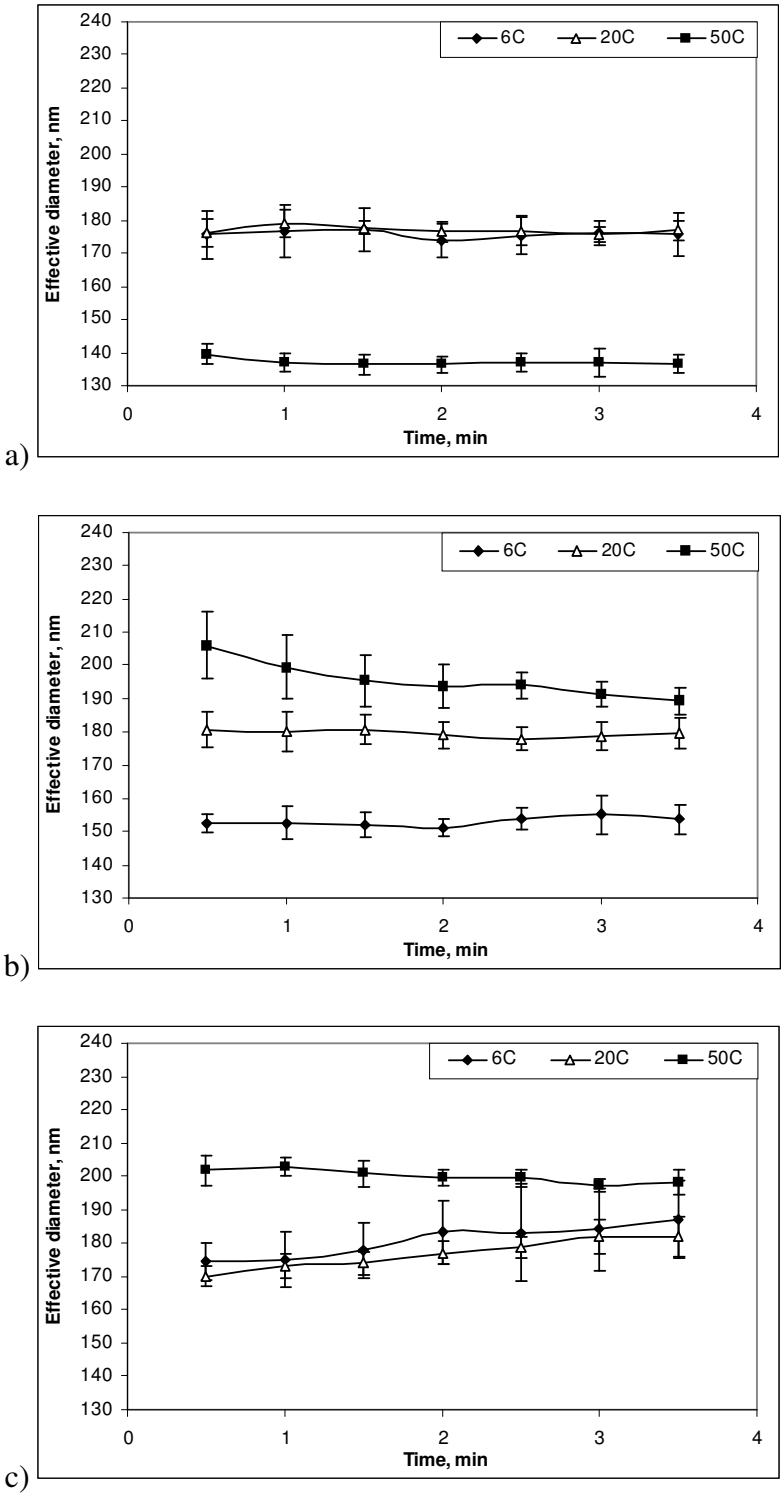
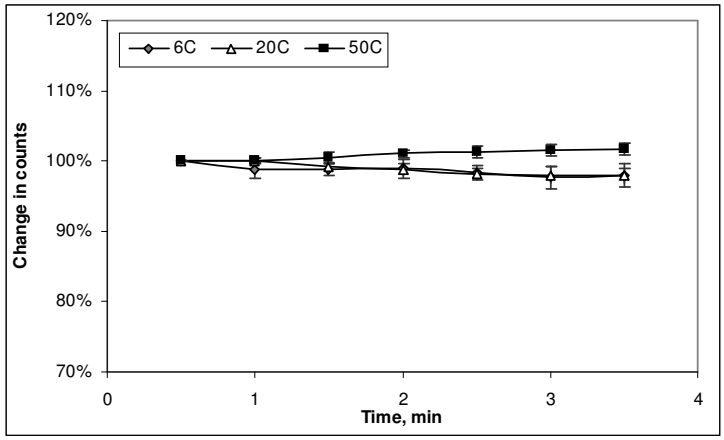


Figure 3.5 Dynamics of the effective diameter of casein micelles in raw skim milk diluted with: a) UF permeate; b) SMUF; and c) water measured at 6, 20 and 50ºC.

a)



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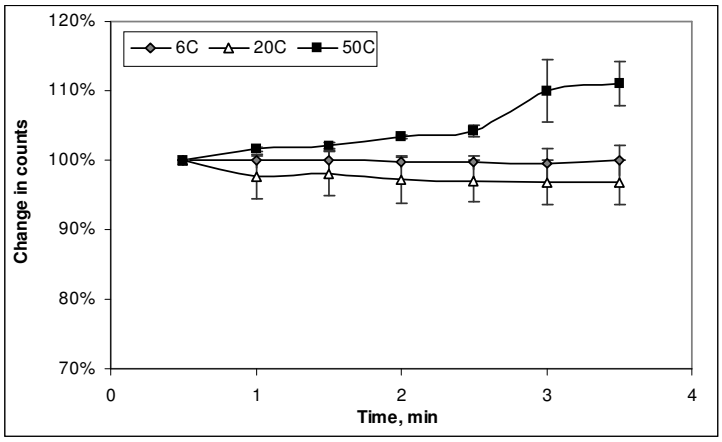

c)

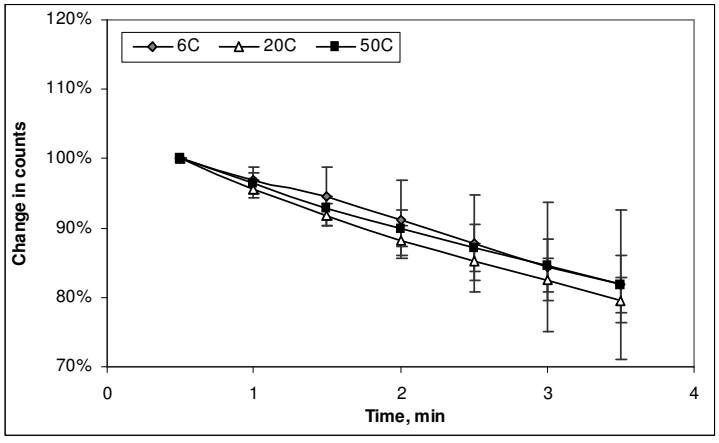

Figure 3.6 Changes in signal intensity (particle counts) during the dynamic light scattering analyses of raw skim milk diluted with: a) UF permeate; b) simulated milk ultrafiltrate; and c) water measured at 6, 20 and 50ºC.

When UF permeate was used as a solvent, the effective diameter was virtually constant throughout the measurement, indicating that the micelles maintained their structure and size over the duration of the experiment (Figure 3.5a). A small decreasing trend was observed at 50°C, but the increase was not statistically significant. Signal intensity was also constant throughout the measurements at 6°C and 20°C, but it did increase slightly over time at 50°C, possibly due to precipitation of amorphous calcium phosphate at that temperature (Figure 3.6a). This increase reflected the small decrease in effective diameter discussed above, but was too small to affect in any significant way the outcome of the measurement.

In SMUF, the solvent with the highest ionic strength (see Table 3.3), the effective diameter of the casein micelles was virtually constant at 6°C and 20°C, but showed a statistically significant decreasing trend at 50°C ($p<0.01$), with a 9.5% decrease taking place over 3.5 min (Figure 3.5b). This decrease in particle size was mirrored by an increase in signal intensity throughout the measurement. This is similar with the phenomenon observed for measurements performed in UF permeate, and it is possible to be caused by the precipitation of amorphous calcium phosphate. In SMUF however these changes had a statistically significant effect on the results of the DLS analyses at 50°C, which changed throughout the duration of the measurement. It is important to note that at 20°C the results of the DLS measurements performed in SMUF were very similar to those obtained when using UF permeate. Differences however were large at the other two temperatures (6°C and 50°C). The increase in the effective diameter with temperature when SMUF was used as a solvent was surprising, and was not consistent with the known effects of temperature on the casein micelles that were discussed above. The reasons for this effect of temperature are still unclear. While precipitation of calcium phosphate and formation of particles of about 200 nm in size (see Figure 3.2) might explain in part the values obtained at 50°C, it is not clear

what led to the decrease in effective diameter by about 20 nm between 20°C and 6°C (Table 3.5).

Measurements were performed in water, the solvent with the lowest ionic strength, also resulted in values that were not consistent with those obtained in UF permeate. At 6°C and 20°C, a statistically significant ($p<0.05$) 7% increase in the effective diameter was observed from the beginning to the end of the measurement (Figure 3.5c). When testing a sample of skim milk in water at 6°C over a longer period of time (45 min), a 42% increase in size was observed, with the effective diameter increasing from 179.2 nm at 30 s to 254.7 nm at 45 min (data not shown). At 50°C no significant changes in size were observed throughout the measurement. The linear, significant decrease in signal intensity observed for all measurements performed in water (Figure 3.6c) suggest dissociation of the casein micelles in water. As a result, it is likely that the contribution of the residual fat globules to the measured effective diameter became more significant, which would explain the increase in size for the measurements performed at 6°C and 20°C. For water, the influence of temperature on the measured effective diameter was similar to the one observed in SMUF, and thus was inconsistent with the expected trend.

When evaluating the influence of the solvents on the particle size of CN micelles, it is worth noting that the influence of their viscosity, although accounted for in the calculations, has not been investigated systematically. This issue deserves further consideration.

Conclusions

The findings of this study illustrate the unsuitability of water as a solvent, and the limitations of SMUF as a solvent at 6°C and 50°C. When water was used as a solvent, significant dissociation of CN micelles occurred, making the results of PSD

analyses inaccurate. In SMUF at 50ºC, precipitation of amorphous calcium phosphate affected the results of the DLS analyses, whereas at 6ºC, the measured effective diameter of the CN micelles was lower than at the higher temperatures, for reasons that are still unclear.

Overall, it can be concluded that in order to obtain accurate measurements of casein micelle sizes it is critically important to use a solvent with a chemical composition as close as possible with the milk serum. In this study, that solvent was identified to be the UF permeate, whose use as a solvent resulted in values of casein micelle sizes at the three different temperatures consistent with those reported in literature. Although relatively minor, the slight overestimation of the effective diameter of CN micelles caused by the presence of residual fat globules in skim milk, in all solvents, must be acknowledged. Another conclusion of this work is the importance of using a clear and consistent protocol for the measurement of particle sizes for casein micelles or any other colloidal particles. Because in literature these important aspects of DLS measurement of casein micelles are, in many cases, mentioned briefly and somewhat inconsistently, this work provides a guide for conducting particle size analyses in milk.

Acknowledgements

The authors would like to thank the NYSMPB for funding this work and Dr. David Barbano's research group for providing the UF permeate and its composition.

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CHAPTER 4

HEAT INDUCED INTERACTIONS BETWEEN MICELLAR CASEIN AND SOY PROTEINS

4.1. EFFECT OF PROTEIN CONCENTRATION AND HEAT TREATMENT TEMPERATURE ON THE PHYSISCAL PROPERTIES OF MICELLAR CASEIN-SOY PROTEIN MIXTURES

Abstract

The objective of this study was to investigate the effect of concentration and temperature on the rheological properties of soy proteins (SP) and micellar casein (MCN) systems. Individual and mixed (1:1) protein systems of 2 - 15% concentration were prepared and heat treated for 5 min at 40° to 90°C. After cooling to 20°C, their rheological properties were determined using steady-shear rheology. Zeta potential and particle size measurements were also conducted. Both proteins were negatively charged under all experimental conditions, but the absolute values of zeta potential and thus the stability of the protein solutions decreased with temperature and concentration. For SP solutions, viscosity and apparent yield stress increased with concentration. Shear thinning behavior was prevalent, becoming more pronounced with increasing concentration. Heat treatments at T≥80ºC induced glycinin denaturation, followed by aggregation and network formation when C≥7.5%. Heat treatment did not significantly affect viscosity of MCN systems, while increasing concentration resulted in a significant increase in apparent viscosity and apparent yield stress. Most MCN systems exhibited Newtonian flow behavior, with the exception of systems with C≥12.5% treated at T≥80ºC, which became slightly shear thickening. Mixed SP-MCN systems mimicked the behavior of SP, with most values of rheological parameters intermediate between SP and MCN-only systems. Mixtures of

7.5% to 12.5% concentration treated at 90°C displayed local phase separation, low viscosity and apparent yield stress, while 15% mixtures treated at 90°C showed protein aggregation and incipient network formation. The data generated in this study can be used to develop a range of protein based products with unique flow characteristics and storage stability.

Introduction

Despite scientifically proven health benefits of soy, many Western consumers are reticent to adopt soy products in their diet, due to undesirable sensory properties. One way to enhance the acceptability of soy proteins could be to incorporate them in dairy products, which have both highly acceptable sensory properties and health benefits. The consumer studies conducted by Drake and Gerard (2003) indicated consumer interest and significant market potential for soy-fortified dairy foods. In recent years there have been several reports about the fortification of dairy products with soy proteins (Mandal, Bandyopadhyay, & Ghatak, 1996; Biswas, Chakraborty, & Choudhuri, 2002; Abdullah, Rehman, Zubair, Saeed, Kousar, & Shahid, 2003; Gokce and Gursoy, 2003). In most of these cases however, the physical and sensory properties of the final products were negatively affected by the addition of soy. For instance, Drake, Chen, Tamarapu and Leenano (2000) reported that the addition of soy protein to dairy yogurts resulted in an increase in sensory chalkiness even at 1% addition.

A critical element in designing milk protein-soy protein blends with palatable textures is establishing the range of compositional and processing conditions that promote miscibility and homogeneity. While the interactions between soy proteins and milk serum proteins have been previously studied (Roesch and Corredig, 2005), the interactions between soy proteins and casein, the major milk protein, have not yet been

investigated.

Soy proteins are represented by two classes of globular proteins: globulins (90%), which can be extracted using dilute salt solutions, and albumins (10%), which can be extracted by water (Fukushima, 1991). According to their sedimentation rates in a 0.5 M ionic strength buffer at pH 7.6, soybean globulins can be classified in four fractions: 2s (15%), 7s (34%), 11s (41.9%) and 15s (9.1%) (Koshiyama, 1969). The 11s and 15s fractions consist of glycinin and polymers of glycinin (Wolf, 1970), while the 7s fraction contains mostly β-conglycinin but also γ-conglycinin, lipoxygenases, α-amylases and hemaglutenins (Nielsen, 1985). The 2s fraction consists of Bowman-Birk and Kunitz trypsin inhibitors, cytochrome C, and α-conglycinin (Wolf, 1970). β-conglycinin and glycinin represent approximately 80% of the total proteins in soy, and thus have a significant influence on the functional properties of soy protein systems. Glycinin exists as a hexamer with a molecular mass of 360 kDa (Renkema, Knabben, & van Vliet, 2001), while β-conglycinin is a trimeric glycoprotein with a molecular mass of 150-200 kDa (Utsumi, Matsumura, & Mori, 1997). Soy proteins denature upon heating, and in the denatured state they can form gels. In the case of glycinin, gel formation takes place via disulfide bonds and noncovalent bonds such as hydrophobic interactions, ionic and hydrogen bonds (Mori, Nakamura, & Utsumi, 1986). Gelation of β-conglycinin is caused by hydrophobic interactions and hydrogen bonds, with no contribution from disulfide exchange reactions (Nakamura, Utsumi, & Mori, 1986).

Caseins are thermally stable phosphoproteins that precipitate at an isoelectric pH of 4.6. They make up 82% of the true protein in milk and are composed of four individual casein proteins, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, in a weight ratio of 3:0.8:3:1 (Schmidt, 1982). Individual casein molecules are disordered and very flexible, mostly due to their high proline content, whose cyclic structure prevents the formation of a highly organized secondary structure (Holt, 1992). In milk, casein is

present as micelles, which are spherical and polydisperse particles with a weighted average diameter of about 200 nm (de Kruif, 1998; Beliciu and Moraru, 2009). Approximately two thirds of the micellar volume in solution is caused by hydration of the porous structure, which explains the generally high viscosity of casein suspensions (Dalgleish, 1997). Several casein micelle models have been developed over the last 50 years, but there still is no consensus as to the validity of any singular model (Horne, 2006). Despite some fundamental differences that exist among these models, particularly related to the existence of casein submicelles, there is general agreement related to certain aspects of the casein micelle structure, including the location of κ-casein at the surface of casein micelles and their role in stabilizing the casein micelles (Walstra, 1990; Fox, 2003). This is relevant to the current work, since κ-casein molecules can provide interaction sites between the casein micelles and other molecules. For instance, in heat treated milk the denatured serum proteins bind to κ-casein via disulfide bonds (Singh and Fox, 1985; Dannenberg and Kessler, 1988; Dalgleish, 1990; Singh and Latham, 1993; Beaulieu, Pouliot, & Pouliot, 1999). Under certain conditions, similar interactions could potentially occur between casein and soy proteins.

An important factor that can facilitate or impede the interactions between casein micelles and soy proteins is their miscibility. Miscibility of biopolymers in general and of proteins in particular is thermodynamic in nature and depends on many factors, such as concentration, temperature, and molecular weight (Tolstoguzov, 1991; Moraru, Lee, Karwe, & Kokini, 2002; Zimeri and Kokini, 2003a, b, c). Incompatibility is typical for proteins belonging to different classes within the Osborne classification, such as casein and soy globulins. Even proteins of the same class are incompatible when they differ in their conformations (i.e. native and denatured forms) (Polyakov, Grinberg, & Tolstoguzov, 1997). It has been reported

that, even when incompatible, most mixed protein solutions in water have a rather high phase separation threshold, with the minimum total concentration of proteins at which phase separation of their mixed solution occurs ranging from 10 to 20% (Polyakov et al., 1997).

The objective of this study was to evaluate the effect of protein concentration and heat treatment temperature on the rheological properties of soy proteins - micellar casein mixtures, in a concentration range from 2 to 15%, and a weight ratio of 50:50. The knowledge gained from this study could then be used as a basis for the development of high protein foods with unique structure and functionality.

Materials and methods

Materials

The study was conducted on protein preparations obtained by membrane separation followed by spray drying, in order to ensure that the effects of the preparation methods on the native structure and properties of the two proteins are minimal. Soy protein isolate (SPI-6000; protein 90.9%, fat 3.3%, ash 5.8% on dry solids basis, moisture 5.4%) (Protient Inc., St. Paul, MN) was used as a source of soy proteins. Micellar casein powder (MCN-85, American Casein Company, Burlington, NJ) was used as a source of casein. The composition of the MCN powder is as follows: protein 84.93%, fat 2.1%, ash 9.5%, lactose 3.2% on dry solids basis, and moisture 4.8%. The following minerals were quantified in the MCN powder by testing at the Dairy One Forage Analysis Laboratory (Ithaca, NY), using the method described by Beliciu and Moraru (2009): calcium - 2.53% (d.b.), phosphorus - 1.47% (d.b.), magnesium - 0.10% (d.b.), potassium: 0.10% (d.b.), sodium - 0.07% (d.b.), and chloride 0.12% (d.b.).

Differential scanning calorimetry (DSC)

Thermal analysis was performed to evaluate thermal denaturation of the proteins before subjecting them to the heat treatment, using a Differential Scanning Calorimeter/DSC 220 (Seiko Instruments Inc.). Sixty-microliter, high pressure, stainless steel crucibles with rubber O-rings (Perkin Elmer, Waltham, MA) were used for the analyses. A crucible containing 50 μL ultra pure water (the solvent) was used as reference. Calibration of the instrument was performed using Indium as a standard, at a heating rate of 5°C/min. Individual (casein and soy proteins, respectively) and mixed solutions of 12.5% (w/w) concentration were carefully weighed (45-50 mg), sealed in crucibles and scanned between 20-110°C, at a heating rate of 5°C/min. Data analyses consisted of manual peak integration of the DSC thermograms, followed by calculation of the enthalpy of denaturation (ΔH) and denaturation temperatures (denaturation onset temperature and the denaturation peak temperature, T_d) using the instrument's software.

Sample preparation

This study was performed in parallel on both individual (micellar casein and soy protein) and mixed protein systems; the latter contained the two proteins in a weight ratio of 50:50. Solutions[⊗] of micellar casein and soy protein of concentrations ranging from 2% to 15% (on a dry matter basis) were obtained by weighing the appropriate amount of protein powder and adding them to 100 ml of ultra pure water. The method used for preparing the protein solutions (i.e. mixing with water) mimics the way in which dry ingredients are rehydrated for use in the food industry. The

[⊗] Since casein is not water soluble, the casein-water mixtures are suspensions, not true solutions. However, in order to use a uniform terminology throughout the manuscript, the term "solution" will be used for all protein preparations, including casein.

following preparation procedure was used for all protein solutions. To ensure that no clumping occurred, the protein powder was added slowly to the water in a beaker placed on a Fisher Thermix 310T stirring plate (American Instruments Exchange, Inc., Haverhill, MA), set at 500 rpm (dial speed 5). The water-protein mixture was kept on the stirring plate for 30 minutes at 25°C under moderate agitation at 400 rpm (dial speed 4), in order to allow the powder to disperse well and the proteins to become hydrated. A second dispersion and hydration step consisted in pouring the solution in a Mojonnier bottle, in order to control foaming, and subjecting it to high shear agitation using an UltraTurrax Model T25 fitted with a S25N–18G dispersion tool (IKA Works Inc., Wilmington, NC), for 5 min at 21,500 rpm. The mixing time for the high-speed dispersion step was established by monitoring the evolution of particle size in the protein solution. The effect of the duration of the high speed dispersion step on the effective diameter measured in micellar casein solutions is shown in Figure 4.1. A mixing time of 5 min ensured that the particle sizes in solution reached the known particle size for casein micelles (Beliciu and Moraru, 2009). The soy protein powder dispersed and solubilized faster than casein, but the same mixing time was used for consistency. The procedure used for particle size analyses is described later in this manuscript. After the high-speed dispersion, the solutions were kept under continuous stirring at 300 rpm (dial speed 3) on the stirring plate until further analyses, which occurred within maximum one hour. The mixed casein-soy protein solutions were prepared immediately before being tested, by adding equal volumes of casein and soy proteins solutions of the same concentration in a test tube and agitating the mixture using an analog vortex mixer (Fisher Scientific, Pittsburg, PA). Much care was taken to eliminate air bubbles from the protein solutions, since bubbles could have led to non-uniformities during the heating step.

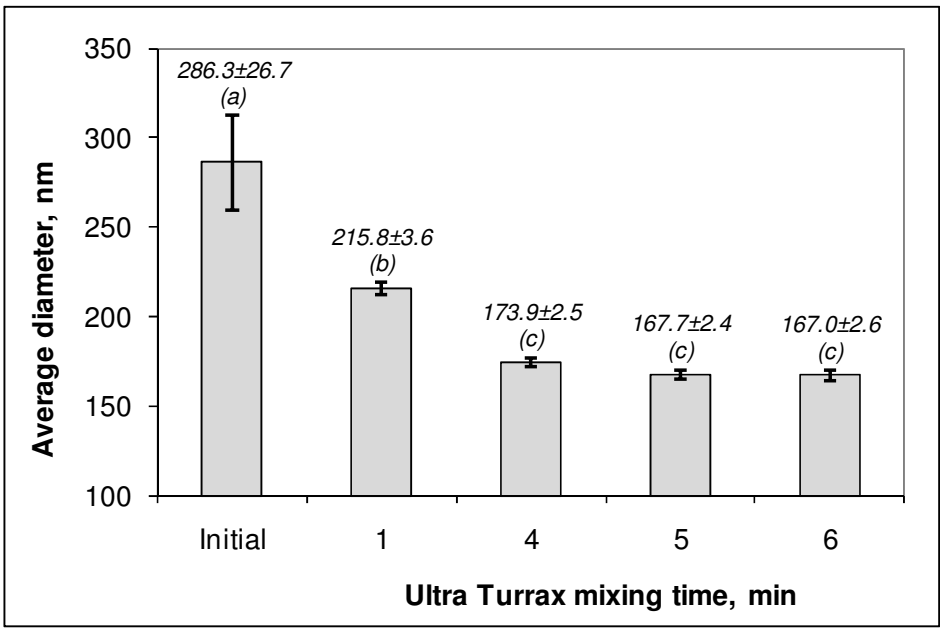


Figure 4.1 The effect of the duration of the high shear mixing step (21,500 rpm) on the effective diameter measured for a micellar casein solution of 10% (w/w) concentration.

Heat treatments

In order to have a good control of the treatment time and temperature and heating uniformity, the protein solutions were subjected to the heat treatment between the parallel plates of an Advanced Rheometric Expansion System (ARES) strain-controlled rheometer (TA Instruments, New Castle, DE), which is equipped with a Peltier system able to achieve quick heating and cooling. A parallel plate geometry with 50 mm diameter Teflon plates and an interplaten gap of 1 mm was used. A volume of 2 ml of protein solution was loaded on the lower plate, avoiding the formation of air bubbles. The gap was set and, in order to avoid dehydration during the heat treatment and subsequent measurement, the exposed area of the sample was thinly coated with mineral oil. An isothermal chamber was placed around the parallel plate, in order to prevent variations in water vapor pressure at the air-sample interface.

A relaxation step of 1 min was allowed before heating. The rapid and controlled heating of the solutions was performed using the Peltier temperature control system of the rheometer. Heat treatments of 5 minutes were independently performed for each protein system at each of the following temperatures: 40º, 50º, 60º, 70º, 80º and 90ºC. A fresh sample was used for each heat treatment. The temperature come-up time varied between a few seconds for the 40ºC treatment and about 2 min for the 90º C treatment. After the respective heat treatment, each sample was cooled to 20ºC and subjected to rheological analyses. All heat treatments were performed in triplicate.

Rheological analyses

An ARES strain-controlled rheometer with dual transducer in conjunction with the Orchestrator data collection and analysis software (TA Instruments, New Castle, DE) was used to evaluate the rheological properties of the protein solutions. A parallel plate geometry with 50 mm diameter Teflon plates and an interplaten gap of 1 mm was used in all measurements. The heat treated and cooled samples were immediately evaluated using steady shear rheological measurements. Strain-controlled, steady-shear rate sweep tests were conducted on each of the heat treated protein solutions, at shear rates from 1 to 10^3 s^{-1}. All rheological measurements were performed in triplicate.

Particle size analyses

In order to perform particle size analysis, the solutions were first heat treated as described above. After the sample was cooled to 20ºC, the isothermal chamber was removed, the upper fixture was lifted and a small amount of the heat treated protein was sampled for particle size analyses. The heat treated protein samples were diluted to very low concentrations (~0.0033 µg/ml) in ultra pure water, immediately prior to

performing the particle size analysis. Particle size measurements of the diluted samples were conducted using a Brookhaven 90Plus Nanoparticle Size Analyzer equipped with a Peltier temperature control system (Brookhaven Instruments Corp., Holtsville, NY), at a temperature of 20°C, a fixed 90 degree angle, and a wavelength of 658 nm. Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp., Holtsville, NY), which converted the experimental data into size distributions. The software contains a Dust Filter algorithm that improves the quality of the measurements by rejecting data corrupted by scattering from random particles such as air bubbles or dust. The dust filter cut-off parameter was set at 30, which is the value recommended by the manufacturer for situations when the expected average particle size is in the range of hundreds of nm. Sample dilution was adjusted in order to achieve the manufacturer recommended signal intensity of 700-900 kilocounts per second (kcps). Each measurement consisted of 8 individual runs of 30 s duration. For each measurement, the relative particle size distribution, the intensity weighed effective diameter ($\overline{d_{eff}}$) and the polydispersity index (p) were determined. Three replicate measurements were performed for each sample.

Zeta potential measurements

In order to perform zeta-potential analysis, the solutions were heat treated, sampled and diluted according to the same procedure used for the particle size analysis. Zeta potential measurements were performed on both untreated and treated solutions using the ZetaPlus option of the 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Measurements were performed using a 35 mW solid state laser, λ = 660 nm, in the "High Precision" mode at 20°C, and setting "water" as solvent. Protein dilutions were adjusted in order to achieve an

optimum ratio (0.1-0.5) between the instrument count rate and the reference count rate (1454 kcps). The measurement consisted of 30 cycles/run, with an intercycle delay of 5 s.

Concentration of free Ca^{2+} and pH of the MCN solutions

The concentration of free Ca^{2+} in the reconstituted casein solutions was measured directly using an Accumet XL25 meter (Fisher Scientific, Pittsburg, PA) fitted with a Calcium ionplus® Sure-Flow® Plastic Membrane Combination ISE electrode (Model 9720BNWP, Thermo Fisher Scientific, Waltham, MA), in the "Ion" mode.

Calibration of the calcium electrode was performed both in the low and high concentration range. Two calcium calibration standards of 10 ppm and 1000 ppm concentration were prepared by diluting 0.25 mL, respectively 25 mL of a 0.1 M calcium standard (Thermo Fisher Scientific, Waltham, MA) to 100 mL with ultra pure water. The standards were diluted in a 50:1 ratio with an ionic strength adjustor (ISA) consisting of a 4 M KCl solution (Fisher Scientific, Pittsburg, PA). Calibration was performed immediately prior to the measurements. The slope of the electrode was between 25 and 30 mV/decade.

The MCN solutions were prepared as described before, then placed in flat bottom Pyrex tubes (Model 9850-25, Corning Incorporated, Corning, NY), equilibrated for 1 hr in a water bath (Model 202-2, National Appliance Co, Portland, OR) at 20ºC and stirred at an uniform rate during the measurement using a Fisher Thermix 310T stirring plate (American Instruments Exchange, Inc., Haverhill, MA). The protein samples were also diluted in a 50:1 ratio with the ionic strength adjustor, and the procedure was validated by testing skim milk (Cornell Dairy, Ithaca, NY) and comparing the results with data from literature (Lin, Lewis, & Grandison, 2006).

pH measurements

The pH of the protein solutions was measured using an Accumet XL25 meter (Fisher Scientific, Pittsburg, PA) fitted with an AccuCap^{TM} Combination electrode (Model 13620131, Fisher Scientific, Pittsburg, PA) in the pH mode. Calibration was performed immediately before the measurements.

Statistical analysis of data

One way analysis of variance (ANOVA) and two way ANOVA with an interaction effect were performed in order to determine significant differences in experimental results ($p < 0.05$). Differences between means were compared using the Tukey-Kramer HSD test and the statistical software package JMP version 7.0 (SAS Institute Inc., Cary, NC).

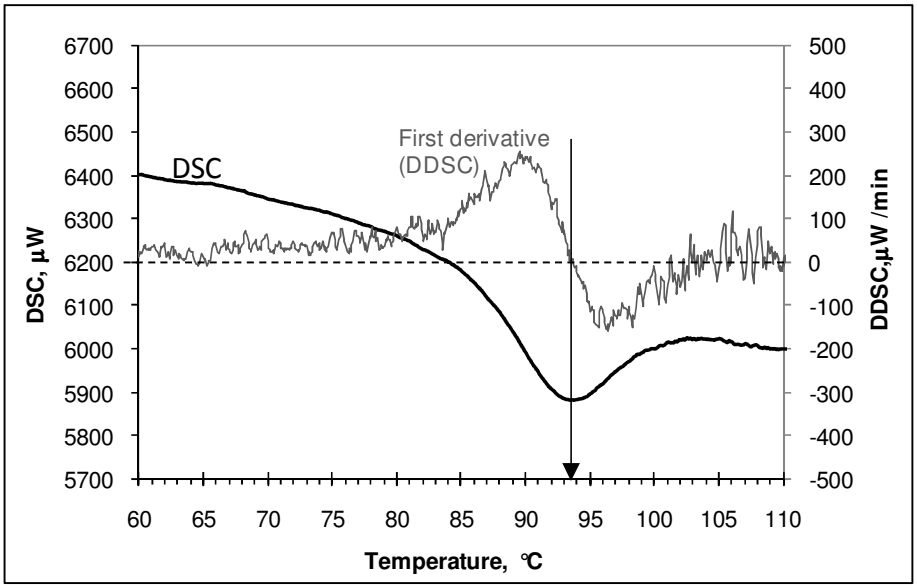
Results and Discussion

Effect of temperature on soy proteins and casein

Thermal analysis (DSC) was used to evaluate the effect of the thermal history of the protein preparations on the denaturation status of individual proteins. As expected, the DSC thermogram of the 12.5% (w/w) casein solutions showed no thermal transition between 20° and 110ºC (data not shown). This is consistent with previous studies (Paulsson and Dejmek, 1990) and with the general knowledge that caseins do not undergo thermal denaturation. The thermogram for the soy protein solutions showed a significant endothermic peak in the range 80° to 103ºC, which was associated with glycinin denaturation. The value of the denaturation temperature was determined by analyzing the DSC curve and was confirmed by examining the first derivative curve (DDSC), using the instrument's software. The denaturation

temperature (T_d) was defined as the local peak on the DSC curve and the temperature where the DDSC curve crosses zero on the DDSC axis (see Figure 4.2a, b).

a)



b)

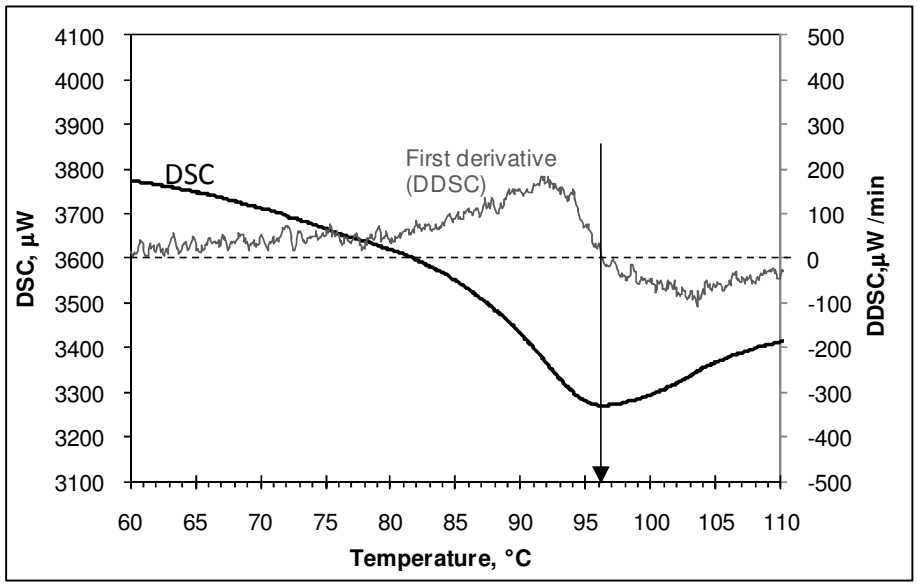


Figure 4.2 DSC thermograms for soy protein (SP) (a) and SP-MCN mixture (b), at 12.5% (w/w) concentration

This peak was characterized by a denaturation temperature of 93.6 ± 0.1ºC and

an enthalpy of 0.64 J/g (Figure 4.2a). These values represent averages of three replicate measurements, and are consistent with data reported previously in literature for glycinin denaturation (Sessa, 1993; Liu, Chang, Li, & Tatsumi, 2004; Zhang, Takenaka, & Isobe, 2004). The thermogram for soy proteins did not show any endothermic peaks that could be associated with β-conglycinin denaturation, which has been reported to take place around 70ºC (Liu et al., 2004). This suggests that the heat sensitive β-conglycinin was already denatured during the manufacturing of the soy protein powder, most probably during spray drying. An endothermic peak in the range 80º to 105ºC was also present on the thermogram for the soy protein–casein mixture (Figure 4.2b). For the mixed SP-MCN solution, the peak was characterized by a lower enthalpy (0.42 J/g) and a slightly higher denaturation temperature (96.3 ± 0.04ºC) as compared to the soy protein alone. The lower enthalpy was due to a dilution effect, i.e. the lower concentration of glycinin molecules in the soy protein–casein solution as compared to the soy protein solution. The higher denaturation temperature in the mixed system as compared to the individual soy protein suggests a thermal stabilization of glycinin by the casein micelles. This could have occurred because of the presence of the casein micelles, which restricted molecular mobility and thus affected the unfolding of glycinin molecules.

Based on the results of thermal analyses, two heating regimes were defined in this study: treatments at temperatures below 80°C, where glycinin is still undenatured, and treatments at temperatures above 80°C, where the unfolding of glycinin is initiated. The highest heat treatment temperature used in the study was 90°C, since above this temperature significant dehydration and bubbling of the protein solutions during heating in the rheometer could not be avoided.

Rheological properties of the heat treated protein solutions

Since many of the protein systems were non-Newtonian, with viscosity changing as a function of shear rate, the term "apparent viscosity" was used to accurately describe their resistance to flow. Figures 4.3a, b and c show the apparent viscosity vs. shear rate graphs for soy protein, micellar casein and mixed soy protein-micellar casein solutions, respectively, after heat treatment at 60°C. Similar trends in apparent viscosity vs. shear rate were observed for soy protein and the SP-MCN mixture for all other heat treatments (data not shown).

Based on the observed rheological behavior, two distinct regimes were identified for the protein solutions evaluated in this study: dilute ($C \leq 5\%$), and concentrated ($C \geq 7.5\%$). The following discussion will refer to these two concentration regimes.

For dilute soy protein solutions ($C \leq 5\%$) apparent viscosity was either not affected or changed only slightly with shear rate, while for solutions with concentration ≥7.5% a clear decrease in apparent viscosity with shear rate was observed (Figure 4.3a). Shear thinning behavior is caused by a decrease in entanglement density and increased orientation of three-dimensional macromolecular structures during flow (Varesano, Aluigi, Vineis, & Tonin, 2008).

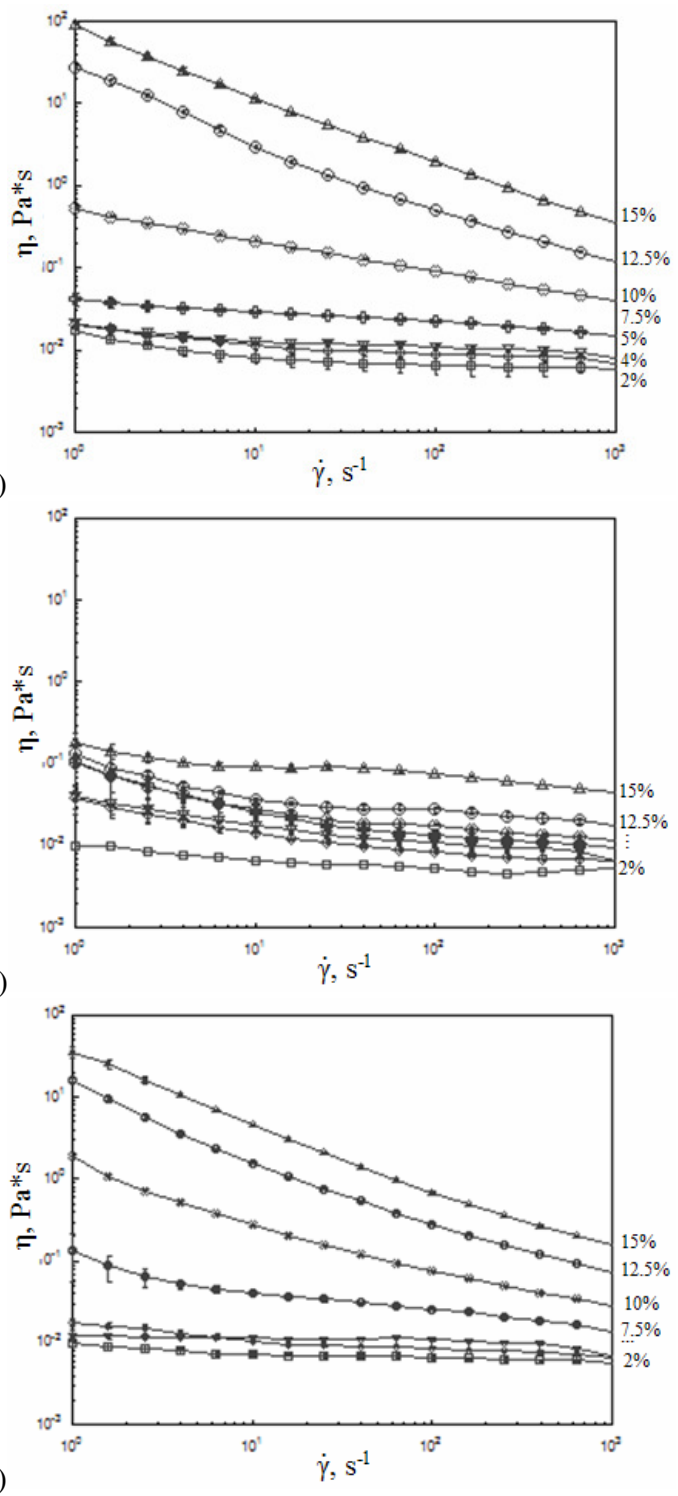


Figure 4.3 Apparent viscosity as a function of shear rate for protein solutions of 2% to 15% concentration after treatment at 60ºC: a) Soy protein (SP); b) Micellar casein (MCN); c) Soy protein-micellar casein (SP-MCN) mixtures.

Most of the micellar casein solutions displayed Newtonian flow (apparent viscosity was not affected by shear rate), but occasional departures from this behavior, either shear thinning or shear thickening were observed.

The viscosity of dilute SP-MCN solutions ($C \leq 5\%$) was relatively constant across the entire shear rate domain (close to Newtonian behavior), but it decreased significantly with shear rate at concentrations above 7.5% (shear thinning behavior).

In order to make direct comparisons among the different protein solutions, the apparent viscosity at a shear rate of 100 s^{-1} (η_{100}) was used. This value of shear rate was selected as it is associated with stirring, pumping, pipe flow, spraying and other processing operations, even mastication (Steffe, 1996a; Carr, Southward, & Creamer, 2003). As observed in Figure 4.4, for all three protein systems η_{100} increased with concentration (C). The increase followed an exponential relationship, according to the equation:

$$\eta_{100} = ae^{bC} \qquad (1)$$

where a and b are numerical coefficients.

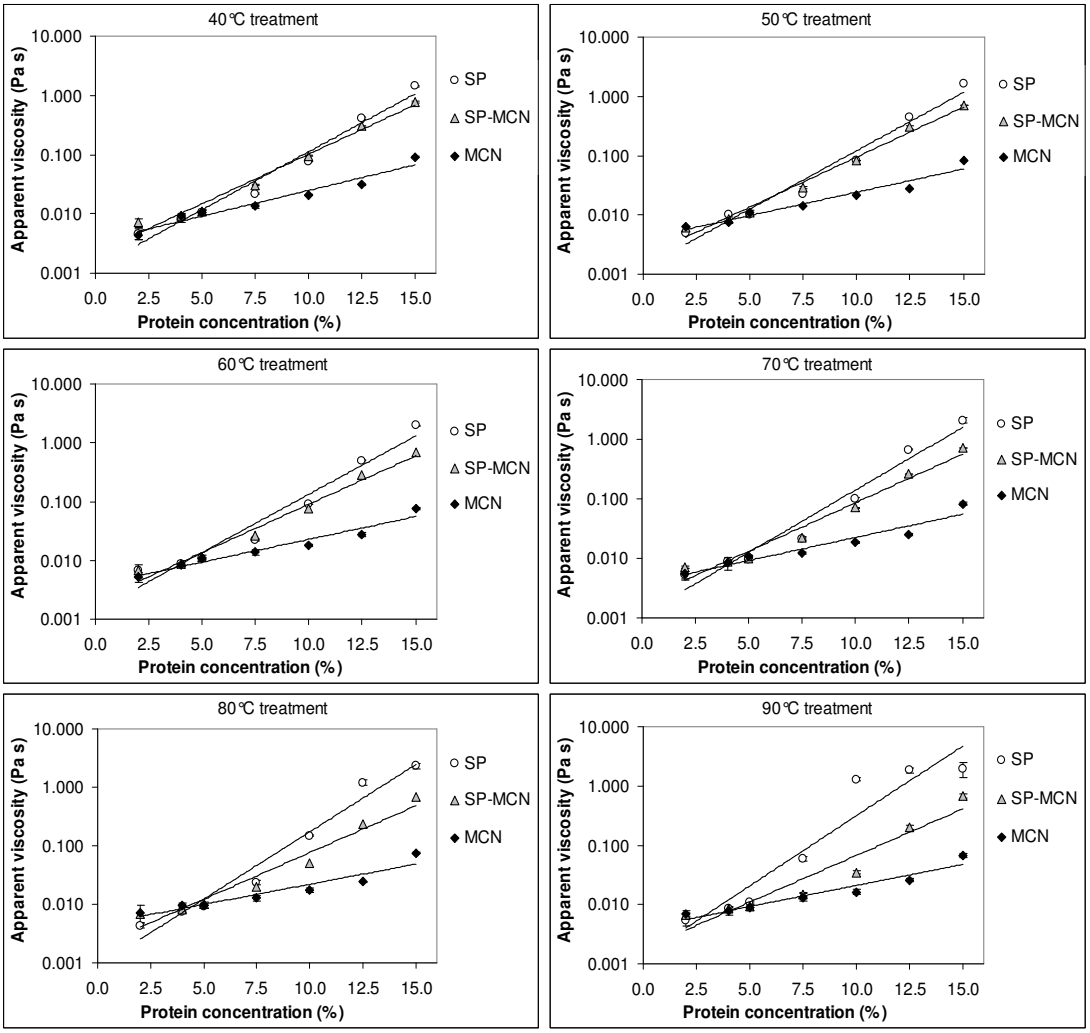


Figure 4.4 Apparent viscosity at $\dot{\gamma}$=100 s^{-1} for protein solutions of 2% to 15% concentration after treatment at 40°C to 90ºC.

As it can be observed in Table 4.1, the value of *a*, which is a constant related to the viscosity of the solvent, did not vary significantly with the intensity of the heat treatment. This constant had different values for each protein solution, and the values for the mixtures were intermediate between the values for the two individual protein systems. The second coefficient, *b*, which is a measure of the effect of concentration on the apparent viscosity of the solution, varied with the heat treatment temperature. In case of the soy protein solutions, *b* increased with treatment temperature; this reflects a larger impact of protein concentration on the viscosity of the protein system as the intensity of the heat treatments is increased. For micellar casein solutions, the coefficient *b* decreased slightly with treatment temperature, indicating a lower impact of protein concentration on viscosity after high temperature treatments. In case of the mixed SP-MCN solutions, *b* remained constant over the entire range of temperature treatments.

Table 4.1. Numerical coefficients for the exponential dependency of apparent viscosity (η) on concentration (C) for all protein solutions and heat treatments ($\eta = ae^{bC}$). The exponential function was fitted to the average of apparent viscosity for three replicate measurements.

Temperature of heat treatment / Coefficient	**40°C**	**50°C**	**60°C**	**70°C**	**80°C**	**90°C**
Soy protein solutions						
a	0.0012	0.0013	0.0014	0.0011	0.0009	0.0013
b	0.45	0.45	0.46	0.48	0.53	0.54
R^2	0.97	0.97	0.96	0.96	0.97	0.92
Casein solutions						
a	0.0034	0.0040	0.0038	0.0038	0.0045	0.0040
b	0.20	0.18	0.18	0.18	0.16	0.16
R^2	0.95	0.95	0.94	0.92	0.89	0.93
Mixed soy protein-casein solutions						
a	0.0022	0.002	0.002	0.002	0.0019	0.0018
b	0.38	0.39	0.38	0.37	0.37	0.36
R^2	0.98	0.99	0.98	0.97	0.96	0.92

When comparing the three protein systems, it was observed that soy protein solutions had higher apparent viscosity than micellar casein solutions, at all concentrations and for all heat treatments. For the soy protein solutions, apparent viscosity increased significantly with the temperature of the heat treatment, especially at $C \geq 7.5\%$ and for heat treatment temperatures ≥80°C (Figure 4.4). This was attributed to the onset of glycinin denaturation and initial stages of network formation, and is consistent with both visual observations and with previously published data that indicates that the critical concentration for gelation of soy proteins is about 6.6% (Bikbow, Grinberg, Antonov, Tolstoguzov, & Schmandke, 1979). It has to be noted though that the relatively short heating time and the fact that the heating temperature was just above the onset of denaturation as determined by DSC did not allow for full gel development in any of the heat treated soy protein samples. The highest value of apparent viscosity for soy protein systems (~1.8 Pa×s) was registered at 10-15% concentration after the 90°C treatment.

The apparent viscosity of the micellar casein solutions was not affected significantly by heat treatment (Figure 4.4), which was expected based on the known stability of casein micelles to heating. The apparent viscosity values for the SP-MCN systems were generally intermediate between those of soy proteins and micellar casein at the same concentration and heat treatment temperature, although exceptions from this behavior were also observed (Figure 4.4).

In order to verify if the viscosity of the mixtures could be predicted based on the viscosity of the individual protein systems, the logarithmic additive rule (Eq. 2) was used. This rule is the most commonly used mixing rule for polymeric mixtures, and it allows the calculation of the viscosity of a mix based on the following equation (Song, Mathias, Tremblay, & Chen, 2003).

$$\ln \eta_{mix} = \sum_i w_i \ln \eta_i \qquad (2)$$

where η_i represents the apparent viscosity and w_i the weight fraction of component "*i*".

Mixtures that follow Eq. 2 are known as additive or compatible (Vankan, Fayt, Jérôme, & Teyssié, 2004; Nakason, Saiwari, & Kaesaman, 2006). Mixtures with strong interphase interactions show positive deviation from the log-additivity rule; incompatible mixtures or mixtures where interactions are weak are characterized by negative deviations; mixtures in which the phase structure changes with composition exhibit both positive and negative deviations (Vankan et al., 2004).

The apparent viscosity for the mixed protein systems at all concentrations and heat treatment conditions was calculated using Eq. 2, and then the deviation of apparent viscosity from the log-additive rule was calculated as:

$$\text{Dev}_\eta = (\eta_{\text{measured}} - \eta_{\text{predicted}})/\eta_{\text{measured}}, \% \qquad (3)$$

As seen in Figure 4.5, SP-MCN mixtures of concentration ≤5% complied with the log-additive rule ($\text{Dev}_\eta \cong 0$). The slight positive deviation for the lowest concentration (2%) was probably caused by the large variability in the experimental values, since the viscosity measurements at this concentration approached the sensitivity threshold of the rheometer's transducer.

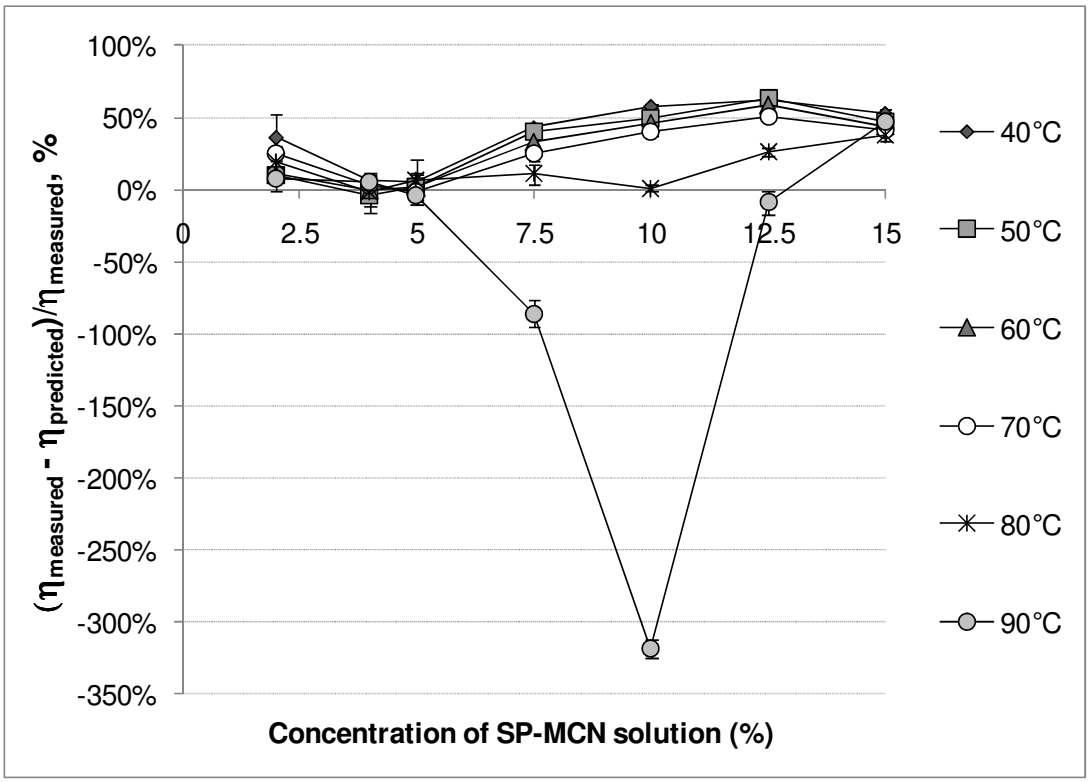


Figure 4.5 Departure from the log additive rule of apparent viscosity at a shear rate of 100 s^{-1} for soy protein-micellar casein (SP-MCN) solutions treated at 40° to 90°C.

At concentrations ≥7.5%, clear positive deviations from the log-additive rule ($Dev_\eta > 0$) were observed for most mixtures, suggesting the presence of intermolecular interactions between casein micelles and soy proteins. Since the deviations were the highest for the mixtures treated at the lowest temperature (40°C), where no chemical interactions are expected, and decreased systematically in amplitude with heat treatment temperature, it is likely that the interactions between the two proteins were physical in nature. The occurrence of interactions and the nature of these interactions require further investigation and will be the focus of a separate study. The only exception from this behavior was observed for the SP-MCN systems of 7.5% to 12.5% concentration subjected to 90°C treatment, which showed a negative deviation from Eq. 2 ($Dev_\eta < 0$), which means that the mixtures' viscosity was lower than the sum of the viscosity of the individual components. Negative deviations of viscosity with respect to the log-additive mixing rule have been reported for many incompatible polymers pairs (Utracki, 1989; Vankan et al., 2004). Some researchers attributed such a behavior to local phase segregation leading to a slip between the components of the mixture (Brochard, de Gennes, & Troian, 1990; de Gennes, 1992). This explanation is plausible for the mixed SP-MCN system heat treated at 90°C, since this temperature causes partial denaturation and unfolding of soy protein molecules. This will lead to a mixture that contains both compact, globular structures (casein micelles) and (partially) denatured soy proteins, with conformations closer to a random coil. It has been proposed before that unfolding of protein molecules is able to enhance incompatibility in protein mixtures, since lower values of phase separation thresholds are typical of flexible chain polymers as compared to compact, globular proteins (Polyakov et al., 1997). The reason why the 15% mixture treated at 90°C did not display a negative deviation from the log-additive rule is that at this concentration, a network of soy proteins started to form in the mixture, which restricted the overall

molecular mobility in the mixed system.

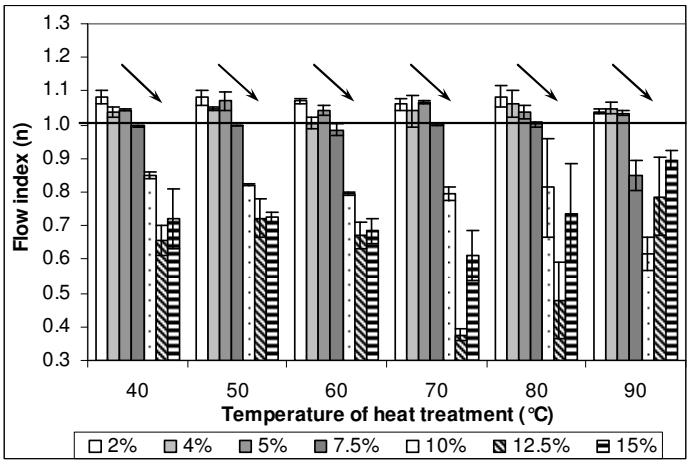
Flow behavior analysis

The flow behavior of the protein systems was characterized using the Herschel-Bulkley constitutive model:

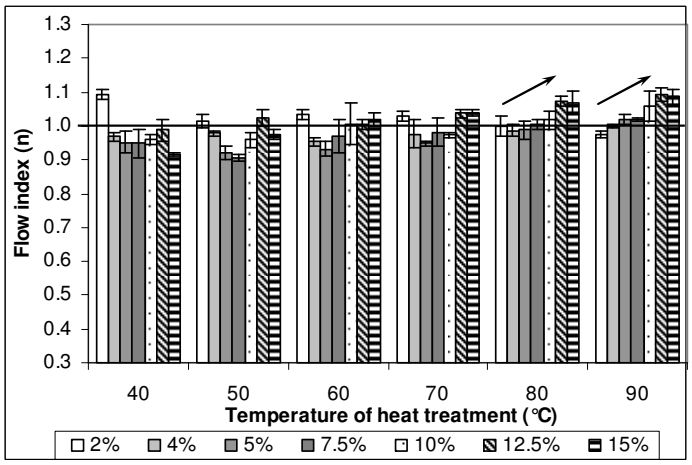
$$\sigma = K\left(\dot{\gamma}\right)^{n} + \sigma_0, \qquad (4)$$

where K is the consistency coefficient ($Pa \times s^n$), $\dot{\gamma}$ is the shear rate (s^{-1}), n is the flow behavior index, and σ_o is the apparent yield stress (Pa). The parameters of the model were obtained by linearizing Eq. 4, with n being determined as the slope and K as the intercept of the $\ln(\sigma)$ vs. $\dot{\gamma}$ curve. The minimum values of the coefficient of determination (r^2) obtained when fitting the Herschel-Bulkley constitutive model ranged from 0.985 to 0.865 for SP solutions of 2% to 15%, respectively; from 0.986 to 0.932 for MCN solutions of 2% to 15%, respectively; and from 0.987 to 0.868 for SP-MCN mixtures of 2% to 15%, respectively.

In case of soy protein systems, both concentration ($F[6, 84]=244.91$, $p<0.0001$) and heat treatment temperature ($F[5, 84]=5.67$, $p=0.0002$) had a statistically significant effect on the flow behavior index *n.* A transition from a near-Newtonian or slight shear thickening behavior ($n \geq 1$) to a shear thinning behavior ($n \leq 1$) occurred at concentrations above 7.5% (Figure 4.6a).



a)



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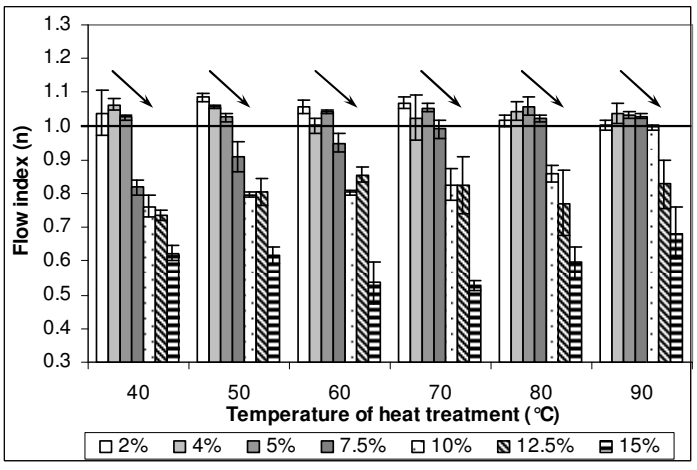


c)

Figure 4.6 Flow behavior index (*n*) for protein solutions (C= 2% to 15%) after treatment at 40°C to 90ºC. a) Soy protein (SP); b) Micellar casein (MCN); c) SP-MCN.

Although the flow behavior index *n* is used to measure the departure from Newtonian flow, a better way to view it is as an indication of the rate of structure change with shear rate (Ferguson and Kemblowski, 1991). Thus, the inverse proportionality between SP concentration and flow index *n* (r^2=0.85) indicates that the three-dimensional protein lattice becomes increasingly structured with increasing protein concentration. Higher heat treatment temperatures seemed also to lead to more pronounced shear thinning behavior. It is important to note that the flow behavior index for the 15% solution treated at 90°C was significantly higher as compared to the 12.5% solution treated at the same temperature. Visual observations of the samples, as well as rheological data indicated an onset of network formation in both of these systems, but a weaker network (more sensitive to shear) was formed when heating the 12.5% solution.

Micellar casein solutions generally displayed a flow behavior close to Newtonian (n~1), although under certain conditions either slight shear thinning or slight shear thickening has been noticed (Figure 4.6b). A two-way ANOVA test indicated that both protein concentration ($F[6, 84]=24.42$, $p<0.0001$) and heat treatment temperature ($F[5, 84]=23.51$, $p<0.0001$) had a statistically significant effect on the flow behavior index of the MCN solutions. However, the only clear trend observed was an increase in *n* with concentration for the MCN solutions subjected to heat treatment at 80°C and 90°C. For those heat treatments, the flow behavior varied from slightly shear thinning at low concentration to slightly shear thickening at high concentration. Shear thickening behavior has been previously reported in concentrated dispersions of charged rigid spheres (Shenoy, Wagner, & Bender, 2003), and was attributed to the formation of hydroclusters at shear rates where the hydrodynamic forces overcome the repulsive interparticle forces (Bender and Wagner, 1996; Shenoy et al., 2003). The possible reasons for the shear thickening behavior of the

concentrated MCN systems require further investigation and will be addressed in a separate study.

The flow behavior of the mixed SP-MCN systems resembled relatively closely the behavior of the soy protein solutions (Figures 4.3c, 4.6c). Protein concentration had a statistically significant effect on the flow behavior index *n* of the mixtures ($F[6, 84]=355.14$, $p<0.0001$); heat treatment temperature also significantly influenced the flow index *n* ($F[5, 84]=9.36$, $p<0.0001$). The dilute SP-MCN solutions ($C\leq5\%$) displayed a slightly shear thickening or Newtonian behavior ($n\geq1$), but the flow behavior became predominantly shear thinning at concentrations above 7.5%. There was a significant correlation between protein concentration and flow index ($r^2=0.92$), with the more concentrated SP-MCN solutions displaying a more pronounced shear thinning behavior. A correlation was also found between treatment temperature and flow index *n* ($r^2=0.82$), with less pronounced shear thinning behavior in SP-MCN mixtures that have been treated at higher temperatures. These observations on flow behavior suggest that the mixtures behave like a soy protein continuum, with casein micelles acting as filler.

Another important parameter that was determined using the Herschel-Bulkley model was the apparent yield stress ($\sigma_{0,app}$), which represents the stress value that must be exceeded in order to make a structured fluid flow. The $\sigma_{0,app}$ value was determined according to the method proposed by Steffe (1996b), as the intercept of the linear region of the shear stress vs. shear rate curve, extrapolated to zero shear rate (Figure 4.7). Most protein systems in this study manifested apparent yield stress, and the $\sigma_{0,app}$ value increased with concentration. For soy protein solutions $\sigma_{0,app}$ has reached values in the range of 100 Pa at 15% concentration (Figure 4.8). High heat treatment (80°C and 90°C) resulted in higher $\sigma_{0,app}$ values at concentrations above 7.5%, probably due to incipient network formation occurring in those systems. For MCN systems, the

$\sigma_{0,app}$ values were much lower, ranging between 0.01 Pa (at 2% concentration) and 1 Pa (at 15% concentration); no significant influence of the heat treatment on the magnitude of $\sigma_{0,app}$ was observed.

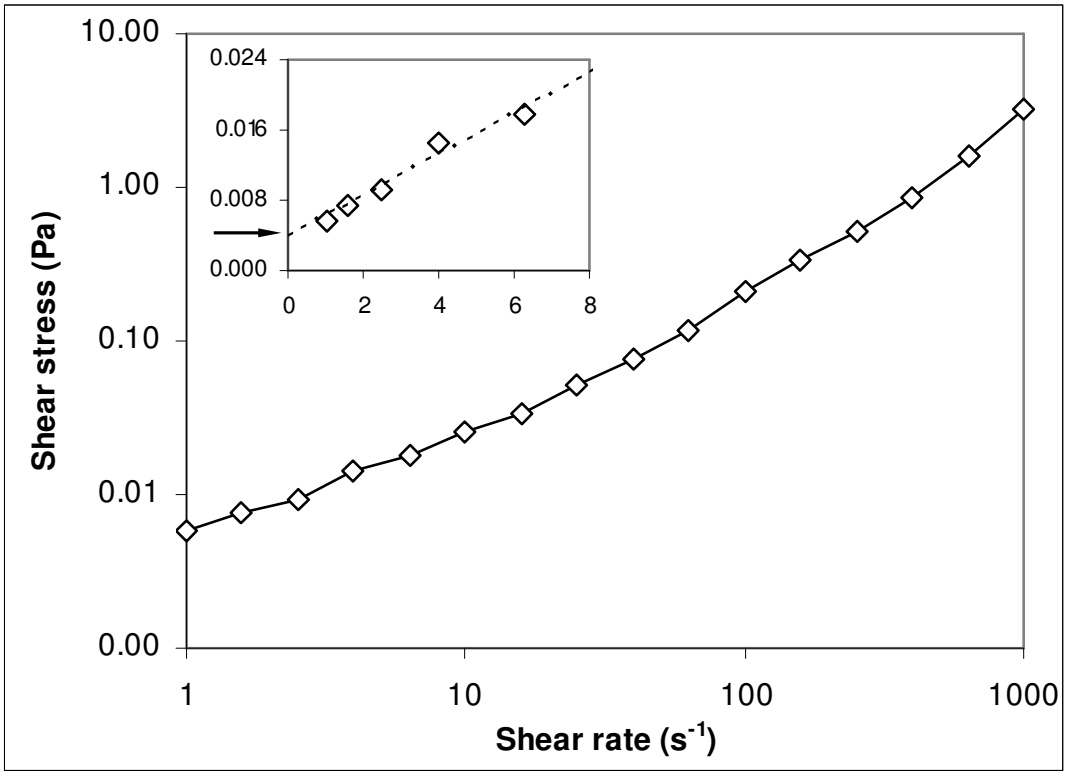


Figure 4.7 Example of yield stress determination from shear stress-shear rate curve for a 2% micellar casein (MCN) solution after treatment at 50°C.

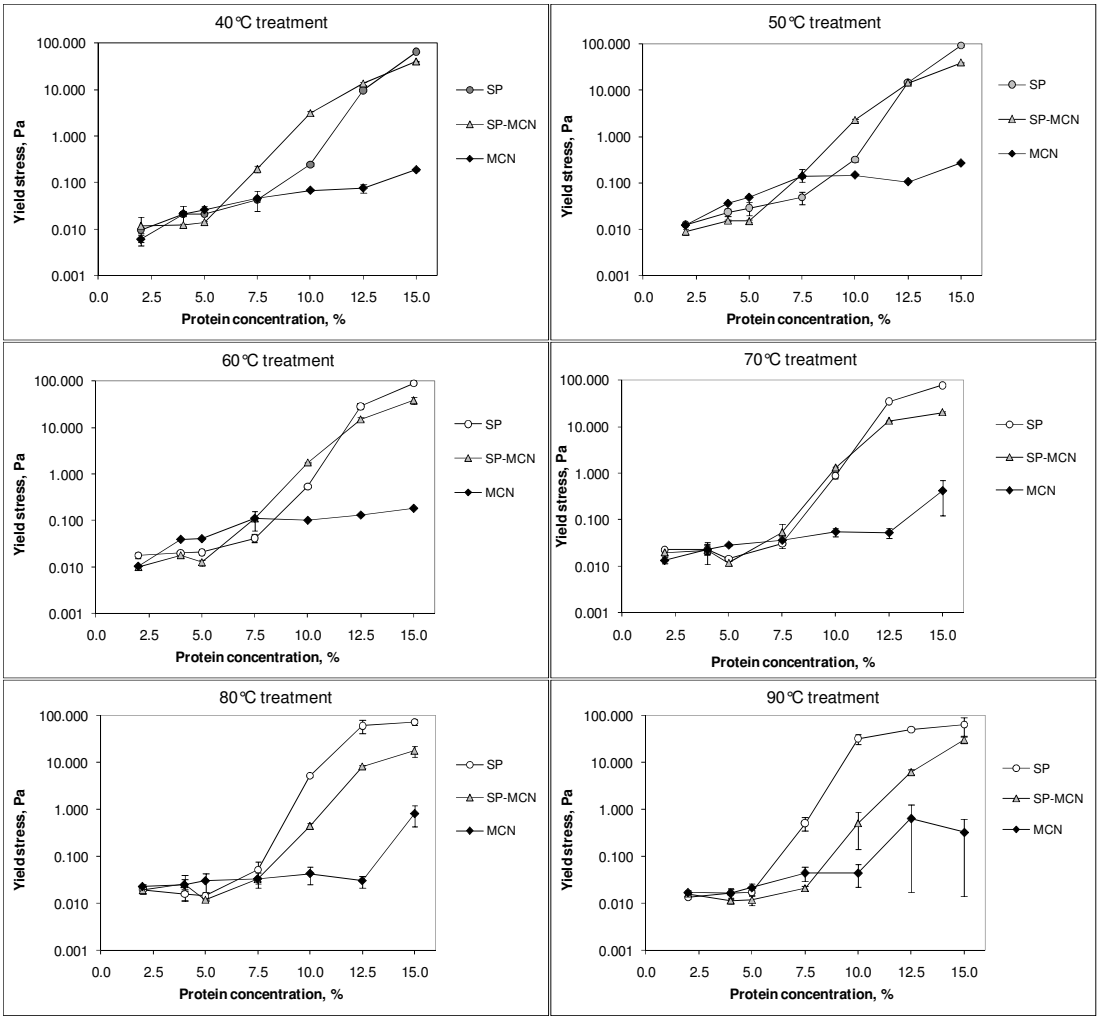


Figure 4.8 Yield stress for protein solutions of 2% to 15% concentration after treatment at 40°C to 90℃.

SP-MCN mixtures generally showed values of $\sigma_{0,app}$ intermediate between those of the individual proteins at the same concentration, although exceptions were also observed (Figure 4.8). The effect of protein concentration on $\sigma_{0,app}$ was statistically significant ($F[6, 84]=188.6$, $p<0.0001$), with the highest values of $\sigma_{0,app}$ being observed for the highest concentration systems. For dilute mixtures ($C \leq 5\%$), $\sigma_{0,app}$ was not significantly affected by the intensity of heat treatments, while for concentrated mixtures ($C \geq 7.5\%$) the heat treatment temperature had a significant effect on $\sigma_{0,app}$. An observation with practical implications is that $\sigma_{0,app}$ of concentrated protein mixtures (7.5 to 12.5%) was substantially lower (by 55-90%) after heat treatments at T≥80ºC as compared to mixtures treated at 40ºC. This is possibly due to the fact that, after high temperature treatments, the looser, more flexible structure of the denatured glycinin chains lowered the resistance to flow of the mixture, as discussed before. An exception to this trend were the 15% SP-MCN solutions that were heat treated at 90ºC, for which the apparent yield stress was higher than at 80ºC. This was due to the fact that in that system the concentration of soy protein molecules exceeded the critical concentration for gelation, which resulted in some network formation, as discussed previously.

Zeta potential analyses of the heat treated protein systems

Zeta potential (ζ) was used as measure of the electrical charge of proteins and as a relative indicator for the colloidal stability of the protein systems. If particles in a mixture have large negative or positive zeta potential values they repel each other, which inhibits aggregation and enhances the stability of the system; if particles have low absolute values of the zeta potential then they will agglomerate and the dispersion becomes unstable (Morrison and Ross, 2002). Riddick (1968) defined the stability of dispersions with relation to zeta potential as follows: (i) 0 to ±3 mV: maximum

agglomeration and precipitation; (ii) +5 to -5 mV strong agglomeration and precipitation; (iii) -10 to -15 mV threshold of agglomeration; (iv) -16 to -30mV threshold of delicate dispersion; (v) -31 to -40 mV moderate stability; (vi) -41 to -60 mV fairly good stability; (vii) -61 to -80 mV very good stability and (viii) -81 to -100 mV extremely good stability.

When interpreting the zeta potential results, the pH of the solutions must also be considered. A small increase of pH from the highest to the lowest protein concentration occurred, as a result of dilution with ultra-pure water. For soy protein solutions, the pH varied from 6.74 in the 15% SP solution to 7.02 in the 2% SP solution; for micellar casein solutions the pH ranged between 6.86 in the 15% MCN solution to 7.21 in the 2% MCN solution, while for the mixed SP-MCN systems the pH ranged from 6.84 in the 15% SP-MCN solution to 7.15 in the 2% SP-MCN solution. Since all pH values were close to neutral, it is not expected that they affected in any significant way the zeta potential values for the protein solutions.

Unheated soy proteins and micellar casein were both negatively charged, with ζ values before heat treatment of -26.3 ± 0.5 mV and -18.8 ± 0.6 mV, respectively.

For soy proteins of various concentrations that had been treated at 40° to 90°C, ζ ranged from -32.7 mV to -19.2 mV, with no statistically significant effect of concentration and heat treatment temperature on the ζ values (Figure 4.9).

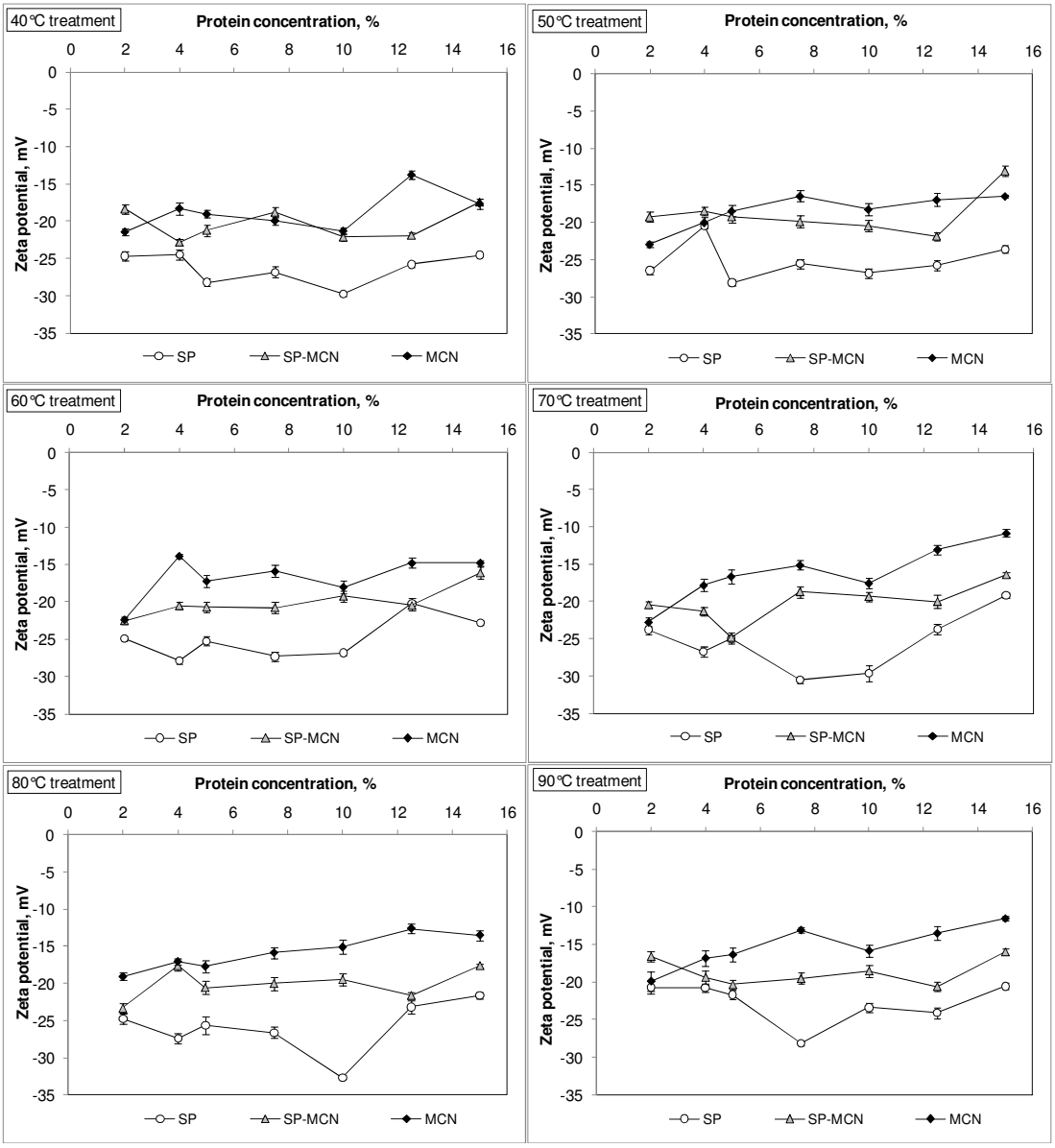


Figure 4.9 Measured zeta potential values for protein solutions of 2% to 15% concentration after treatment at 40°C to 90ºC.

These values indicate a low to moderate stability for these systems. As a note, it was not possible to measure ζ for the 15% soy protein after treatment at 90°C, due to the onset of gelling.

Micellar casein had lower absolute values of ζ as compared to soy proteins, ranging from -22.9 mV to -10.8 mV. These values are similar with those reported by Anema and Klostermeyer (1996). While such values of ζ might indicate low stability of micellar casein solutions, it is known that casein micelles are further stabilized through steric hindrance by the presence of the "hairy layer" of κ-casein at the surface of the micelles (Horne and Davidson, 1986; Fox, 2003). Concentration had a statistically significant effect on the ζ of micellar casein systems ($F[6, 84]=54.8$, $p<0.0001$), as did the heat treatment temperature ($F[5, 84]=20.5$, $p<0.0001$). A clear trend of decreasing negative values of ζ with increased protein concentration and treatment temperature was observed (see Figure 4.10). For example, 2% MCN treated at 40°C had ζ = -21.4 ± 0.4 mV, while 15% MCN treated at 90°C had ζ = -11.6 ± 0.3 mV, indicating a much lower stability of the latter.

Increasing MCN concentration from 2% to 15% resulted in lower negative values of ζ, even for the low treatment temperatures (Figure 4.10). This is possibly a direct result of a neutralizing effect of ionic calcium present in solution (Ca^{2+}) at the surface of the casein micelles.

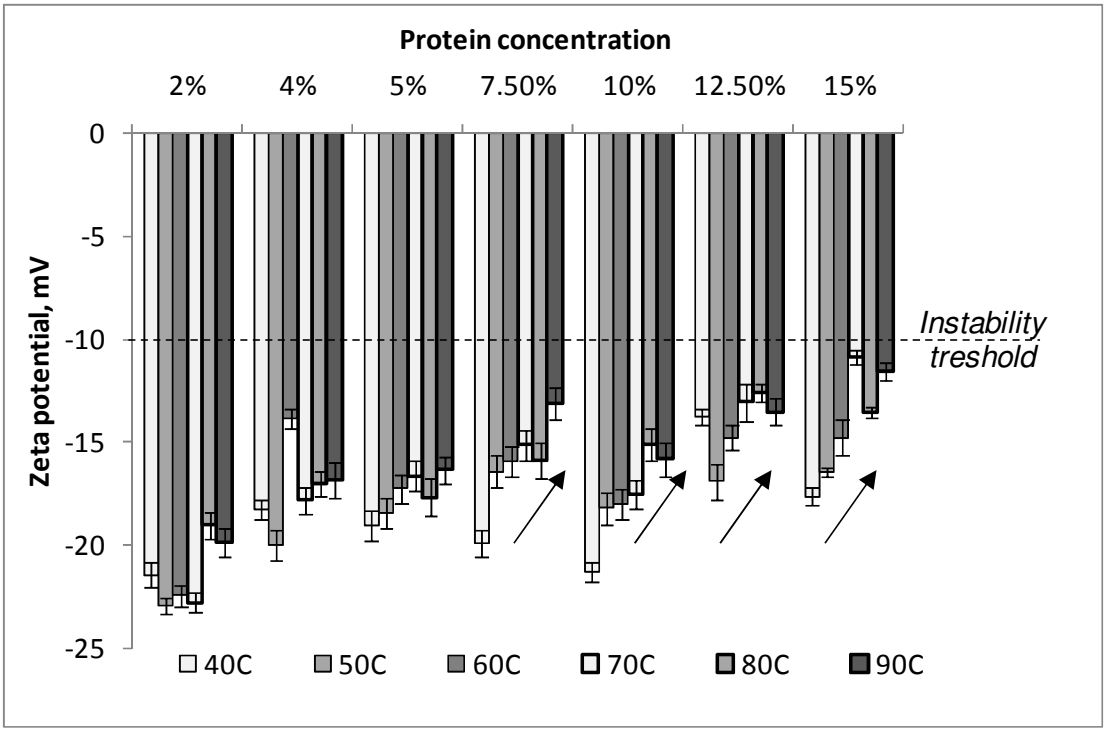


Figure 4.10 Measured zeta potential values for micellar casein (MCN) solutions of 2% to 15% concentration after treatment at 40°C to 90ºC.

The total calcium in the MCN samples increased linearly with concentration, from 0.57 g/kg in the 2% samples to 4.25 g/kg in the 15% samples. Yet, the concentration of free Ca^{2+} did not follow the same linear increase with MCN concentration. As seen in Table 4.2, the concentration of free Ca^{2+} in the samples varied between 1.48 mM (59.5 mg/kg) in the 2% MCN solution to 4.09 mM (164 mg/kg) in the 15% MCN solution. This indicates a decrease in the proportion of free Ca^{2+} from 10.9% in the 2% MCN solution to only about 3.9% in the 15% MCN solution. The main reason for the decrease in % free Ca^{2+} is suspected to be its precipitation out of solution, due to the known limited solubility of calcium in water. It is also possible that the measured Ca^{2+} values in the higher concentration samples were affected by the high viscosity of these solutions, which may have slowed down

the diffusion of Ca^{2+} through the water phase, thus affecting the accuracy of the measurement. Nonetheless, the hypothesis regarding the precipitation of Ca^{2+} out of solution would explain well the change in zeta potential values as the MCN concentration increased. Some of the calcium ions could have come out of solution and onto the surface of the micelles, neutralizing some of the negative charges on the surface of the micelles, thus reducing their zeta potential. While this hypothesis needs further testing, it is consistent with previous observations by Horne (1998), who reported that lower absolute values of the zeta potential were caused by partial neutralization of the negatively charged posphoserine clusters on the surface of the casein micelles by positive calcium ions.

Table 4.2 Free Ca^{2+} concentration of casein solutions at 20ºC.

Micellar casein concentration, %	**Free Ca^{2+}, mM**	**Free Ca^{2+}/Total Ca, %**
Milk[1]	2.05 ± 0.01	7.9[2]
2	1.48 ± 0.01	10.9[3]
4	2.17 ± 0.09	7.7[3]
5	2.52 ± 0.01	7.1[3]
7.5	2.91 ± 0.02	5.5[3]
10	3.33 ± 0.02	4.7[3]
12.5	3.62 ± 0.04	4.1[3]
15	4.09 ± 0.01	3.9[3]

[1] Data reported in literature: 2.1 ± 0.06 mM (Lin, Lewis, & Grandison, 2006)

[2] Calculated based on total Ca in milk reported by Gaucheron (2005) (1043 mg/kg)

[3] Calculated based on total Ca content of MCN powder (2.53% d.m.)

The effect was even more pronounced at high treatment temperatures (≥70°C), where decreases in ζ by about 10mV were observed, as compared to decreases of only about 5mV after treatment at lower temperatures. A possible reason for the significant decrease in ζ after heat treatment at temperatures ≥70°C could be the precipitation of calcium phosphate on the surface of the casein micelles, which was reported to occur upon heating at high temperatures (Wahlgren, Dejmek, & Drakenberg, 1990; Anema and Klostermeyer, 1997). This data is consistent with the report by Faka, Lewis, Grandison, & Deeth (2009), who established a direct correlation between the free Ca^{2+} content of milk and dairy products and the decrease in the absolute value of the zeta potential as a result of high heat treatment.

Mixed SP-MCN systems had ζ intermediate between those of micellar casein and soy proteins, with values ranging between -24.9 mV and -13.0 mV for the range of experimental conditions tested (Figure 4.9). Both the protein concentration ($F[6, 84]=21.47$, $p<0.0001$) and the temperature of the heat treatment ($F[5, 84]=4.59$, $p=0.0007$) had a statistically significant effect on the zeta potential values of the mixed systems.

Overall, the zeta potential analysis indicates that in most cases zeta potential values were above the threshold of agglomeration (-10mV), suggesting that the studied protein systems were relatively stable under the experimental conditions of this study. Stability of the protein solutions decreased however with temperature and concentration, and systems of $C>12.5\%$ heated at temperatures above 80°C seemed prone to aggregation.

Particle size analyses

The occurrence of aggregation in the heated protein systems was investigated using dynamic laser light scattering (DLS). The results of the particle size

measurements were evaluated based both on multimodal size distribution and lognormal distribution. The lognormal distribution was used to calculate a single effective diameter for each analyzed sample, which allowed direct comparisons among the different protein systems; the multimodal size distribution on the other hand offered information regarding the presence of groups of particles or molecules of different sizes (Beliciu and Moraru, 2009).

Measurements were performed for proteins at concentrations ≥5% and temperatures ≥60°C, where aggregation phenomena and subsequent changes in particle sizes were more likely to occur. Deionized water was used as a solvent for the DLS analyses. While this is not an ideal solvent for measuring the size distribution of casein micelles (Beliciu and Moraru, 2009), it allowed the use of a single solvent for both classes of proteins studied in this work.

As seen in Figure 4.11, both caseins and soy proteins showed a bimodal size distribution, with soy proteins being slightly smaller in size than casein micelles. The results of the particle size measurements are shown in Figure 4.12.

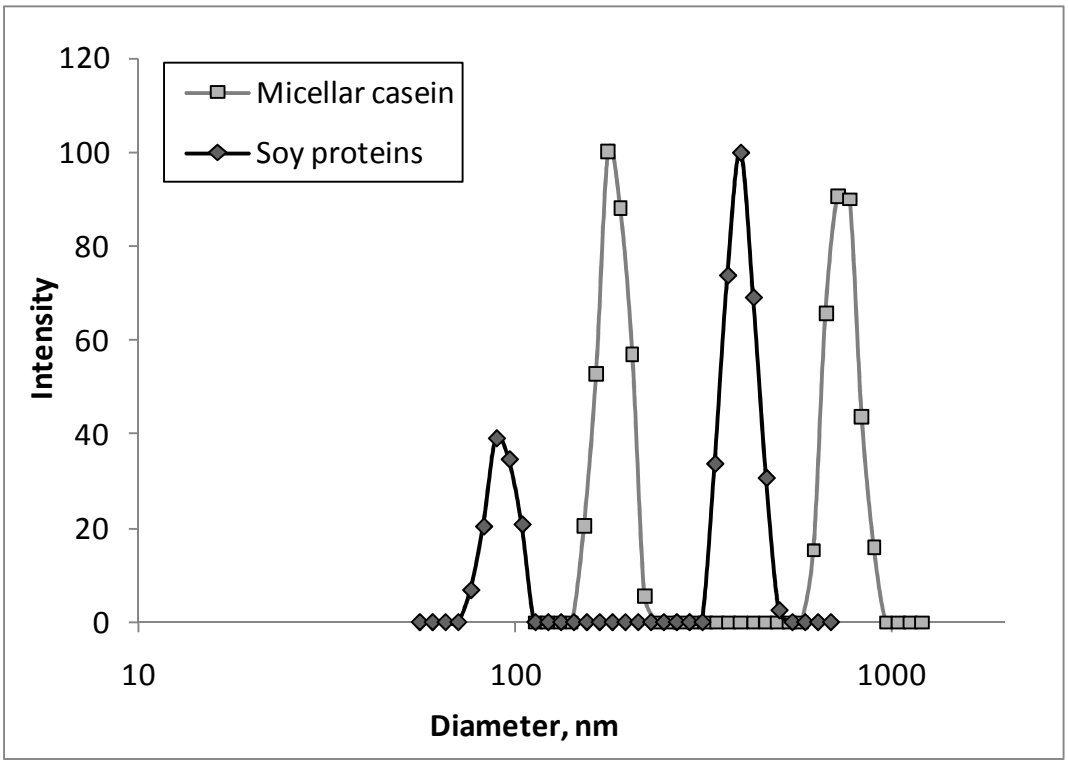


Figure 4.11 Example of particle size distribution for soy proteins and micellar casein after heat treatment at 60ºC in 10% solution.

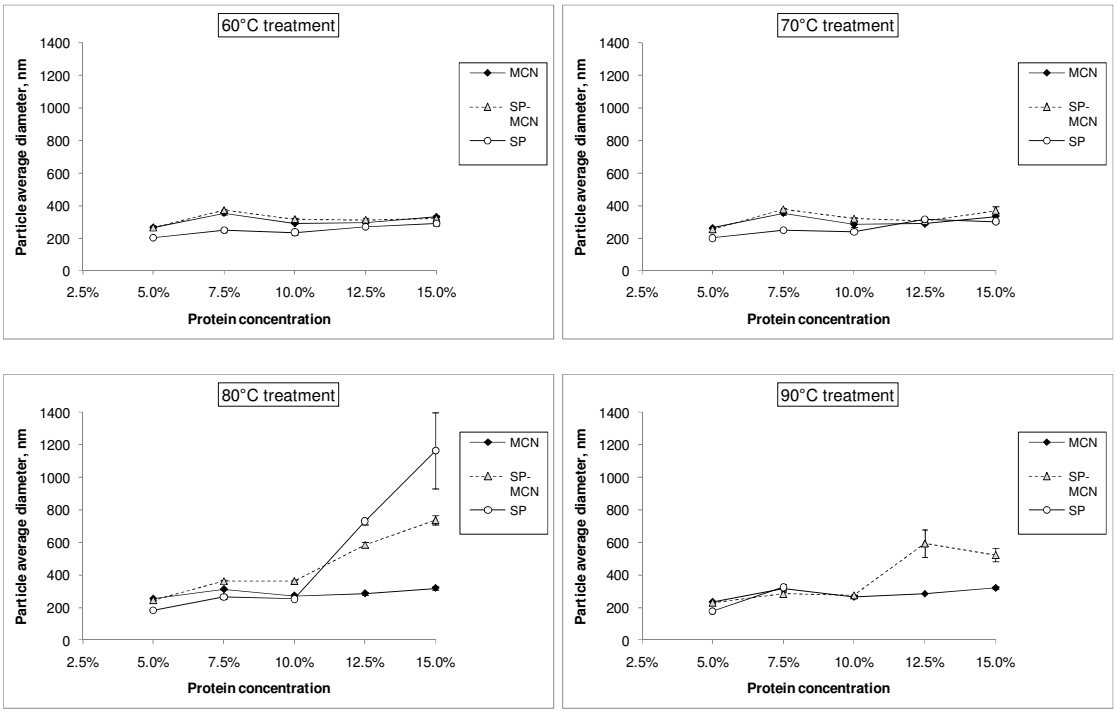


Figure 4.12 Particle sizes of the heat treated protein solutions measured after the heat treatments, at 20ºC.

The average particle size of soy proteins was in the 200-300nm range after non-denaturing heat treatments (T<80°C). After the denaturing heat treatments (T≥80ºC), concentrated soy proteins systems (C≥7.5%) showed a drastic increase in the measured particle size. In the 12.5% and 15% soy proteins solutions heat treated at 80°C, particle sizes of 729 ± 19 nm and 1163 ± 234 nm were measured. For soy protein solutions with C≥10% heat treated at 90°C, particle size analyses could not be performed accurately due to the formation of macroscopic aggregates and inhomogeneity of the system, which made acquiring a representative sample and subsequent particle size measurement difficult. These observations are consistent with the rheological data, and clearly indicate aggregation phenomena and network formation in concentrated protein systems heated at denaturing temperatures.

Casein micelles had slightly larger particle sizes than non-aggregated soy proteins, and typically ranged between 250-400nm. It must be noted that the $\overline{d_{eff}}$ values for casein micelles reported in this study are larger than the sizes previously reported for casein micelles. The main reason for this discrepancy is the use of water as a solvent, which affects the hydration and voluminosity of casein micelles, and thus their measured particle size (Beliciu and Moraru, 2009). While there were some differences in particle size of casein micelles as a function of heat treatment temperature and concentration, these changes will not be addressed in this paper, since the choice of solvent may have contributed to the magnitude of these differences.

Generally, the particle sizes measured in SP-MCN protein systems were closer to the values measured for casein micelles than soy proteins. This is expected, since the casein micelles (the larger particles in the mixed systems) will contribute more to the intensity of the signal than the smaller soy protein particles. Under treatment conditions that were conducive of soy protein denaturation (T≥80°C), aggregation phenomena became noticeable in the mixtures with protein concentrations ≥12.5%, in

which the soy protein concentration was above the critical limit for gelation (6.6%). This emphasizes once again that, besides treatment temperature, another parameter that is pivotal for the initiation of aggregation phenomena is the soy protein concentration in the system.

Conclusions

The findings of this study illustrate the complex behavior of mixed soy protein-micellar casein systems and the effect of total protein concentration and heat treatment on this behavior. The viscosity and flow behavior of the studied protein systems were affected by concentration and temperature in a different manner. For micellar casein, viscosity and apparent yield stress increased exponentially with concentration, but were not significantly affected by heat treatment. Most micellar casein systems exhibited a nearly Newtonian flow behavior, with the exception of systems with concentration higher than 12.5% treated at temperatures above 80ºC, which displayed a slight shear thickening flow behavior. Viscosity and apparent yield stress of soy protein systems also increased with concentration, and generally exhibited a shear thinning behavior. Heat treatments at temperatures above 80ºC induced glycinin denaturation, which resulted in protein aggregation and network formation in soy protein systems with concentration above 7.5%, which was above the critical limit for gelation.

Mixed SP-MCN systems had a rheological behavior closer to that of soy proteins than of micellar casein. The mixtures that had the most interesting properties were those of concentration higher than 7.5%. The structure and rheological behavior of these mixtures was controlled by the denaturation and concentration of soy proteins. Heat treatment of SP-MCN mixtures at temperatures above 80ºC led to partial denaturation and unfolding of soy proteins (glycinin) molecules, and the rheological

behavior of these mixtures was highly dependent on concentration. For mixed protein systems of intermediate concentration (7.5% to 12.5%), data suggested that a local phase separation between the spherical CN micelles and the unfolded SP molecules took place. These results are particularly interesting from a practical point of view, since through controlled denaturation of soy proteins it would be possible to develop beverages with a high protein concentration, relatively low shear viscosity and almost Newtonian flow behavior. The good storage stability observed for these mixtures (no visible sedimentation observed after 2 months of storage at 4ºC) is also very interesting and deserves further investigation. Higher concentration mixtures (15%) treated at temperatures >80ºC showed protein aggregation and incipient network formation, but CN micelles physically restricted the formation of a strong SP network.

Overall, the findings of this study demonstrate an interesting rheological behavior both in the individual protein systems as well as in the mixed soy protein-micellar casein systems, and suggest the presence of complex interactions between these two proteins. The nature of the interactions between the two classes of proteins is of high interest, and will be the subject of a follow-up investigation. The findings of this study could be used as a basis for the development of high protein foods with unique structure and functionality.

Acknowledgements

This research was supported by the Cornell University Agricultural Experiment Station federal formula funds, Project No. NYC 143425, received from Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

4.2. EVALUATION OF INTERMOLECULAR INTERACTIONS IN HEAT TREATED MICELLAR CASEIN-SOY PROTEIN MIXTURES

Abstract

In this study, structural changes and intermolecular interactions induced by heating in mixed micellar casein (MCN) - soy proteins (SP) were investigated, using a combined rheological and chemical approach. Individual and mixed (1:1) protein systems of 10 and 15% concentration were prepared and heat treated for 15 min at 40°, 60º and 95°C. After cooling to 20°C, their rheological properties were determined using steady-shear and dynamic rheology. Viscosity and yield stress of MCN, SP and mixtures increased with concentration. Treatment of 10% MCN-SP at 95ºC resulted in a solution characterized by Newtonian flow behavior, lower apparent viscosity and yield stress than the 40ºC treated solution, as well as high long term stability. Most heat treated mixtures had a liquid-like behavior, except the 15% MCN-SP mixture treated at 95ºC, which formed a gel and showed a solid-like behavior. A differential solubility method was used to identify the nature of intermolecular interactions, and native-PAGE analysis was used to identify specific protein fractions in the heat treated protein solutions. The high temperature treatments induced disulfide bonding in the mixed MCN-SP systems, which most likely occurred exclusively between soy proteins and only when the critical concentration for gelation was exceeded. The findings of this study can be used in the development of high protein foods with unique characteristics. By controlling thermal denaturation of soy proteins in MCN-SP mixtures of specific concentrations, it is possible to obtain mixtures with desired structure, rheological properties and storage stability.

Introduction

Proteins are very important both for their nutritional value, as well as their functionality, which make them very desirable food ingredients. Casein, the major milk protein, and soy proteins currently have many uses in the food industry. An interesting prospect is using mixtures of these two proteins.

The results of a recent study on the effect of concentration and heat treatment temperature on the miscibility and rheological properties of mixtures of micellar casein (MCN) and soy proteins (SP) suggest that heat treatment can induce certain intermolecular interactions between the two proteins. The nature and strength of such interactions are expected to be dictated by the molecular structure and conformation of the individual proteins. Caseins are phosphoproteins that precipitate at an isoelectric pH of 4.6, and they make up 82% of the protein content of cow's milk. In native milk, they are associated into casein micelles, which are aggregates that contain α_{s1}, α_{s2}, β and κ-casein in a weight ratio of 3:0.8:3:1 (Schmidt, 1982) and 6% (d.b.) phosphate and calcium ions (Horne, 2006). The exact structure of casein micelles is still debated (Walstra, 1990; Horne, 2006; Qi, 2007). However, it is commonly accepted that they are roughly spherical core-shell particles with outer diameters ranging from 50 to 500 nm (de Kruif, 1998; McMahon and McManus, 1998; Dalgleish et al., 2004; Beliciu and Moraru, 2009). In recent works, the core is described as a homogeneous web of individual casein molecules in which calcium phosphate nanoclusters are uniformly distributed (Horne, 2002; Marchin et al., 2007; McMahon and Oomen, 2008). The outer shell of the micelle is considered to be made of κ-caseins that extend into the aqueous phase as a polyelectrolyte brush, producing short-range repulsions among micelles (de Kruif and Zhulina, 1996; Tuinier and de Kruif, 2002). Caseins are thermally stable on their own, but in the presence of other molecules (i.e. serum proteins) they can be affected by heat, due to the fact that κ-casein contains two

cysteine residues that can participate in sulphide interchange reactions with other molecules (Carr et al., 2003). It has been shown that heating milk above 70°C induces the denaturation of serum proteins and subsequent complex formation with the κ-casein located at the casein micelle surface (Rose, 1963; Creamer et al., 1978; Singh and Fox, 1985; Dannenberg and Kessler, 1988; Dalgleish, 1990; Singh and Latham, 1993; Beaulieu et al., 1999).

The structure and properties of soy proteins are quite different from those of casein. Soy proteins contain four protein fractions that are classified according to their sedimentation properties: 2s, 7s, 11s and 15s which represent, respectively, 8%, 35%, 52% and 5% of the total protein content (Kinsella, 1979; Barać et al., 2004). The 11s and 15s fractions consist of glycinin and polymers of glycinin (Wolf, 1970), while the 7s fraction contains mostly β-conglycinin but also γ-conglycinin, lipoxygenases, α-amylases and hemaglutenins (Nielsen, 1985). The 2s fraction consists of Bowman-Birk and Kunitz trypsin inhibitors, cytochrome C, and α-conglycinin (Wolf, 1970). β-conglycinin and glycinin represent more than 80% of the total proteins in soy, and thus have a significant influence on the functional properties of soy protein systems. Glycinin exists as a hexamer with a molecular mass of 360 kDa (Renkema et al., 2001), while β-conglycinin is a trimeric glycoprotein with a molecular mass of 150-200 kDa (Utsumi et al., 1997). Upon heat treatment, the bonds that maintain the secondary and tertiary structure of soy proteins weaken, leading to denaturation and exposure to the solvent of hydrophobic moieties that were buried in the native conformation. This can further lead to aggregation of the partially unfolded protein molecules; at concentrations above a critical value (6.6%, according to Bikbow et al., 1979) a network is formed as a result of aggregation (Clark and Ross-Murphy, 1987; Aguilera, 1995; Berli et al., 1999). In case of glycinin, gel formation takes place via disulfide bonds and noncovalent bonds such as hydrophobic interactions, ionic and

hydrogen bonds (Mori et al., 1986). Gelation of β-conglycinin is caused by hydrophobic interactions and hydrogen bonds, with no contribution from disulfide exchange reactions (Nakamura et al., 1986). In mixed soy protein solutions, gel properties are greatly affected by the glycinin: β-conglycinin ratio and the environmental conditions, including temperature, pH and ionic strength (Utsumi et al., 1997).

The main objective of this study was to investigate the nature of the interactions that occur between soy proteins and micellar casein upon heating, using a combined rheological and chemical approach. Knowledge of such interactions could be used as a basis for the development of protein based foods with unique structure and functionality.

The investigation was conducted on two protein concentrations and three heat treatment temperatures, which were selected based on both practical considerations and on the conclusions of previous work. Protein concentrations of 10% and 15% (w/w) were chosen, so that soy protein concentrations both below and above the critical soy protein concentration for gelling (6.6%) are achieved in MCN-SP mixtures (at a 1:1 ratio of the two proteins). For comparison purposes, individual protein solutions (micellar casein and soy protein) of 10% and 15% concentration were also evaluated.

The heat treatment temperatures used in this study were chosen as follows: a low temperature treatment of 40°C was chosen as a baseline or point of reference; an intermediate heat treatment at 60°C was chosen since under these conditions a positive departure from the log-additivity rule of viscosity of the mixtures was observed in previous work, while a high temperature heat treatment of 95°C was chosen in order to exceed the denaturation peak of glycinin, which was reported to be 93.6°C. A treatment time of 15 minutes was selected based on the conclusions of Mori et al.

(1986), who established this time interval as the time when a heat-induced glycinin gel is fully developed.

Materials and Methods

Materials

In order to ensure that the effects of the preparation methods on the native structure and properties of the two proteins are minimal, this study was conducted on protein preparations obtained by membrane separation followed by spray drying. Micellar casein powder (MCN-85, American Casein Company, Burlington, NJ) was used as a source of casein. The composition of the MCN powder is as follows: 84.93% (d.b.) protein, 2.1% (d.b.) fat, 9.5% (d.b.) ash, 3.2% (d.b.) lactose, and 4.8% moisture. The mineral profile of the MCN powder is as follows: 2.53% (d.b.) calcium, 1.47% (d.b.) phosphorus, 0.10% (d.b.) magnesium, 0.10% (d.b.) potassium, 0.07% (d.b.) sodium - and 0.12% (d.b.) chloride. Soy protein isolate (SPI-6000 Protient Inc., St. Paul, MN) was used as a source of soy proteins. The composition of the soy protein isolate was: 90.9% (d.b.) protein, 3.3% (d.b.) fat, 5.8% (d.b.) ash, and 5.4% moisture.

Sample preparation

Casein and soy protein powders were weighed and added to 100 ml of ultra pure water to obtain individual protein solutions of the desired concentrations (10% and 15% on a dry matter basis). To ensure good dispersion, the protein powder was added slowly to the water in a glass beaker placed on a stir plate (Cimarec Model SP 131325, Thermo Scientific, Waltham, MA) set at speed 5. The water-protein mixture was kept on the stirring plate for 30 minutes at 25°C under moderate agitation (speed 4), in order to allow hydration of proteins. The solution was poured in a Mojonnier bottle in order to control foaming, and the complete dispersion of the proteins was

achieved using an UltraTurrax Model T25 fitted with a S25N–18G dispersion tool (IKA Works Inc., Wilmington, NC) for 5 min at 21,500 rpm. The mixing time for the high-speed dispersion step was established by monitoring the evolution of particle size in the protein solution, as explained in detail in section 4.1. After the high-speed dispersion step, the solutions were kept under continuous stirring at speed 3 on the stirring plate for 1 hour. The mixed casein-soy protein solutions were prepared by mixing together equal amounts of the individual protein solutions of the respective concentration (10% or 15%) in a flat bottom Pyrex tube (Model 9850-25, Corning Incorporated, Corning, NY) placed on an analog vortex mixer (Fisher Scientific, Pittsburg, PA) (30 s at speed 6 then continuously increase speed over 1 min from 6 to 10, lower from 10 to 6 during 1 min and final 30 s at 6). Much care was taken to eliminate air bubbles, which could lead to non-uniformities during the heating step.

Heat treatments

Both individual and mixed protein solutions were placed into flat bottom Pyrex test tubes (Model 9850-25, Corning Incorporated, Corning, NY) and heat treated for 15 minutes at 40ºC, 60ºC or 95ºC, respectively, using a water bath (Model 202-2, National Appliance Co, Portland, OR). Protection against dehydration of the protein solutions during treatment was insured by covering the test tubes with Parafilm and heavy duty aluminum foil. Ice water was used to rapidly cool down the protein solutions to 20ºC immediately after the heat treatments.

Rheological analyses

The rheological properties of the protein solutions were evaluated using an Advanced Rheometric Expansion System (ARES) strain-controlled rheometer with dual transducer in conjunction with the Orchestrator data collection and analysis

software (TA Instruments, New Castle, DE). Large-deformation, steady shear analyses and small-deformation dynamic analyses were performed in order to characterize the heat treated samples. The large-deformation tests were used to obtain information on flow behavior, which is relevant for practical applications (mixing, pipe flow, etc.), while the dynamic tests were used to obtain insight into intermolecular interactions and stability of the heat treated protein solutions.

A parallel plate geometry with 50 mm diameter Teflon plates and an interplaten gap of 1 mm was used for measurements of all heat treated protein solutions that did not form gels. 2 ml aliquots of solution were loaded on the lower plate, avoiding the formation of air bubbles; a relaxation step of 1 min was allowed before starting the measurements. For the samples that produced gels as a result of the heat treatments, a parallel plate geometry with 25 mm diameter Teflon plates and an interplaten gap of 2 mm was used. The gels were carefully sliced using a disposable scalpel (Feather Safety Razor Co., Ltd., Osaka, Japan) to produce 2 mm thick slices, which were then placed between the plates of the rheometer.

All measurements were performed at 20ºC. The temperature control during the measurements was ensured using a Peltier system. In order to avoid dehydration during the measurements, all samples were thinly coated with mineral oil at the air-sample interface. Additionally, an isothermal chamber was used to prevent variations in vapor pressure and humidity at the air-sample interface.

Strain-controlled, steady-shear rate sweep tests were conducted for each of the protein solutions, with shear rates ranging from 1 to $10^3 s^{-1}$, in logarithmic sweep mode, with two-directions per measurement. From these tests, viscosity as a function of shear rate data was obtained.

Dynamic, oscillatory rheological testing was also performed. The main dynamic rheological parameters determined were the storage modulus (G') and loss

modulus (G"). The storage modulus characterizes the solid-like response, while the loss modulus (G") characterizes the liquid-like response of the system (Stokes and Frith, 2008). Other parameters determined included the loss tangent (tan δ = G"/G'), complex modulus ($G^* = \sqrt{(G')^2 + (G'')^2}$) and complex viscosity (η^* = G*/ω, where ω = frequency).

Dynamic strain sweeps were conducted for each of the samples in order to identify their linear viscoelastic region (LVR). For liquid samples, the tests were performed at a frequency of 1 rad/s, over the strain interval of 0.05-7% with a strain increment of 0.2%. For samples that displayed heat-induced gelation, the tests were performed at a frequency of 1 rad/s, over the strain interval of 0.1%-1% with a strain increment of 0.02%. Frequency sweeps were then performed for all samples, using a strain value located within the LVR, over the frequency range of 0.1-100 rad/s. All measurements were performed in triplicate.

Chemical evaluation of intermolecular interactions. Differential solubility method and the RC DC assay

The nature of molecular interactions induced by heat treatments in the mixed MCN-SP solutions was evaluated using a modified version of the method developed by Hager (1984). This method uses four different solvents in order to solubilize different protein fractions. *Solvent 1* (35 mM K_2HPO_4, pH 7.5) is known to dissolve protein molecules that remain in their native state (Liu and Hsieh, 2008). *Solvent 2* (35 mM K_2HPO_4 and 8M urea) additionally dissolves denatured, but not highly aggregated, molecules and small aggregates (such as protein monomers rendered insoluble by hydrogen bonding or hydrophobic interactions, or small aggregates of protein molecules joined by intermolecular covalent bonds). *Solvent 3* (35 mM K_2HPO_4 and 0.1M Na_2SO_3), due to the use of sodium sulfite, can cleave disulfide

bridges that make large aggregates insoluble. *Solvent 4* (35 mM K_2HPO_4, 8M urea and 0.1M Na_2SO_3) is able to dissolve the protein segments cleaved by the sodium sulfite, due to the presence of urea. It was critical that all solvents were freshly prepared for each experimental replication, in order to avoid crystallization phenomena, especially in those solvents that contained urea (solvents 2 and 4).

In order to measure protein solubility for the heat treated samples, a dilution of 1.0 mg/mL protein was prepared, for all protein solutions. For 10% (w/v) MCN-SP mixtures, 0.4 mL of solution were pipetted to a 50 mL plastic centrifugation tube (Fischer Scientific, Pittsburg, PA) and solvent was added to achieve a total volume of 40 mL. For 15% (w/v) MCN-SP mixtures, 0.267mL of solution were necessary to achieve a concentration of 1.0mg/mL protein. The tubes were vortexed using an analog vortex mixer (Fischer Scientific, Pittsburg, PA) to homogenize the protein dilutions (30 s at speed 6). Subsequently, the tubes were loaded in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DW) and centrifuged for 1h at 10,000g and 20°C. The supernatant of the centrifuged protein dilution was separated from the precipitate and tested for protein content using the standard RC DC (reducing agent compatible, detergent compatible) protein assay (Bio-Rad Laboratories, Hercules, CA).

The assay uses the principles put forth by Lowry (1951), and is based on the correlation between the amount of tyrosine, tryptophan, and cysteine residues in a protein and the absorbance of the protein solution treated with Folin-Ciocalteau's phenol reagent. The protein content is calculated based on the absorbance of the protein solution measured at 750 nm. First, the proteins solubilized in the solvent were denatured by using trichloroacetic acid, after which the denatured protein pellet was separated by high speed centrifugation (15 min at 15,000g, at 20ºC). In order to minimize interference from supernatant carryover, it was necessary to apply two

washing steps to the protein pellet, each constituting of resolubilization with triclhloroacetic acid and high speed centrifugation. The pellet was then dissolved in an alkaline (pH 10) SDS-NaOH solution containing 1% sodium tartrate and 0.1% copper sulfate, incubated for 5 minutes at room temperature and vortexed (30 s at speed 6); under alkaline conditions, the divalent copper ions form a complex with the peptide bonds and are reduced to monovalent ions. In the next step, diluted Folin reagent was added and samples were immediately vortexed before incubation for 15 minutes at room temperature. At this stage, the monovalent copper ions and the radical groups of tyrosine, tryptophan, and cysteine react with the Folin reagent to produce molybdenum/tungsten blue. A Genesys^{TM} 20 Spectrophotometer (Thermo Spectronic, Rochester, NY) was used to measure the absorbance of samples at 750nm.

This method is subject to protein-to-protein variation due to the correlation of color intensity dependent on a protein's tyrosine, tryptophan and cysteine content. Therefore, the same protein as that being analyzed should be used as a standard (Stoscheck, 1990). For this work, the standard curve was built using 5 predetermined dilutions (0.6, 0.8, 1.0, 1.2, 1.4 mg/mL) of the untreated MCN-SP protein mixture in the solvent being tested. The standard curve was then used to quantify the amount of protein that was soluble in each solvent.

Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE testing was performed at the Proteomics and Mass Spectrometry Laboratory of the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY). The protein solutions were diluted 1:1 (vol/vol) with sample buffer containing 12.5mM Tris-HCl pH 6.8, 1% SDS, 20% glycerol, and 0.02% (w/v) bromophenol blue (for tracking). No dithiothreitol (DTT - a reducing agent) was used and the boiling

step was eliminated, so as to not induce any new sulfhydryl interactions (Zhang et al., 2004) and avoid breaking down disulfide bonds that were already present in the samples. The samples were loaded at 25°C, at a protein concentration of 10µg of protein/well. The stacking SDS-PAGE gel contained 5% acrylamide, 0.25M Tris pH 6.8, 1% SDS, 1% ammonium persulfate ((NH4)2S2O8) and 0.04% tetramethylethylenediamine (TEMED) as a catalyst for the polymerization of acrylamide; the resolving SDS-PAGE gel contained 15% acrylamide, 0.4M Tris pH 6.8, 1% SDS, 1% ammonium persulfate ((NH4)2S2O8) and 0.04% tetramethylethylenediamine (TEMED). The running buffer contained 0.193M Glycine, 25mM Tris and 0.1% SDS. An Ettan DALT Twelve Vertical Gel System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used by applying 50 mA/gel until the tracking dye was visible in the stacking gel, then 75 mA/gel until the tracking dye reached the bottom of the gel. A protein Standard marker was used (Wide Range Marker, Mark 12, Invitrogen Corporation, Carlsbad, CA). After running, the gels were fixed for 30 min and stained overnight using a solution of 6mM HCl and 0.23% Colloidal Coomassie Blue G-250. The gels were destained using a 6 mM HCl in 25% (v/v) methanol for 5 hours. The gels were scanned using a Typhoon 9400 scanner (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Statistical analysis of data

The JMP software, version 8 (JMP, Cary, NC) was used for performing one way analysis of variance (ANOVA) and two way ANOVA with an interaction effect, in order to determine significant differences in experimental results ($p < 0.05$). Differences between means were compared using the Tukey-Kramer HSD test.

Results and Discussion

Viscosity and flow behavior of the heat treated protein solutions

A comprehensive evaluation of the effect of heat treatment on the viscosity and flow behavior of micellar casein, soy proteins and their mixtures has been presented in section 4.1. In this work, only the rheological properties of the 10% and 15% protein solutions treated for 15 minutes at the 40°C, 60°C and 95°C were determined.

The results of steady shear measurements indicated that all protein solutions studied in this work are non-Newtonian, with viscosity dependent on shear rate. Therefore, the term "apparent viscosity" was used to describe their resistance to flow. In order make direct comparisons between the different protein solutions, the measured values of apparent viscosity at a shear rate $\dot{\gamma} = 100 s^{-1}$ (η_{100}) were used, as this shear rate is relevant for mastication, pipe flow and a score of processing operations (Steffe, 1996a).

Figure 4.13 shows the values of η_{100} for micellar casein, soy protein and mixed micellar casein-soy protein solutions, after heat treatments at 40ºC, 60ºC and 95°C. No values are available for the 10% and 15% SP and for 15% MCN-SP solutions heat treated at 95ºC for 15 minutes, since in those cases gels were formed as a result of the heat treatment.

As reported previously in section 4.1, protein concentration had an effect on η_{100} for all three protein solutions (MCN, SP and MCN-SP).

The 10% MCN solutions heat treated at 95ºC had a significantly lower η_{100} ($F(2,6)=177.8$, $p<0.0001$) as compared to the MCN solutions heat treated at 40ºC and 60ºC. This can be attributed to precipitation of calcium phosphate on the surface of the casein micelles, which was reported to occur upon heating at temperatures above 50ºC (Tziboula and Horne, 2000; Beliciu and Moraru, 2009). The result is a decreased surface charge of the casein micelles, as reported in section 4.1, which leads to

reduced repulsion between the casein micelles and consequently lower resistance to flow (i.e. lower η_{100}) of the 10% MCN after the 95ºC treatment.

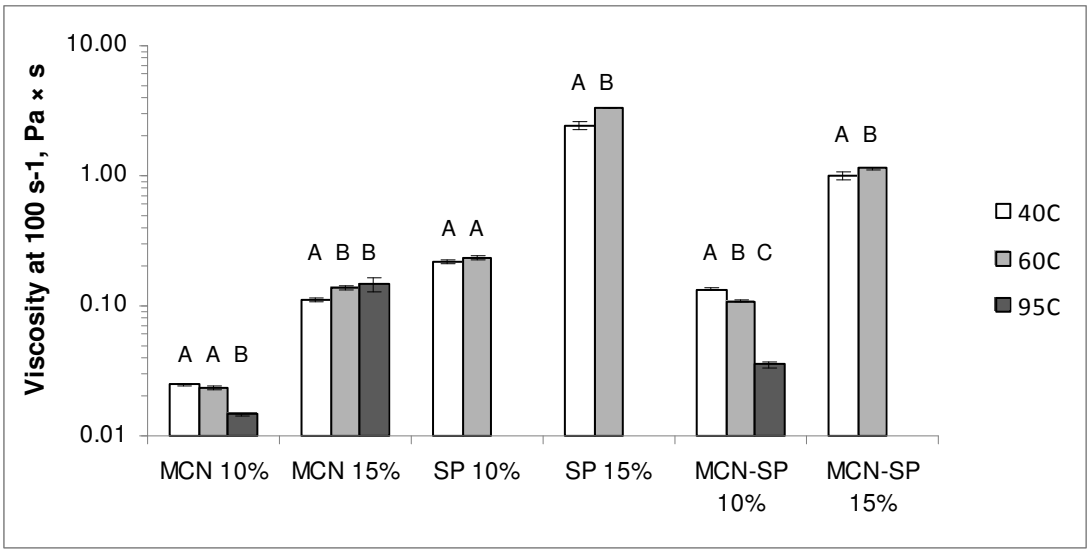


Figure 4.13 Apparent viscosity at $\dot{\gamma} = 100 s^{-1}$ of the heat treated protein solutions. Different letters represent statistically significant differences ($p<0.05$), within a given protein concentration.

For 15% MCN solutions however, η_{100} values significantly increased with heat treatment temperatures ($F(2,6)=9.7$, $p=0.013$), despite the decrease in surface charge of the casein micelles as a result of the heat treatment. This is likely due to the fact that at such a high protein concentration, casein micelles are close together to the point where repulsion is significant even though the surface charge is lower (Walstra, 2003).

Another reason that could be responsible for the higher viscosity of the concentrated MCN solutions with heat treatment temperature could be related to the rigidity of the micelles. Larson (1999) noted that in particulate suspensions, in systems of equal effective volume fraction $\phi>0.4$, the viscosity of a system containing "squishy" spheres is lower than the viscosity of more rigid, harder spheres. Based on

the water holding capacity of micellar casein (2.7g of water/1g of micellar casein at pH 6.7 (Walstra, 1990) and a density of the hydrated casein micelles of 1.0632 g/cm^3 (Kirchmeier, 1973; McMahon and Brown, 1984), the volume fraction of micellar casein solutions can be calculated as ϕ=0.35 for the 10% MCN solution and ϕ=0.52 for the 15% MCN solution. Casein micelles in the 15% MCN solution treated at 40ºC could be considered "squishy" spheres due to the diffuse hairy κ-casein layer that sterically stabilizes the micelles. The precipitation of calcium phosphate on the surface of the casein micelles that occurred after heat treatments at 60ºC and 95ºC might increase the rigidity of the micelles' surfaces. This could also explain, at least in part, why the 15%MCN solution treated at high temperatures (ϕ>0.4, with "rigid" micelles) is higher than the viscosity of the 15% MCN solution treated at lower temperatures (ϕ>0.4, with "squishy" micelles).

For soy protein solutions, the intensity of the heat treatments also affected viscosity. Both the 10% and 15% SP solutions that were heat treated at 95ºC could not be subjected to large deformation testing, since they formed gels. This happened since the soy protein concentration in this solution was higher than the critical concentration for gelation of soy proteins (6.6%) and the heat treatment temperature was above the temperature of glycinin denaturation.

In the case of mixed micellar casein-soy protein mixtures, the behavior of the 10 and 15% solutions was very different from each other. In the 10% MCN-SP solution, the total SP concentration was 5%, which was lower than the critical concentration for gelation. As a result of the intense heat treatment, glycinin molecules denatured and unfolded, leading to a solution that contains spherical structures (casein micelles) and looser, more flexible chains of denatured glycinin). Compact and denatured protein molecules tend to be thermodynamically incompatible and exhibit phase separation (Polyakov et al., 1997), which can lead to slippage between the

components of the mixture (Brochard et al., 1990). As a result, η_{100} of the 10% mixture was significantly lower (by 74%) after the denaturing heat treatment (95°C) as compared to the undenaturing heat treatment.

The viscosity of the 15% MCN-SP mixture was also affected by the intensity of the heat treatments. Since in this sample the absolute concentration of SP was above the critical concentration for gelation, after the heat treatment at 95°C a gel was formed, and thus large deformation testing was not possible.

Flow behavior analysis

The flow behavior of all protein solutions was characterized using the Herschel-Bulkley constitutive model:

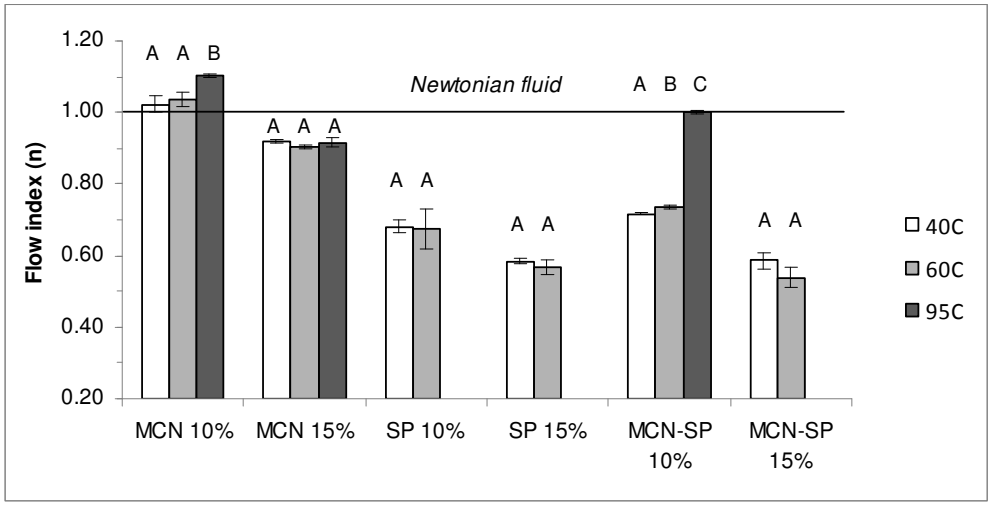
$$\sigma = K\left(\dot{\gamma}\right)^{n} + \sigma_0 \qquad (1)$$

where K is the consistency coefficient ($Pa \times s^n$), $\dot{\gamma}$ is the shear rate (s^{-1}), n is the flow behavior index, and σ_0 is the yield stress (Pa). Within the confines of the Herschel-Bulkley model rheological behaviors such as Newtonian (n=1, σ_0=0), power law (shear thinning when $0<n<1$, or shear thickening when $1<n<\infty$) and Bingham plastic (n=1, $\sigma_0>0$) can all be considered as special cases.

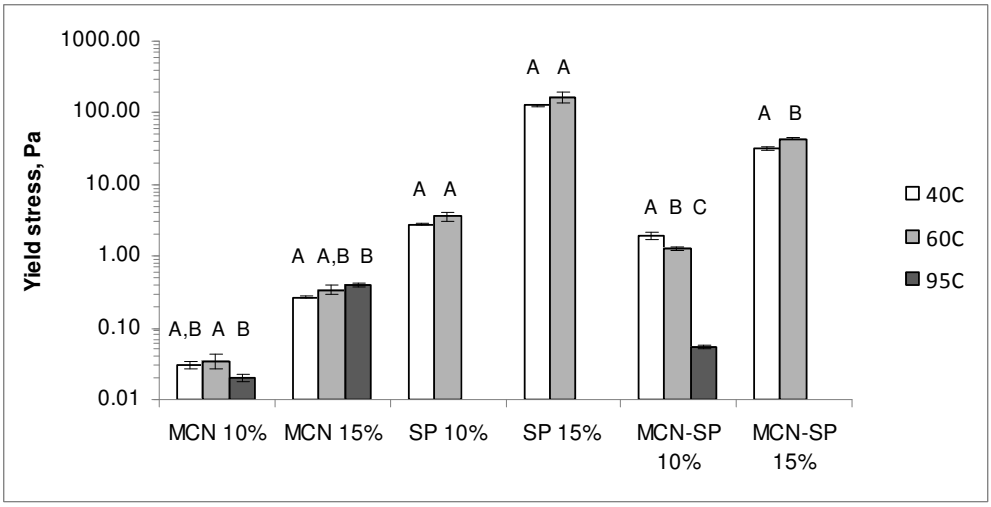
The 10% MCN solution displayed a behavior very close to Newtonian, with slight shear thickening tendencies ($1.00 \leq n \leq 1.05$) after the lower temperature heat treatments (40ºC and 60ºC). Heat treatment at 95ºC of the 10% MCN solution led to a significantly higher value of the flow index (n=1.1±0.01). Similar shear thickening behavior has been previously reported in concentrated dispersions of charged rigid spheres, in the same shear rate ranges as in this study (Shenoy et al., 2003; Kalman et al., 2007), and was attributed to the formation of hydroclusters at shear rates where the hydrodynamic forces overcome the repulsive interparticle forces (Bender and Wagner,

1996; Shenoy et al., 2003). All heat treated 15% MCN solutions displayed shear thinning behavior (n<1) (Figure 4.14a).

Soy protein solutions heat treated at 40ºC and 60ºC displayed shear thinning, which was more pronounced for the 15% solutions as compared to the 10% solutions. For a given concentration, there was no significant difference between the n values for the two different treatment temperatures.



a)



b)

Figure 4.14 Flow behavior parameters of the heat treated protein solutions: a) flow index; b) yield stress. Different letters represent statistically significant differences (p<0.05), within a given protein concentration.

The 10% MCN-SP mixture displayed shear thinning behavior when treated at 40ºC and 60ºC ($0.71 \leq n \leq 0.74$) and, interestingly, Newtonian behavior after heat treatment at 95ºC ($n=1.00 \pm 0.00$). The 15% MCN-SP mixtures displayed shear thinning behavior, with no significant difference between the *n* values for the 40ºC and 60ºC heat treatments.

Another important parameter that was determined was yield stress. The value of yield stress was determined by extrapolating the stress vs. shear rate curve to $\dot{\gamma}=0$ s^{-1} (Steffe, 1996b). All protein solutions in this study manifested yield stress, and the value of yield stress increased with concentration (Figure 4.14b). The yield stress dependence on intensity of heat treatment and protein concentration mimicked trends that were observed for viscosity.

MCN solutions had relatively low values of yield stress, i.e. 0.4Pa at a concentration of 15%. In 10% MCN solutions, statistical analysis showed that samples that were heat treated at 95ºC were characterized by significantly lower ($F(2,6)=5.6$, $p=0.042$) yield stress values than samples heat treated at 60ºC. For 15% MCN solutions, samples that were heat treated at 95ºC were characterized by significantly higher ($F(2,6)=11.2$, $p=0.009$) yield stress values than samples that were heat treated at 40ºC. The explanation for this behavior is the same as the one provided in the previous section, where the effect of concentration and heat treatment temperature on viscosity was discussed.

For soy protein solutions yield stress ranged between 4Pa (at 10% concentration) and 160Pa (at 15% concentration), with no significant influence of the heat treatment on the magnitude of yield stress.

MCN-SP mixtures showed yield stress values intermediate between those of the individual proteins at the same concentration, for the mild heat treatments (40ºC and 60ºC). For the 10% MCN-SP mixtures, treatment temperature had a significant

effect on yield stress ($F(2,6)=147.1$, $p<0.0001$), with the value of yield stress significantly decreasing with treatment temperature. For instance, the 10% mixture treated at 95ºC had a yield stress that was 97% lower than that of the 10% mixture heat treated at 40ºC. The heat treated 15% MCN-SP solutions showed yield stress values that were about one order of magnitude higher than for the 10% mixtures.

Dynamic rheological properties of the heat treated protein solutions

Oscillatory measurements were used to gain information about intermolecular interactions, system stability and storage behavior in the heat treated protein solutions. First, oscillatory strain sweeps were used to identify the linear viscoelastic region (LVR) of material response, i.e. the range of strain where the rheological properties of the samples are independent of the applied strain. Figure 4.15 shows examples of strain sweeps for 10% protein solutions heat treated at 95°C. Since those samples showed both predominant solid-like behavior (SP) and liquid-like behavior (MCN and MCN-SP), the complex modulus (G^*) was used to make direct comparisons among samples.

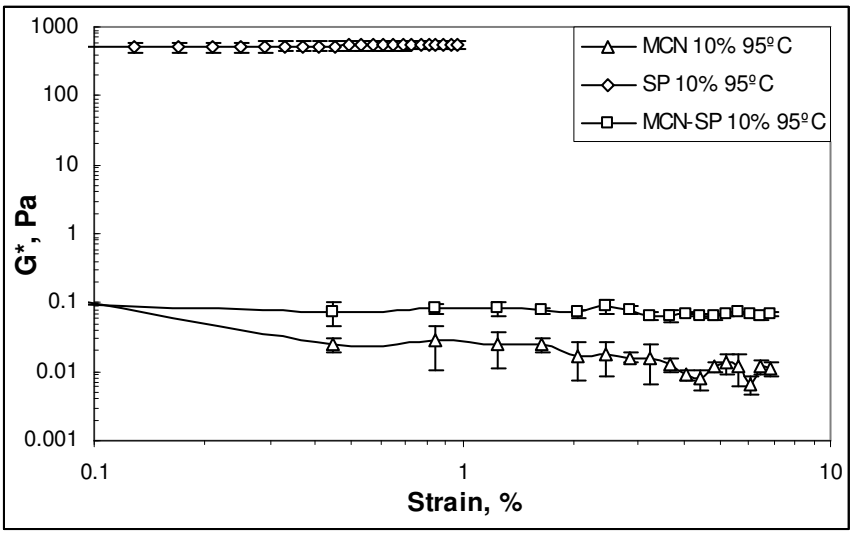
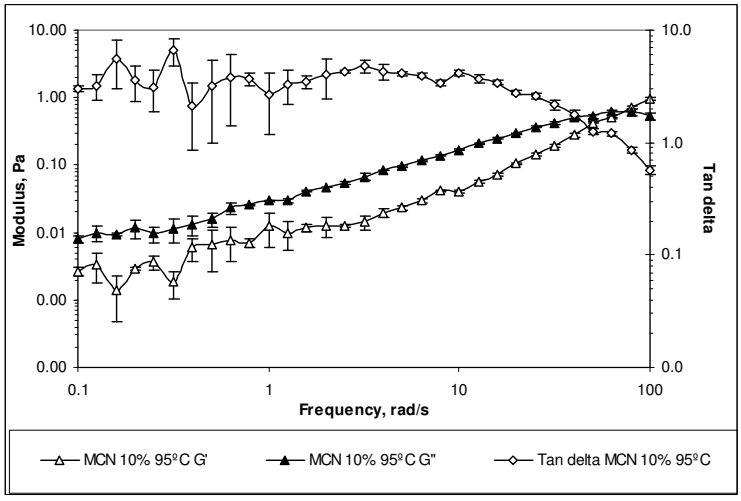


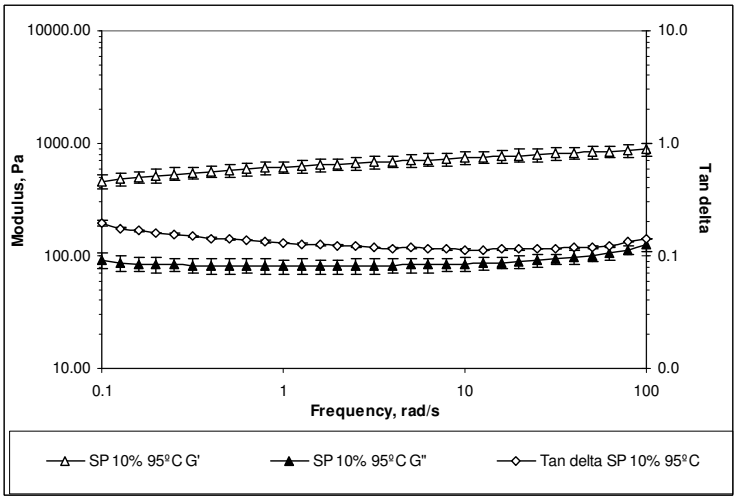
Figure 4.15 Strain sweep results for 10% solutions.

Frequency sweeps were then performed for all samples, using a strain value located within the LVR. A frequency sweep enables the viscoelastic properties of a sample to be determined as a function of timescale. For food systems, the frequency dependence of the moduli and their ratio (tan δ) can range in response from that of a viscoelastic liquid, where the moduli are strongly dependent on frequency, to that of a soft solid, where the moduli are relatively independent or weakly dependent on frequency over several orders of magnitude, and G'>G". Whether a material is a viscoelastic liquid or soft solid depends on the time scale of observation, but classification is usually dictated by what is measurable over a frequency range of 0.1 to 100 rad/s (Stokes and Frith, 2008). In order to perform direct comparisons of the frequency dependence of the protein samples evaluated in this work, a frequency dependence parameter (*m*) was determined, and represents the slope of the prevailing modulus vs. frequency curve. The prevailing modulus was chosen as G' for the samples with solid-like behavior and G" for those with liquid-like behavior.

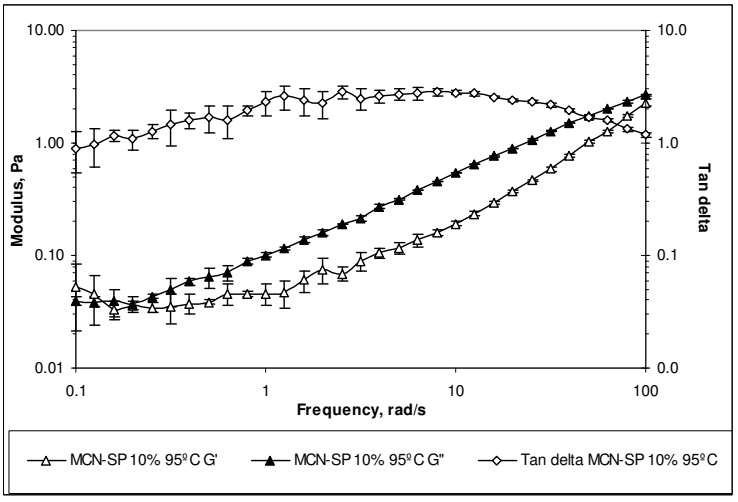
Figure 4.16 shows examples of frequency sweeps for micellar casein, soy proteins and MCN-SP mixtures of 10% concentration that have been heat treated at 95ºC.



a)



b)



c)

Figure 4.16 Frequency sweep results of a) 10% MCN; b) 10% SP; c) 10% MCN-SP.

Micellar casein solutions at both 10% and 15% concentration were characterized by a dominant viscous modulus over the entire frequency range (0.1-100 rad/s), for all three heat treatments, indicative of liquid-like behavior. For the 10% MCN solutions, the temperature of the heat treatment had a statistically significant effect ($F(2,6)=18.3$, $p=0.0028$) on the frequency dependence of the prevailing modulus (G"). G" became more frequency dependent (higher values of *m*, see Table 4.3) as the temperature of the heat treatment increased. Another observation is that in the lower frequency range (below 1 rad/s), which indicates behavior over long time frames, the tan δ values were above 3, with higher values being recorded for the higher temperature treatments. According to Rohn (1995), systems in which tan $\delta > 3$ are non-associated particle dispersions in which sedimentation is likely to occur. Overall, the increase in frequency dependence, coupled with an increase in the value of tan δ (Table 4.3), suggest that the higher intensity heat treatments are inducive of sedimentation of the 10% MCN solutions during long term storage.

Table 4.3 Comparison of some dynamic rheological parameters for the heat treated protein solutions. Different letters represent statistically significant differences ($p<0.05$) among *m* values, within a given protein concentration.

Sample	**Protein content, %**	**Temperature of the 15 min heat treatment, ºC**	**Relationship between G' and G"**	**ω-dependence (m) of highest modulus ($1 \leq \omega \leq 100$ rad/s)**	**Range of tan δ values (min - max)**
MCN	10	40	G'<G"	0.75 ± 0.04 (A)	1.1 - 7.5
		60	G'<G"	0.84 ± 0.05 (A)	1.2 - 8.1
		95	G'<G"	0.97 ± 0.05 (B)	0.5 - 8.5
	15	40	G'<G"	0.79 ± 0.01 (A)	2.3 - 4.5
		60	G'<G"	0.78 ± 0.01 (A)	2.5 - 4.5
		95	G'<G"	0.77 ± 0.02 (A)	1.5 - 4.9
SP	10	40	G'≥G"	0.45 ± 0.02 (A)	0.7 - 0.9
		60	G'≅G"	0.53 ± 0.01 (B)	0.9 - 1.2
		95	G'>>G"	0.08 ± 0.00 (C)	0.1 - 0.2
	15	40	G'>G"	0.22 ± 0.01 (A)	0.3 - 0.4
		60	G'>G"	0.20 ± 0.00 (B)	0.3 - 0.4
		95	G'>>G"	0.09 ± 0.00 (C)	0.1 - 0.2
MCN-SP	10	40	G'≥G"	0.44 ± 0.01 (A)	0.7 - 0.9
		60	G'≅G"	0.52 ± 0.01 (B)	0.8 - 1.2
		95	G'<G"	0.87 ± 0.03 (C)	0.5 - 3.2
	15	40	G'>G"	0.17 ± 0.00 (A)	0.3 - 0.4
		60	G'>G"	0.17 ± 0.00 (A)	0.3 - 0.4
		95	G'>>G"	0.12 ± 0.00 (B)	0.1 - 0.3

The heat treated 15% MCN solutions had also a liquid-like behavior, with a frequency-dependent prevailing modulus (G"). For these systems, the temperature of the heat treatment did not have a significant effect on *m* (Table 4.3). Overall, the 15% MCN solutions did behave as weakly associated systems, with tan δ values close to 3 and frequency dependent moduli, in which sedimentation may still occur over time.

In the case of soy protein solutions, the viscoelastic behavior was more varied, depending on the heat treatment temperature and the protein concentration. For the 10% SP solutions that were subjected to low heat treatments (40°C and 60°C), the elastic modulus was very close in value to the viscous modulus, and its values were moderately dependent on frequency (slope ~0.5), while their tan δ values were relatively constant and lower than 3 (Table 4.3). This behavior is indicative of a relatively high level of association and good long-term stability. The visual consistency of these samples was of concentrated dispersions.

In case of the 15% SP solutions, the storage modulus prevailed over the loss modulus (solid-like behavior), with low tan δ values (0.3-0.4 for the samples treated at 40°C and 60°C, and 0.1-0.2 for those treated at 95°C). There was a statistically significant difference between the *m* values for the 15% SP solutions treated at 40°C and 60°C ($F(2,6)=771.7$, $p<0.0001$). These observations indicate that the 15% SP heat treated at these two temperatures behave as highly associated suspensions, with a high level of intermolecular interactions. The visual consistency of these samples was that of a paste, and under large deformation testing they manifested high viscosity and a measurable yield stress.

Both 10% and 15% SP solutions formed gels after being heat treated at 95°C. The storage modulus was much higher than the loss modulus and both were almost independent on frequency. For the 10% SP gel, G' at ω=1rad/s was 615±78 Pa, while for the 15% SP gel G' at ω=1rad/s was 3121±284 Pa (Figure 4.16b).

The 10% MCN-SP mixtures that were subjected to low heat treatments (40ºC and 60ºC) had a concentrated dispersion behavior, with G'≅G" and tan δ values of 0.7-1.2. The elastic modulus was moderately dependent on frequency (m~0.5), indicating a relatively high level of intermolecular interactions which will most likely prevent sedimentation during storage. There was a statistically significant difference between the m values for the two heat treatment temperatures ($F(2,6)=411.5$, $p<0.0001$). Heat treatment at 95°C of the 10% mixture had a dramatic effect, transforming the system into a non-associated dispersion with a highly frequency-dependent, dominant viscous modulus, but tan $\delta < 3$ in the low frequency range (Figure 4.16c). A close examination of the data in the low frequency range (0.1-1rad/s) shows that the elastic modulus is higher than viscous modulus in this range, after which there is a crossover point and G" exceeds G'. Over this entire decade $0.9 \leq \tan \delta \leq 1.9$, which indicates that the system is stable over long time frames (i.e. no sedimentation will take place), which is relevant from a storage behavior standpoint. This could be caused by the unfolded molecules of denatured glycinin physically preventing casein micelles from sedimenting out of solution. These results are in agreement with visual observations of the samples, which did not display any noticeable sedimentation even after being stored under refrigeration for over 2 months. By contrast, the 10% MCN sample treated under the same conditions (frequency sweep results shown in Figure 4.16a) manifested visible sedimentation after less than 1 day of refrigerated storage.

All 15% MCN-SP mixtures had a dominating elastic modulus over the entire frequency range. For the samples treated at 40°C and 60°C, the tan δ had values of 0.3-0.4, characteristic for highly associated suspensions. The samples had the appearance of a paste and, as discussed in the previous section, displayed shear thinning behavior and high yield stress. The relative frequency independence of the elastic modulus suggests the presence of high intensity of intermolecular interactions.

The 15% MCN-SP mixture formed a gel after being heat treated at 95°C which, as discussed before, was caused by the fact that the SP concentration in the system was 7.5%, which is higher than the critical concentration for gelation for soy proteins (Bikbow et al., 1979). The storage modulus was much higher (496 ± 65 Pa at ω=1rad/s) than the loss modulus, and both moduli were virtually independent on frequency.

Chemical evaluation of intermolecular interactions in the heat treated protein mixtures

While previous experiments have suggested the presence of intermolecular interactions in some of the heat treated micellar casein – soy protein solutions, a definite answer about the existence of such interactions can only be obtained using chemical methods.

The nature of the heat induced molecular interactions within the mixed micellar casein-soy protein solutions was evaluated using a modified version of the differential solubility method developed by Hager (1984), which uses four different solvents: solvent 1, which dissolves protein molecules that remain in their native state; solvent 2, which also dissolves denatured, but not highly aggregated, molecules and small aggregates (such as protein monomers rendered insoluble by hydrogen bonding or hydrophobic interactions, or small aggregates joined by intermolecular covalent bonds); solvent 3, which can cleave disulfide bridges, thus being able to dissolve even large aggregates; and solvent 4, which can dissolve most protein fractions.

The amount of protein solubilized by each solvent was then determined colorimetrically, using the RC DC assay. Due to intrinsic interactions between the solvents used and the reagents employed by the RC DC assay, the experimental data was expressed using the "additional protein solubilization" (APS) value, defined as:

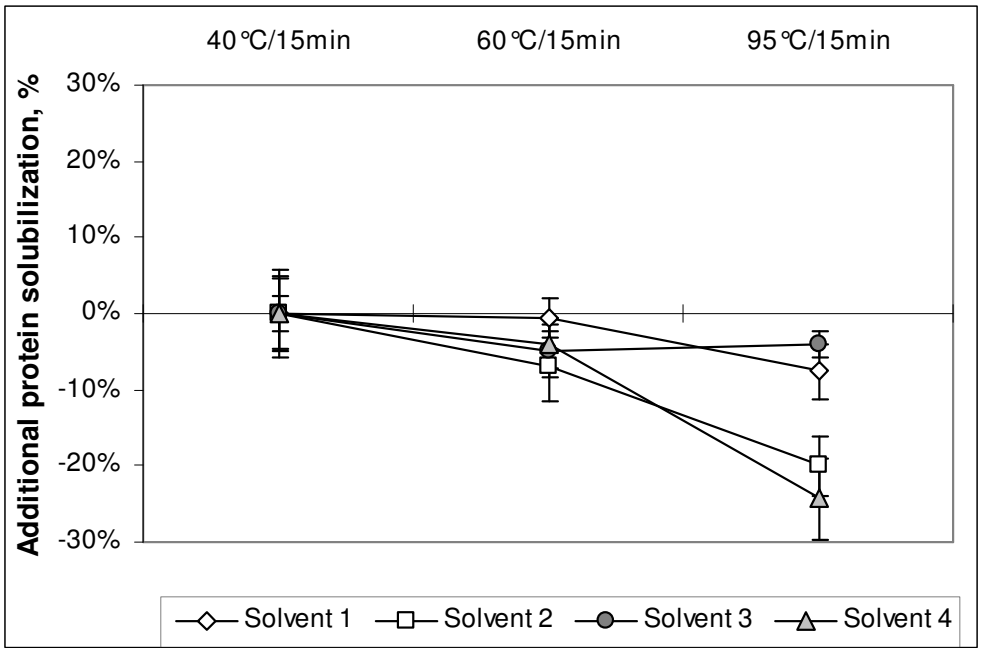
APS = (Protein solubilized at temperature "t"/ Protein solubilized at 40°C) × 100 (%) (2)

An APS value was calculated separately for each of the four solvents and heat treated mixtures, and the results are shown in Figure 4.17. It must be noted that when interpreting the data in Figure 4.17, the trends are more important than the absolute values.

Both for the 10% and 15% MCN-SP mixtures, the amount of protein solubilized by solvent 1 was significantly lower after the 95°C treatment as compared to the 40°C and 60°C treatments (see Figure 4.17a, b). This indicates that the amount of protein that becomes insoluble increases significantly after the heat treatment at 95°C, which can be attributed to the denaturation of the soy proteins present in the mixtures.

For the other 3 solvents, the observed trends were different for the 10% MCN-SP mixture as compared to the 15% MCN-SP mixture. For the 10% mixture a significant decrease in the amount of protein solubilized by solvents 2, 3 and 4 was observed after heat treatment at 95°C (Figure 4.17a), which simply indicates that increased protein denaturation and consequently a loss of solubility occurs as a result of the heat treatment.

a)



b)

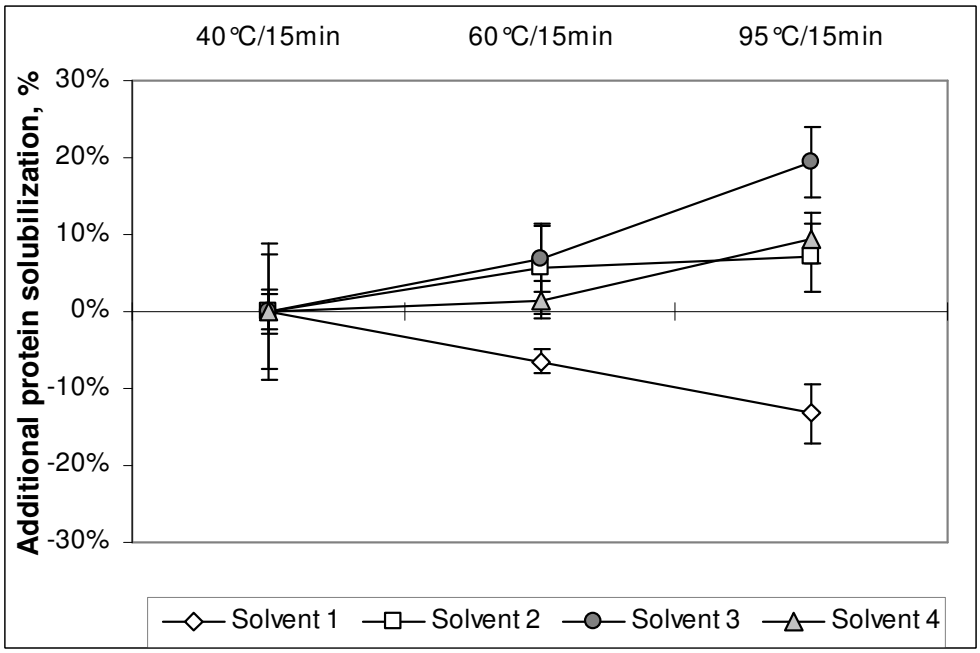


Figure 4.17 Differential solubilization of heat-treated mixed micellar casein-soy proteins solutions: a) 10% and b) 15% protein concentration.

It is interesting to note that, for solvent 3, no statistically significant differences were observed between the 10% MCN-SP samples treated at the three temperatures, which indicates that no disulfide bonds were formed in this mixture, even after the 95°C treatment. In case of the 15% mixture, slight increases in solubility were noticed for solvent 2 for the samples treated at 95°C as compared to the 40°C and 60°C treated samples, although these increases were not statistically significant. Denaturation of the soy proteins has definitely occurred upon subjecting the 15% mixture to the 95°C treatment, which would have resulted in decreased protein solubility, similar to what was observed for the 10% mixture. The increase in the amount of protein solubilized by solvent 2 can only be attributed to the formation and subsequent solubilization of small aggregates after the high heat treatment. This is consistent with the increase in particle size in 15% MCN-SP mixtures, but not in the 10% MCN-SP mixtures, which was observed in previous work after heat treatment at temperatures above 80°C (see section 4.1). The results for solvent 4 were similar with those obtained for solvent 2, most likely due to the interference of the high concentration of urea present in both solvents.

For solvent 3, which is known to break down aggregates formed via disulfide bonds, a significantly higher amount of protein was solubilized for the 95°C heat treated 15% MCN-SP mixture as compared to the lower heat treatment temperatures, which can be explained by the presence of a significant amount of aggregates held together by disulfide bridges. This is consistent with the findings of the rheological testing, visual observations of the samples and with the known fact that at such concentrations and temperatures gel formation by soy proteins takes place, with disulfide bond formation playing a significant role.

Non-reducing SDS-PAGE

The differential solubility data presented in the previous section indicates that

heat treatment for 15 min at 95°C induced the formation of a significant amount of disulfide bonds in the 15% MCN-SP mixture, which seemed to occur exclusively between soy protein molecules. To further investigate the aggregation phenomena (presence and molecular size of aggregates), non-reducing SDS-PAGE analysis was also performed for the protein mixtures. As controls, individual protein solutions (MCN, SP) of the same absolute concentration as in the mixed 10% and 15% MCN-SP solutions were used. Since the 60°C treatment did not seem to induce any significant intermolecular interactions as compared to the 40°C treated mixtures, the non-reducing SDS-PAGE analysis was only performed on samples which have been treated at 40°C and 95°C for 15 min. The results of the non-reducing SDS-PAGE analyses are shown in Figure 4.18. For better viewing of the relevant protein bands, the portion of the gels corresponding to molecular weights below 21.5 kDa is not shown, as it did not present any clear features.

The protein bands shown on the gels for the individual proteins were first identified, and are shown in Figure 4.18. For caseins, it is important to note that identification of the various fractions (α_s, β and κ) was based on existing literature (Jovanovic et al., 2007), since it is known that caseins do not migrate on electrophoresis gels according to their molecular weight.

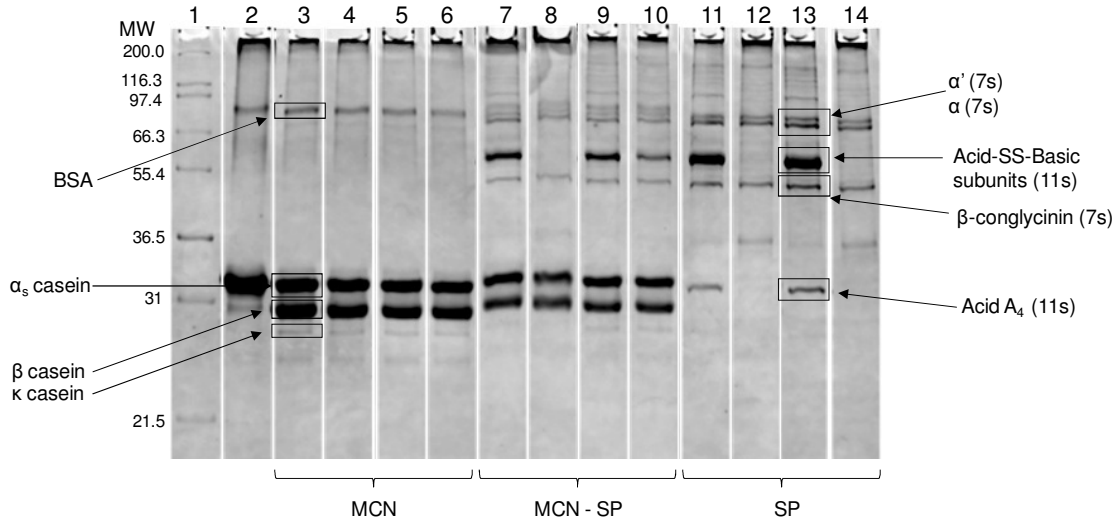


Figure 4.18 Non-reducing SDS-PAGE gels for the protein solutions treated at 40°C and 95°C for 15 minutes:

Lane 1. Wide range Marker: Mark 12, Invitrogen

Lane 2. α casein (1µg)

Lane 3. MCN 5% 40ºC/15 min

Lane 4. MCN 5% 95ºC/15 min

Lane 5. MCN 7.5% 40ºC/15 min

Lane 6. MCN 7.5% 95ºC/15 min

Lane 7. MCN-SP mix 10% 40ºC/15 min

Lane 8. MCN-SP mix 10% 95ºC/15 min

Lane 9. MCN-SP mix 15% 40ºC/15 min

Lane 10. MCN-SP mix 15% 95ºC/15 min

Lane 11. SP 5% 40ºC/15 min

Lane 12. SP 5% 95ºC/15 min

Lane 13. SP 7.5% 40ºC/15 min

Lane 14. SP 7.5% 95ºC/15 min

For soy proteins, the bands were identified based on the molecular weight standard and on knowledge about the various soy protein fractions. Glycinin has a molecular weight of 360 kDa in its hexamer state (Renkema et al., 2001). Each hexamer is made up of six A-SS-B subunits, each of them composed of an acid polypeptide (~38 kDa) and a basic polypeptide (~20 kDa) linked by a single disulfide bond, with the exception of the acid polypeptide A_4 (Staswick et al., 1981; Barac et al., 2004; Bittencourt et al., 2007). β-Conglycinin is a trimeric glycoprotein with a molecular weight of 150-200 kDa, which is composed of three subunits (α', α and β), with molecular weights of 72 kDa, 68 kDa and 52 kDa, respectively (Tanh and Shibasaki, 1977; Utsumi et al., 1997).

Non-reducing SDS-PAGE profiles of micellar casein samples were not affected by heat treatments or sample concentration (lanes 3-6 in Figure 4.18), while the non-reducing SDS-PAGE profiles of soy protein solutions at both protein concentrations were significantly affected by the heat treatments at 95°C for 15 min. In soy protein solutions treated at 40ºC four distinct groups of bands were identified (lanes 11 and 13 in Figure 4.18): the α' and α subunits of the 7s fraction (72 and 68 kDa), a pronounced band at 60 kDa containing A-SS-B subunits of the glycinin hexamer (11s fraction) (the A-SS-B units were not broken down into their constitutive polypeptides, as no reducing agents were used), a band corresponding to the β subunit of β-conglycinin (52 kDa) and a band corresponding to the acid polypeptide A_4 (32 kDa). As a result of heat treatment at 95ºC of the soy only protein solutions (lanes 12 and 14 in Figure 4.18) the bands representing the A-SS-B subunits and polypeptide A_4 have disappeared. These results are in agreement with the observations by Mori et al. (1981), according to which as a result of heating glycinin at a low concentration, a temporary soluble aggregate forms, which then disaggregates into the constitutive acidic and basic polypeptides. In the heat treated soy protein solutions (lanes 12 and

14 in Figure 4.18) a faint band becomes apparent, corresponding to the molecular weight of 36 kDa, very close to the MW of the acid polypeptide of A-SS-B subunits of glycinin (38 kDa). There were no noticeable bands attributable to basic polypeptides (~20 kDa). The bands corresponding to β-conglycinin (α, α' and β) remained unchanged after the heat treatment. This is in agreement with the results of DSC analysis reported in section 4.1, which showed that the 7s fraction was already denatured in the starting material, most likely due to the ingredient manufacturing process.

The gels for the mixed protein solutions generally showed a combination of the bands found in the individual protein solutions, with a few exceptions. For the 10% MCN-SP samples (lanes 7 and 8 in Figure 4.18), the band corresponding to the glycinin subunits disappeared in the heat treated sample (lane 8). Correlating this observation with the results of the differential solubility work, it can be concluded that glycinin subunits denature and disaggregate in the 10% MCN-SP solution as a result of the high heat treatment.

In case of the 15% MCN-SP mixtures (lanes 9 and 10, Figure 4.18), the SP concentration was high enough to lead to gel formation as a result of heat treatment at 95ºC, via disulfide bonding. The disulfide bond formation most likely occurred between the A-SS-B subunits which, according to Wolf (1993), contain at least 2 free SH groups per subunit. The band representing A-SS-B subunits did not disappear completely in the heat treated samples (lane 10), since not all glycinin underwent denaturation and subsequent aggregation. While significant denaturation of soy proteins was expected to take place at a treatment temperature of 95ºC, denaturation is completed only at temperatures above 100ºC, as demonstrated by the DSC results for the soy protein concentrate used in this study reported previously in section 4.1.

One of the important conclusions of the electrophoresis analysis is that no new

bands were observed on the gels of mixed solutions, which could have indicated the formation of complexes between the soy proteins and the casein micelles. In order to ensure that such complexes were not observed due to of the upper limit of molecular weight used for these gels (200 kDa), another set of experiments were conducted with 3-5% Tris Acetate gels and Spyro Ruby staining (for 6.5 hours), covering a MW range of 150-460 kDa. Even on those gels, there were no noticeable new bands showing for the MCN-SP samples treated at 95°C/15min, as compared to the mixtures treated at 40°C/15 min (data not shown). Therefore, it can be concluded that, while protein-protein interactions and aggregate formation does occur in the heat treated MCN-SP mixtures, these due to interactions between soy proteins only.

Conclusions

The results of this work revealed a complex rheological behavior of the heat treated mixed micellar casein – soy protein mixtures, which could be useful in designing foods of desired texture and mouthfeel. Heat treatments at a temperature above the denaturation point of glycinin induced aggregation and gelling via disulfide bonding in both soy protein and micellar casein – soy protein mixtures, when soy protein concentration exceeded the critical concentration for gelling. Below this critical concentration, controlled thermal denaturation of soy proteins in micellar casein – soy protein mixtures resulted in a Newtonian liquid with lower viscosity and yield stress, and improved storage stability as compared to the mixture subjected to non-denaturing heat treatment.

The wide range of rheological characteristics displayed by the heat treated micellar casein – soy protein mixtures has potential practical applications in the development of food products with unique textural properties and a healthy image.

Acknowledgements

This research was supported by the Cornell University Agricultural Experiment Station federal formula funds, Project No. NYC 143425, received from Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

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CHAPTER 5

THE EFFECT OF COMMERCIAL STERILIZATION REGIMES ON MICELLAR CASEIN CONCENTRATES

Abstract

This work focused on evaluating the effects of two commercial sterilization regimes (continuous-flow UHT treatment and in-container retorting) on the stability and physical properties of MCCs with 5% - 10% casein concentration. Both the UHT treatment and the retorting achieved the same microbial inactivation effect, at a cumulative value of the lethality factor $F_0 = 9.9$. Sterilization affected the stability, viscosity and flow behavior of MCCs, mainly due to loss in solubility of calcium phosphate at the micelle level. Additional mechanisms such as κ-casein dissociation may also play a significant role in the changes induced by sterilization. Retorting resulted in slight aggregation of casein micelles, while UHT caused the formation of visible aggregates. The UHT treated MCCs had higher viscosity than retorted MCCs, and displayed a solid-like behavior, indicative of structure formation. Drying of MCCs affected their sterilization behavior, as reconstituted micellar casein concentrates (R-MCCs) were more unstable to UHT sterilization than MCCs. The calcium load (calcium concentration per gram of casein) of the MCCs was identified as a critical parameter for their heat stability. The results of this study provide valuable information about the heating behavior and physical properties of micellar casein concentrates obtained by membrane separation, particularly for the manufacture of shelf stable, milk protein based beverages.

Introduction

Casein ingredients are widely used in foods due to their availability, high nutritive value, blandness and physico-chemical and functional properties (Southward, 1985; Chandan, 1997). The number of new products containing casein/caseinates launched in the US has grown on average by about 22% per year between 2000 and 2008 (Affertsholt, 2009). Due to its exceptional water binding capacity (Walstra, 1990), emulsifying and foaming properties (Phillips et al., 1994; Damodaran, 1997), and viscosity (Konstance and Strange, 1991), casein can be used in a variety of applications. In dairy products, casein ingredients have been used to adjust the protein content and enhance the sensory properties of low-fat dairy products (Mulvihill and Ennis, 2003). Their whipping and foaming properties make them good candidates for applications in beverages or dessert-type products. The emulsification and water-binding properties of casein and caseinates are useful for confectionery applications and, due to their lysine-rich aminoacid profile they make a great supplement in bakery products and pasta (Crowley et al., 2002). The majority of the casein preparations traditionally used as ingredients in the food industry have been obtained through methods that involve the destabilization or chemical modification of casein micelles at some point in the production process by acidification, renneting or co-precipitation.

Recent developments in membrane filtration technology have allowed the large scale production of micellar casein concentrates obtained by microfiltration (MF) (Le Berre and Daufin, 1996; Nelson and Barbano, 2005). In these preparations, micellar casein is closer to its native state than in the case of ingredients obtained by chemical methods. The differences in the manufacturing process also result in different functionality of the casein preparations obtained by membrane separation as compared to the traditional casein ingredients. As the availability and affordability of these ingredients increase, there is a need to understand their processing behavior and

stability. One of the potential applications of micellar casein concentrates (MCC) obtained by membrane separation is the manufacture of shelf-stable , high protein beverages.

Casein micelles are remarkably stable to heat treatments, their integrity being caused by strong linkages with calcium phosphate, hydrophobic interactions, hydrogen bonds, salt bridges and entropic forces that cause chain entanglements. Because of the multitude of forces responsible for their association, micelles are capable of responding to environmental changes in a variety of ways and at different rates (de Kruif and Holt, 2003). There have been reports of changes of the casein micelle level as a result of heating, with these changes being somewhat different when heating was done in the presence of all milk components (in milk), or in the absence of some of these components (protein concentrates).

Heating milk at temperatures above 90°C was reported to lead to an increase in casein micelle size (Mohammad and Fox, 1987), which was also confirmed in UHT-sterilized milk and UF-concentrated milk (McMahon, 1996). High heat treatments of skim milk can also lead to a dephosphorylation of casein. Belec and Jenness (1962) have shown that after 20 minutes at 120°C, about 10% of micellar casein in skim milk has undergone dephosphorylation. This reaction has the potential to disrupt the native micellar structure, which is largely held together by colloidal calcium phosphate links (Dalgleish et al., 1987). Dephosphorylation was reported to be responsible for 30% of the heat induced drop in pH in milk heated at 120ºC (Pyne and McHenry, 1955; Singh, 2004). Some proteolysis was also observed after heating milk under sterilization at 120°C for 30 min, which increased the non protein nitrogen content by 35% as compared to untreated milk (Saidi and Warthensen, 1993).

The mechanisms responsible for changes at the casein micelle level depend on the intensity of the heat treatment. For example, it has been reported that calcium

phosphate present in the diffusible fraction becomes less soluble during heat treatment (Holt, 1995). The less soluble phases of calcium formed after heating have not been unequivocally identified. Some authors reported the formation of crystalline β-tricalcium phosphate ($Ca_3(PO_4)_2$) after in-can sterilization of skim milk for 5 minutes at 120ºC (Nelson et al., 1989), while others reported formation of hydroxyapatite ($Ca_5(PO_4)_3(OH)$) after heating skim milk for 15 minutes at 100ºC (Visser et al., 1986).

Deposition of insoluble calcium phosphate onto the casein micelles was identified as a major mechanism responsible for sedimentation of casein micelles in UHT treated milks and UF concentrated milks during storage (Wahlgren et al., 1990, Dalgleish, 1992). It was reported before that heat induced precipitation of primary calcium phosphate ($Ca(H_2PO_4)_2$) and secondary calcium phosphate (brushite, $CaHPO_4 \cdot 2H_2O$) as tertiary calcium phosphate ($Ca_3(PO_4)_2$) is coupled with a concomitant release of protons, which has been shown to contribute 20% of heat-induced acidification; this decrease in pH may lead to dissolution of micellar calcium phosphate and potential destabilization of the casein micelles (O'Connell and Fox, 2003).

In a study by Aoki et al. (1974), protein dissociation as a result of heating was reported. The amount of casein that sedimented by ultracentrifugation decreased when serum protein free casein dispersions were heat treated at temperatures >110°C, as compared to the untreated casein dispersions. About 40% of the total dissociated protein was characterized as κ-casein.

Heat-induced polymerization has also been reported in UHT-treated serum protein-free micellar casein dispersions (Zin El-Din and Aoki, 1993). As causes for heat induced polymerization, crosslinking via Maillard reaction when lactose is present (Kato et al., 1988) or formation of thermally generated colloidal calcium phosphate linkages (Singh, 1994) were suggested.

The effect of heat treatment on serum protein free, lactose free micellar casein has not yet been reported. This work will provide an evaluation of the effect of commercial sterilization regimes (ultra high temperature sterilization and in-container sterilization) and spray drying on the properties and stability of micellar casein concentrates obtained by membrane separation, as well as an investigation of the changes that take place at the micelle level as a result of high heat treatment.

Materials and methods

Production of the micellar casein concentrates

The micellar casein concentrates (MCC) used in this study were produced by Dr. Barbano's group at Cornell University, following the procedure detailed below. As raw material, whole raw bovine milk from the Cornell University teaching and research dairy farm was used. This study was conducted during the months of June and July, when the average true protein content is the lowest in the year (Barbano, 1990). The production of MCC was replicated 3 times, with different batches of raw milk. The chemical composition of skim milk, as determined by infrared spectroscopy, is presented in Table 5.1.

Table 5.1 Chemical composition (% by weight) of skim milk. $^{(1)}$ = calculated, $^{(2)}$ = measured.

Experimental replication	**TP$^{(1)}$, %**	**CN$^{(1)}$, %**	**NCN$^{(2)}$, %**	**NPN$^{(2)}$, %**	**CN%TP $^{(1)}$**	**Lactose$^{(2)}$, %**	**Fat$^{(1)}$, %**	**Ash$^{(2)}$, %**
1	3.13	2.54	0.79	0.19	80.98	4.80	0.06	0.719
2	3.05	2.47	0.79	0.20	80.83	4.78	0.08	0.715
3	3.01	2.44	0.74	0.18	81.23	4.75	0.07	0.712

The timeline for processing was as follows: a batch of raw whole milk (~965 kg) was received on Day 1, weighed in gravity separation tanks and stored overnight at 4ºC. On Day 2, the raw gravity separated skim milk was collected, pasteurized and centrifugally separated at 50ºC, according to procedures described elsewhere (Zulewska et al., 2009). The pasteurized skim milk was subjected to ultrafiltration (UF) in order to reduce the total lactose content, and the UF retentate was re-diluted with water purified by reverse osmosis (RO), cooled to 4ºC and stored overnight. On Day 3, the diluted UF retentate was microfiltered (MF) to obtain a serum protein (SP) reduced retentate and MF permeate, according to procedures described by Zulewska et al. (2009); the retentate and permeate were collected separately, cooled to 4ºC and stored overnight. On Day 4, the MF retentate was further diafiltered (DF) twice in order to produce a highly SP reduced micellar casein concentrate (MCC). The 3rd stage MF retentate underwent a finishing stage designed to increase its protein content to 10.5%. The resulting retentate (from here on to be referred to as 4th stage retentate) was rapidly cooled to 4ºC and stored overnight on ice. The composition of the 4th stage retentate was provided by Dr. Barbano's group, and is shown in Table 5.2.

Table 5.2 Chemical composition (% by weight) of the 4th stage retentate. $^{(1)}$ = calculated, $^{(2)}$ = measured (see Materials and methods). pH measured at 50ºC.

Experimental replication	**TP$^{(1)}$, %**	**CN$^{(1)}$, %**	**NCN$^{(2)}$, %**	**NPN$^{(2)}$, %**	**Lactose$^{(2)}$, %**	**Fat$^{(2)}$, %**	**pH $^{(2)}$**
1	11.01	10.25	0.87	0.10	0.17	0.29	6.81
2	11.11	10.28	0.95	0.12	0.16	0.31	6.83
3	11.15	10.41	0.87	0.13	0.20	0.27	6.69

Treatment of the MCC preparations

On Day 5, the 4th stage MF retentate was treated and analyzed according to the experimental design shown in Figure 5.1.

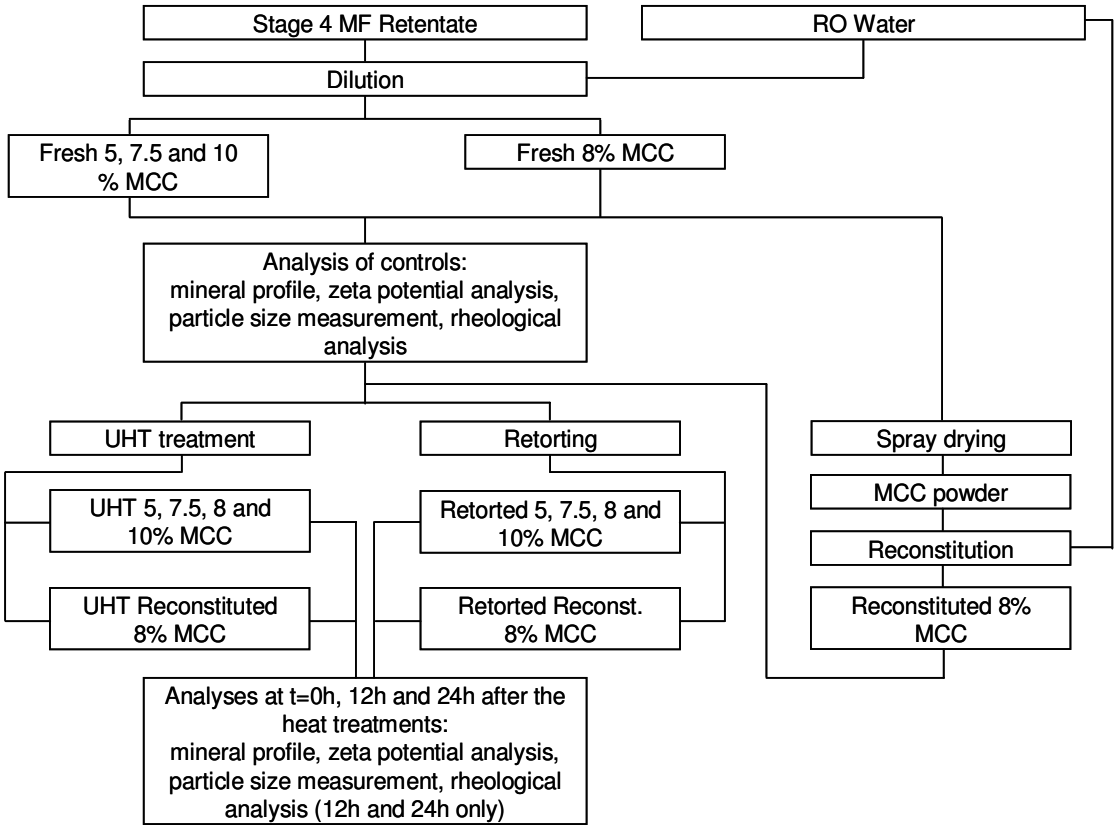

Figure 5.1 Experimental design

Preparation of the MCC dilutions

The study was performed on MCCs with target casein concentration of 5, 7.5 and 10%. A fourth concentration (8%) was added to the experimental design in order to also study the effect of drying. The MF retentate was diluted with RO water to the four fixed casein concentrations (5, 7.5, 8 and 10%). The MF retentate was weighed into milk cans using a bench scale (Model ES 50L, Ohaus Corporation, Pine Brook, NJ) and the pre-determined amounts of RO water were added in order to achieve the

target casein concentrations of 5, 7.5, 8 and 10%. The resulting MCCs were then stirred for 15 min using an air driven mixer (Model Unar-33, Lightnin®, Rochester, NY). When not in use, the MCC was kept under refrigeration at 4°C.

Since the results of the Kjeldahl protein analysis for the 4th stage MF retentate were not available at the time of dilution, the protein content of the retentate was estimated as 98% of the total protein measured by IR analysis, according to previous work by Hurt and Barbano (2010). The estimated protein concentration of the 4th stage MF retentate was used to calculate the theoretical casein concentration, using the percent of true protein (CN%TP) in the pasteurized skim milk (Table 5.1). This resulted in a slight difference between the calculated and measured casein concentration in the MF retentate (i.e. 10.79% calculated as compared to 10.25% measured by Kjeldahl for the first experimental replication). This difference was attributed to the slight underestimation of the casein contents of MF retentates by the Kjeldahl method, which has been previously explained by Nelson and Barbano (2005).

Preparation of reconstituted MCC

In order to evaluate the effect of drying on the sterilization behavior of MCC, a comparison between the processing behavior of fresh MCC (which from now on will be referred to as MCC) and spray dried and reconstituted MCC (which will be referred to as R-MCC), at the same casein concentration (8%), was also performed. The procedure used for obtaining reconstituted MCC is described below.

Spray drying. A portion of the 8% MCC (about 39 kg) was kept overnight in a metal can, at or below 7°C, then spray dried the following day (Day 6) using a Model 1 Niro Atomizer equipped with a FU11 atomizer rotating at 23,000 rpm (Niro Atomizer Inc., Columbia, MD). The feed rate into the spray dryer was 16 kg/h, the inlet air temperature was 200°C and the outlet air temperature was 95°C. The powder

collected during the first 10 min of the drying session was discarded. The dried MCC powder was collected and packaged every half hour in Lightblock Mylar bags (Model PAKVF2.5MB, Impak Corporation, Los Angeles, CA), which were subsequently stored at 21°C. The total time for a drying run was approximately 130 min.

Reconstitution of the MCC powders. The MCC powder was reconstituted to 8% casein concentration by mixing it with a predetermined amount of RO water at 40ºC. The RO water used for reconstitution was heated using a direct steam kettle (Model DN 20, Groen, Plainfield, IL) and weighed into dairy metal cans using a bench top scale (Oxo International, Chambersburg, PA). The MCC powder was added slowly to the water under vortex conditions, using a high-speed Silverson AXR vertical mixer (Silverson Machines, Inc., East Longmeadow, MA) set on speed 5 (1950rpm). A 10 minute re-suspension step was followed by a high-speed dispersion step (20 minutes at 3500rpm, speed setting 9), in order to ensure homogeneity of the reconstituted MCC. Full hydration of the reconstituted MCC was ensured by cooling the reconstituted 8% MCC and storing it overnight at 4ºC.

Preparation for thermal treatment

Each of the MCC dilutions was split into 2 portions: one to be heat treated by UHT, another by retorting. The MCC that was to be retorted was filled into glass jars.

Special attention was paid to filling the jars, in order to ensure a proper seal and avoid overflow of the jars during retorting. The needed amounts of MCC were transferred into 1 L HDPE Nalgene bottles (Thermo Scientific, Waltham, MA), which were then preheated in a water bath (Model 202-2, National Appliance Co, Portland, OR) to 40 ± 1°C. The preheating step was used in order to eliminate some of the air contained by the MCC, since excessive air trapped in the jars could expand and cause overflowing of product during retorting. The 8 oz (237 mL) Mason jars (Ball

Corporation, Broomfield, CO) were filled gravimetrically with 180 ± 1 g product, using a peristaltic pump (Amicon LP-1 pump, Beverly, MA, with Cole-Palmer Masterflex 7015-81 pump head, Vernon Hills, IL). In order to avoid excessive foaming, the transfer tube was placed close to the bottom of the jar. Before sealing the containers, the lids were heated in water to 95°C for 5 min to soften the rubber rings for a better fit, then pressed onto the well cleaned rims of the jars. The filled Mason jars, as well as the MCC needed as a control sample and for UHT processing, were stored overnight at 4°C.

Heat treatments

On Day 6, the MCCs were subjected to continuous flow UHT sterilization and batch retorting. The sterilization regimes used in this study were typical heat treatments used in the Dairy Industry. The temperature-time combinations for the two treatments were selected so that they would ensure an equivalent microbial inactivation effect. For this, the lethality factor F_0 was calculated, according to equation 1 (Bylund, 1995):

$$F_0 = \frac{t}{60} \times 10^{\frac{T-121}{z}} \quad (1)$$

where:

F_0 = the equivalent time at the processing temperature (T) required to produce a given sterilization effect as saturated steam at 121°C (250°F) (minutes)

T = sterilization temperature (ºC)

t = sterilization time (seconds) at temperature T

z = the increase in temperature (°C) needed to obtain the same lethal effect in 1/10 of the time. For dairy products, z = 10ºC.

The cumulative F_0 values were 9.98 for the UHT treatment and 9.89 for retorting. The temperature profiles for the two treatments are presented in Figure 5.2.

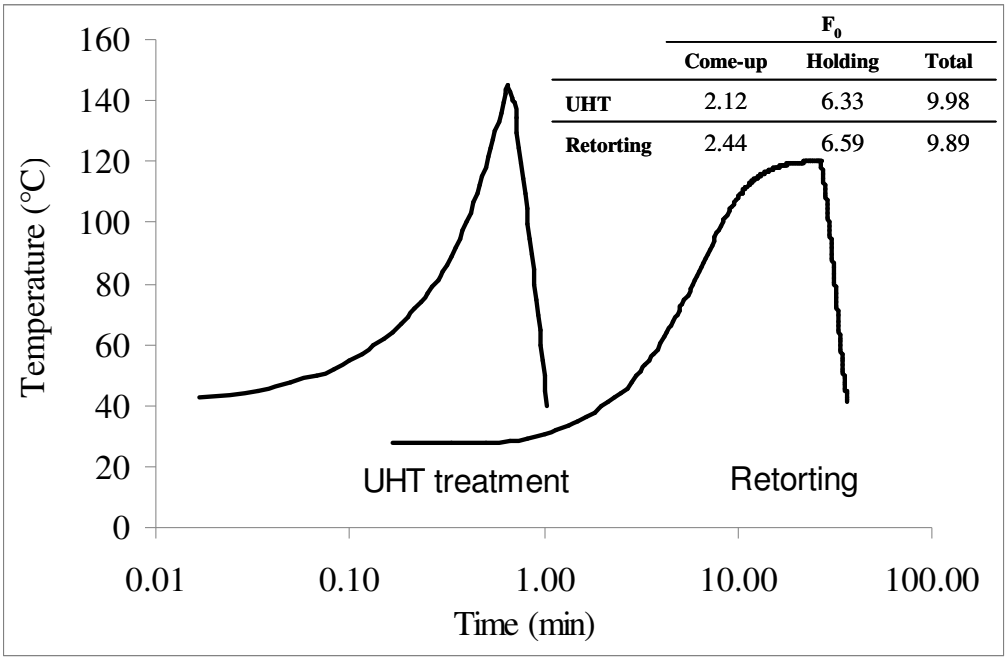

Figure 5.2 Temperature profiles and calculated F_0 values for the sterilization treatments used in this study.

Continuous flow ultra high temperature (UHT) treatment

Both the MCCs (5, 7.5, 8 and 10% casein) and the R-MCC (8% casein) were heat treated under continuous-flow UHT conditions using a pilot scale Microthermics heating system (Model 25 HV, Microthermics, Inc., Raleigh, NC), equipped with an Ultra Clean Fill Hood/Sterile Product Outlet. The four MCCs were treated sequentially, on three separate dates (one experimental replication per day), and the three 8% casein R-MCCs were all treated later, on a separate date.

The UHT unit was subjected to a cleaning-in-place (CIP) cycle the day before the treatments. A high velocity external sanitary centrifugal pump (Model 2045, Waukesha Cherry Burrell, SPX, Charlotte, NC) was used in order to improve cleaning efficiency. The UHT system was first heated to 79.4ºC (175ºF) with water, followed by a caustic cleaning step, which consisted in recirculating a dilute solution

(18.5mL/L) of heavy duty alkaline cleaner (Conquest, Ecolab, St. Paul, MN), for 20 min at a flow rate of 7.6 L/min. The unit was then rinsed with continuous water for 10 minutes and heated to 68.3ºC (155ºF). This was followed by an acid cleaning step, consisting of recirculating a dilute solution (18.5mL/L) of acid detergent rinse (Prestige, Ecolab, St. Paul, MN) for 20 min at 7.6 L/min. The CIP cycle was completed with a 10 minute rinsing step with water at 7.6 L/min. At the beginning of each processing day, the clean fill hood underwent a 30 min steam sterilization cycle at 121.1ºC (250ºF).

After the CIP cycle, the UHT unit was heated up to the sterilization parameters using water in the product stream, until stability of the processing parameters was achieved. The following values of the processing parameters were used: pre-heater temperature: 90ºC (194ºF), steam pressure: 165.5 kPa (24 psi); final heater temperature: 145.5 (294ºF), steam pressure: 331 kPa (48 psi); temperature out of the holding tube: 141.7ºC (287ºF). The MCC preparations were preheated from the refrigeration temperature to 37.8ºC (100ºF) in a tabletop direct steam kettle (Model TDA 5, Groen, Plainfield, IL) and immediately subjected to the sterilization treatment. The UHT treatment consisted of pre-heating the product to 90ºC (194ºF) in the first heater for 20 seconds, final heating of the product to 143.3ºC (290ºF) for another 20 seconds in the final heater, holding 141.7ºC (287°F) for 3 seconds, and cooling to 38.3ºC (101ºF) for 20 seconds (Figure 5.2). The flow rate was 2L/min.

Since a transition from water to product was used at the beginning of each processing run, the refractive index of the output stream was monitored by taking readings every 2-3 seconds using a portable refractometer (r^2mini, Reichert Analytical Instruments, Depew, NY), as an indication of sample concentration. Collection of UHT treated samples was initiated when the refractive index of the output stream became stable, i.e. remained unchanged after 3 consecutive measurements. The UHT

treated product was aseptically filled in pre-sterilized polycarbonate bottles (2000mL Nalgene 2105 Series, Thermo Scientific, Waltham, MA). The final temperature of the UHT treated product was 30ºC. An ice bath was used to quickly cool the product to 4ºC before storing it under refrigeration.

After each processing run, the UHT system was flushed with water for 5 min, followed by a full CIP cycle, performed as described above.

Retorting

A FMC Multipurpose Lab Retort with LogTec Process Management System (Steritort®, FMC, San Jose, CA) was used to batch-sterilize the MCCs. To verify the uniformity of temperature distribution inside the retort during processing, temperature data was collected in a preliminary trial, in which the same number of jars, placed in the same configuration as in the actual trial was used. Thermocouples were attached to 4 jars in the center of the retort cage, and the heating profile was calculated from the recorded temperature data with a modified Ball's formula using CALSoft 32, version 1.0 (TechniCAL Inc., Kenner, LA).

The filled jars were preheated in a water bath to 28°C to ensure the same starting temperature for all processing runs. 60 jars of two different concentrations were randomly placed into the retort cage in two layers, then the cage was loaded and secured inside the retort. The product was treated at 250°F (121.1°C) (product temperature) 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 118.9ºC (246ºF)), a cooking step (9 min, average temperature of 120ºC (248ºF)) and a cooling step (10 min, final temperature 41.1ºC (106ºF)) (Figure 5.2).

At the end of the processing cycle, jars were taken out of the retort and placed

on ice for 30 min. The second batch of 60 jars of the two remaining concentrations were pretreated and processed the same way. The order of the two concentrations processed at the same time was established randomly. For the second batch of reconstituted samples (replication 2), 30 jars filled with water were used to bring the total number of jars to 60.

Analytical evaluation of the MCC samples

All MCC samples (both heat treated and the non-heat treated controls) were subjected to the following analyses: mineral profile characterization, zeta potential analysis, particle size measurement and rheological analysis. All analyses were performed at 0 h (immediately after heat treatment), 12h and 24h, with the exception of rheological characterization, which was only performed at 12h and 24h after heat treatment.

Mineral profile characterization

MCC samples were evaluated for the total mineral content and for soluble minerals. In order to measure the concentration of soluble minerals, samples were ultracentrifuged and the mineral content in the clear supernatant was analyzed.

Ultracentrifugation procedure. This step was performed using the procedure by Philippe, Le Graët, & Gaucheron (2005). Equal volumes of 8.5 mL of sample were pipetted into four 10.4mL polycarbonate centrifuge bottles with cap assemblies (Model 355603, Beckman Instruments, Inc., Palo Alto, CA). The bottles were ultracentrifuged 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. At the end of the ultracentrifugation step, special care was taken to not re-suspend the insoluble pellet while handling the rotor, removing the bottles and collecting the

supernatant. 10 mL syringes (Luer-Lock, Thermo Scientific, Waltham, MA) with 4-in stainless steel needles (Popper Blunt 304 Stainless Steel 20G, Popper & Sons, Inc., New Hyde Park, NY) were used to collect 4 mL of clear supernatant from each bottle, without disturbing the pellet. The needle was only submerged once to avoid disturbing the thin fat layer formed at the top of the centrifugation bottle.

Mineral composition analysis. The mineral composition of the micellar casein concentrates and of the ultracentrifugation supernatants was tested at the Dairy One Forage Analysis Laboratory (Ithaca, NY). The following minerals were quantified: calcium, phosphorous, magnesium, potassium and sodium. 4-5 grams of sample were weighed into 50ml calibrated CEM Xpress Teflon PFA vessels with fiberglass insulating sleeves. The samples underwent a 30 minutes digestion step with 8 mL nitric acid (HNO_3) and 2 mL hydrochloric acid (HCl) at 1600W and 190ºC, in a CEM Microwave Accelerated Reaction System (MARS) (CEM, Matthews, NC) equipped with the MarsXpress Temperature Control option. The vessels were then brought to 50 mL volume with 1.5N HNO_3 + 0.5N HCl solution to match standards, then aspirated and analyzed with a Thermo Jarrell Ash IRIS Advantage HX Inductively Coupled Plasma (ICP) Radial Spectrophotometer (Thermo Scientific, Madison, WI). All mineral analyses were performed in duplicate.

Particle size analysis

Dynamic light scattering (DLS) was used to evaluate particle size and particle size distributions (PSD) in all MCCs, following the procedure by Beliciu and Moraru (2009). 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.

Before analysis, the MCC samples were equilibrated in a water bath (Model

202-2, National Appliance Co, Portland, OR) for 1h at 20ºC. Protection against dehydration during equilibration was ensured by covering the test tubes that contained the samples with Parafilm (Model PM-996, Pechiney Plastic Packaging, Menasha, WI).

In order to maximize the accuracy of the measurements, the MCC samples were diluted before the DLS analysis using UF permeate from the same milk, obtained during the ultrafiltration step in Day 2. UF permeate constitutes the ideal solvent for measuring PSD of casein micelles, since its chemical composition is very close to that of the milk serum (Beliciu and Moraru, 2009). Before testing, the UF permeate was filtered using a 0.2-µm syringe filter (25mm SFCA Syringe Filter, Thermo Scientific, Waltham, MA) and equilibrated in a water bath for 1h at 20ºC. The dilution ratio was adjusted for each sample so that the signal intensity was between 700 and 900 kilocounts per second (kcps), as recommended by the DLS equipment manufacturer.

The refractive index and viscosity of the UF permeate at 20ºC were used to define the solvent. A viscosity of 1.154cP was measured for UF permeate using an ARES strain-controlled rheometer (TA Instruments, New Castle, DE), equipped with a double-wall couette geometry. The temperature was maintained at 20ºC using a Julabo FS18-MW circulator (Julabo USA, Inc., Allentown, PA). The refractive index of the UF permeate was determined using a digital fiber-optic refractometer (Misco Products Division, Cleveland, OH); the measured refractive index for the UF permeate was 1.341.

The particle size measurements were performed at a constant temperature of 20°C. The disposable cuvettes were equilibrated in the temperature-controlled chamber of the particle size analyzer for 3 min, the diluted samples were pipetted in the cuvette and allowed a 2.5 min temperature equilibration step, after which the DLS measurement was promptly started.

Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp.), which converted the experimental data into size distributions. The software contains a Dust Filter algorithm that improves the quality of the measurements by rejecting data that are corrupted by scattering from random particles such as air bubbles or dust. The Dust Filter cutoff parameter was set at 30, which is the optimal value suggested by the manufacturer when the expected particle size is in the range of hundreds of nanometers. Each measurement consisted of 8 subsequent individual runs of 30s duration. For each measurement, the relative particle size distribution, the intensity weighted effective diameter $(\overline{d_{eff}})$, and the polydispersity index (p) were determined. The tests were performed in duplicate.

Zeta potential analysis

Zeta potential analysis was performed using the ZetaPlus option of the 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Before the measurement, samples were diluted using ultra-pure water previously filtered with a 0.2-µm syringe filter (25mm SFCA Syringe Filter, Thermo Scientific, Waltham, MA). The diluted samples were pipetted into a single use cuvette and allowed a 2.5 min temperature equilibration step before initiating the test. Measurements were performed using a 35 mW solid state laser, λ = 660 nm, in the "High Precision" mode, at 20°C, after setting "Water" as solvent. Protein dilutions were adjusted in order to achieve an optimum ratio (0.1-0.5) between the instrument count rate and the reference count rate (1454 kcps). The measurement consisted of 30 cycles/run, with an intercycle delay of 5 s. Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp.).

Rheological analyses

Large-deformation, steady shear rheological analyses. An ARES strain-controlled rheometer with dual transducer in conjunction with the Orchestrator data collection and analysis software (TA Instruments, New Castle, DE) was used to evaluate the viscosity as a function of shear rate for the MCCs. A parallel plate geometry with 50 mm diameter Teflon plates and an interplaten gap of 1 mm was used; 2 mL of the MCC samples were loaded on the lower plate, avoiding the formation of air bubbles, then the gap was set by lowering the upper plate. A relaxation step of 1 min was allowed before starting the measurements. All measurements were performed at 20ºC, and the temperature control was ensured using a Peltier system. In order to avoid dehydration during the measurements, the samples were thinly coated with mineral oil. Additionally, an isothermal chamber was used to prevent variations in vapor pressure and humidity at the air-sample interface.

Strain-controlled, steady-shear rate sweeps, with shear rates ranging from 1 to 10^3 s^{-1} were performed. The measured parameters were viscosity (η) and shear stress (τ) as a function of shear rate. All measurements were performed in duplicate.

Since most of the micellar casein concentrates were non-Newtonian, the term "apparent viscosity" (η_{app}) was used to accurately describe their resistance to flow. In order to make direct comparisons among the different MCCs, the apparent viscosity at a shear rate of 100 s^{-1} (η_{100}) was used. This value of shear rate was selected due to its association with stirring, pumping, pipe flow, spraying and other processing operations, even mastication (Steffe, 1996; Carr et al., 2003).

The flow behavior of the micellar casein concentrates was characterized using the Herschel-Bulkley constitutive model:

$$\sigma = K\left(\dot{\gamma}\right)^{n} + \sigma_0, \qquad (2)$$

where K is the consistency coefficient ($Pa \times s^n$), $\dot{\gamma}$ is the shear rate (s^{-1}), *n* is the flow behavior index, and σ_o is the yield stress (Pa).

Small-deformation, oscillatory rheological analyses. Oscillatory dynamic measurements were performed in order to obtain information about the structural characteristics and viscoelasticity of the MCC samples. The double wall couette geometry was used (interplaten gap = 1 mm) and a constant temperature of 20°C during the measurement was maintained using a Julabo FS 18-MW Heating and Refrigerated Circulator (Julabo USA, Inc., Allentown, PA). For each analysis, 8 mL of MCC sample were loaded into the couette, avoiding the formation of air bubbles. The gap was set and, in order to avoid dehydration during the measurement, the samples were thinly coated with mineral oil. An isothermal chamber was also secured in place above the geometry, in order to prevent variations in vapor pressure and humidity at the air-sample interface. A relaxation step of 1 min was allowed after the sample reached the treatment temperature of 20°C.

The rheological parameters measured were: storage modulus (G'), loss modulus (G"), complex modulus (G*) and the loss tangent (tan δ = G"/G').

First, dynamic strain sweeps were conducted for each of the samples, in order to locate the linear viscoelastic region (LVR) of material response, which represents the range of strain at which the rheological properties are independent of the applied strain. The strain sweeps were performed over the strain interval 0.05-10%, with a strain increment of 0.2%. Frequency sweeps were then conducted, in order to evaluate the viscoelastic properties of the MCC samples as a function of timescale. Frequency sweeps were conducted at 20°C, using a strain value in the LVR region, and a frequency range of 0.1-100 rad/s.

pH measurements

The pH was measured in untreated samples immediately before heat treatments and in the sterilized samples at 0h (immediately after heat treatments), 12hr and 24hr after the heat treatments. All measurements were performed at 20°C using a freshly calibrated Fisher Scientific accumet Excel XL20 pH meter (Fisher Scientific, Pittsburgh, PA).

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 content, treatment and time after treatment) and random effects (experimental replication, experimental replication x product source, experimental replication x product source x treatment, experimental replication x casein content, experimental replication x casein content 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 content, 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 and the Student's t-test. Differences with $p < 0.05$ were considered statistically significant.

Results

Chemical composition of skim milk and micellar casein concentrates

The chemical composition of skim milk is presented in Table 5.1. It must be noted that the casein, lactose and ash content of skim milk decreased from the first experimental replication to the third, due to seasonal variability.

The measured chemical composition of the 4th stage MF retentate can be seen

in Table 5.2. The casein content (calculated as CN = (TN - NCN) x 6.38) in the 4th stage MF retentate was slightly underestimated by the Kjeldahl method. According to Nelson and Barbano (2005), this is due to the incomplete precipitation of casein by acetic acid in the sample preparation stage for determining the NCN in the highly concentrated retentate. As a result of the combination of the UF stage and four MF stages, more than 95% of the initial serum protein content was removed (Zulewska et al., 2009), and more than 96% of the initial lactose content was depleted. The pH of the 4th stage MF retentate measured at 50ºC immediately at the end of the filtration process is also presented in Table 5.2. It can be noticed that the retentate obtained in the third experimental replication was slightly more acidic (pH=6.69) than the retentates obtained in the previous two processing cycles. This is important to note, since it may be one of the causes for the differences in behavior during thermal processing for this experimental replication as compared to the first two.

The mineral composition of the untreated micellar casein concentrates is presented in Table 5.3. The values in the table represent averages of the three experimental replications. The decrease in mineral concentration with the casein concentration of the MCCs was due to the dilution of the retentate with RO water. Experimental replication had a significant effect on the total mineral content of the micellar casein concentrates. The levels of total Ca, P and Mg were significantly lower, while the levels of total K and Na were higher for the third replication MCCs as compared to the first two replications. Also important is the fact that the overall Ca/P molar ratio was significantly lower in the third replication MCCs than for the first two replications. The spray drying and reconstitution had no effect on the total mineral profile of the MCCs used in this study.

The soluble mineral content of MCCs is also presented in Table 5.3. For Ca, P and Mg, which are all partly bound to the casein micelles, the soluble content as a

fraction of total content was much lower in the MCCs as compared to skim milk. In skim milk, the soluble fractions of Ca, P and Mg represent in average 31%, 54% and 65% of the total content of these minerals, respectively (Holt, 1985). In the MCCs, the soluble Ca, P and Mg represented in average 8%, 9% and 19% of the total contents, respectively. For K and Na, which are essentially diffusible, more than 90% of their total content present is present in soluble form (Holt and Jenness, 1984). This was also observed for the MCCs obtained in this study but, at the same time, due to repeated filtration and diafiltration, the total levels of K and Na in the MCCs were lower by 96% and respectively 97% than in skim milk.

The soluble mineral content also decreased with casein concentration, as a result of the dilution with RO water of the 4th stage MCCs. Experimental replication had a significant effect on the soluble mineral fraction of the micellar casein concentrates, particularly for soluble Ca, P and Na, which were significantly lower in case of the third experimental replication MCCs as compared to the first two replications. The Ca/P molar ratio in the supernatants of untreated MCCs from the 3rd replication was significantly higher than the ratios for replications 1 and 2.

Table 5.3 Mineral composition of the micellar casein concentrates

MCC	Ca			P			Mg			K			Na		
	Total, mM	**Sol., mM**	**Sol./ Total, %**	**Total, mM**	**Sol., mM**	**Sol./ Total, %**	**Total, mM**	**Sol., mM**	**Sol./ Total, %**	**Total, mM**	**Sol., mM**	**Sol./ Total, %**	**Total, mM**	**Sol., mM**	**Sol./ Total, %**
5%	42.2±1.7	3.9±0.6	9.2	31.1±1.7	3.0±0.4	9.6	2.5±0.1	0.6±0.2	24.0	2.1±0.2	1.9±0.5	90.5	1.0±0.1	0.8±0.2	80.0
7.5%	65.4±3.3	5.2±0.5	8.0	48.5±2.4	4.2±0.5	8.7	3.9±0.1	0.7±0.0	17.9	2.8±0.3	2.6±0.1	92.9	1.3±0.2	1.1±0.3	84.6
8% MCC	72.2±8.2	5.9±0.6	8.2	53.6±5.4	4.9±0.6	9.1	4.2±0.3	0.7±0.1	16.7	3.1±0.2	2.8±0.1	90.3	1.4±0.1	1.1±0.2	78.6
8% R-MCC	71.2±2.4	5.7±0.3	8.0	54.1±1.8	4.8±0.3	8.9	4.3±0.2	0.8±0.1	18.6	3.3±0.2	2.6±0.2	78.8	1.5±0.1	1.2±0.1	80.0
10%	91.1±5.5	7.5±1.3	8.2	67.2±2.7	6±1.2	8.9	5.3±0.2	1±0.2	18.9	4.0±0.2	3.1±0.6	77.5	1.8±0.1	1.5±0.3	83.3

The spray drying and reconstitution had no statistically significant effect on the soluble mineral profile of the untreated MCCs used in this study. The exception to this rule was Mg, which was present at a significantly higher level in the soluble fraction of R-MCCs as compared to the MCCs. The reason for this difference is not clear, but this is not expected to have a significant effect on the MCCs' behavior.

Mineral composition

Sterilization had no significant effect on the total mineral profile and the overall Ca/P molar ratio of micellar casein concentrates.

Soluble Ca, P and Mg levels were lower in the sterilized MCCs as compared to the controls (untreated samples), while Na and K levels were not significantly affected by sterilization (Figure 5.3). Soluble Ca and P were affected differently by retorting and UHT, with significantly lower levels of soluble Ca and P being recorded for the UHT treated samples as compared to the retorted samples.

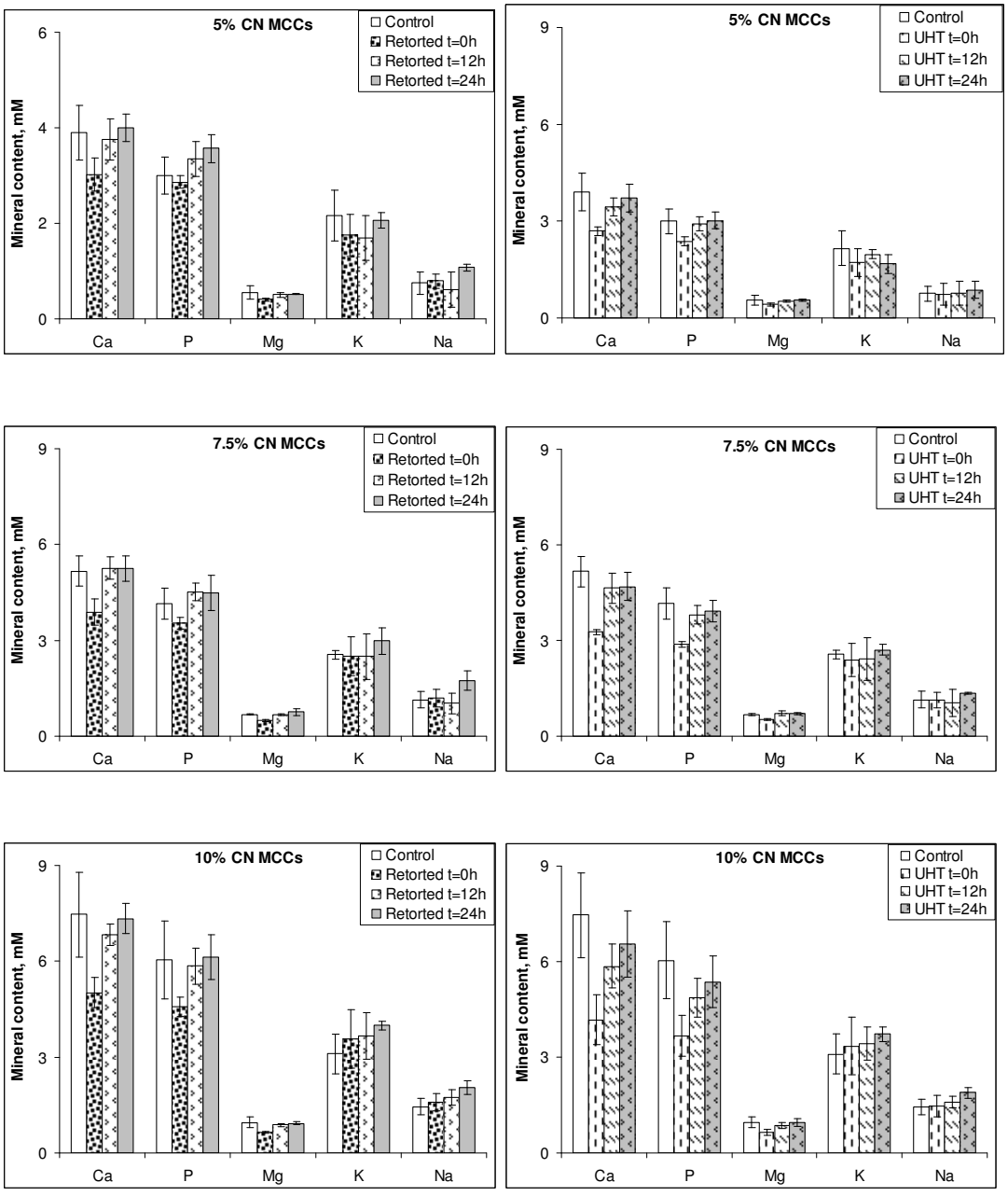

Figure 5.3 The time dependence of soluble minerals for retorted and UHT treated samples at 5%, 7.5% and 10% micellar casein concentrates.

The effect of the different sterilization treatments on the Ca/P molar ratio in the soluble phase can be assessed in Figure 5.4, for all MCCs and R-MCCs. The soluble Ca/P molar ratio was highest in the untreated samples. The soluble Ca/P ratio for the UHT treated samples was significantly higher than in the soluble fraction of the retorted samples. The concentration of soluble Mg was not affected by the type of heat treatment.

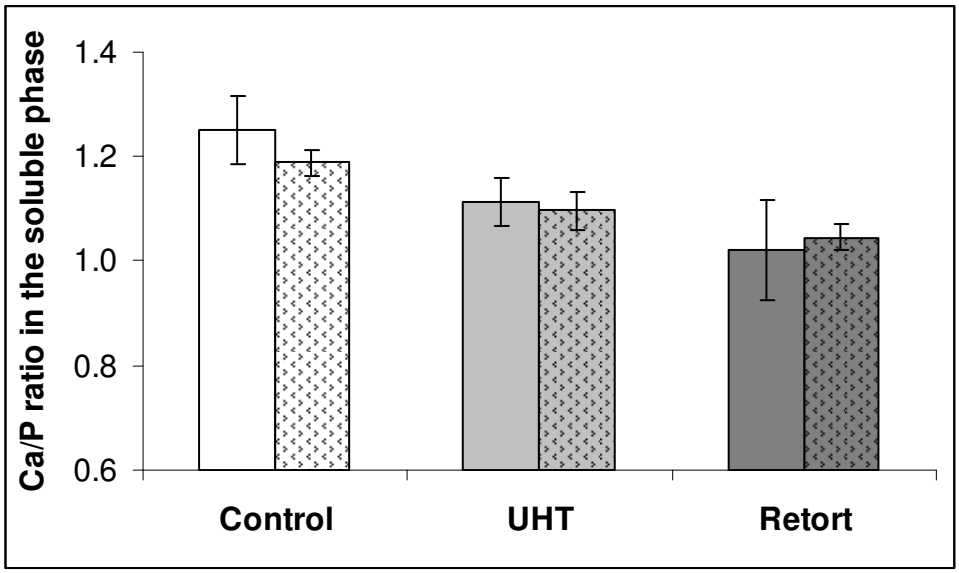

Figure 5.4 The Ca/P molar ratio in the soluble phase for untreated MCCs (□) and R-MCCs (▨), UHT-treated MCCs (▭) and R-MCCs (▨), retorted MCCs (■) and R-MCCs (▩) immediately after the treatment (t=0h).

Time after heat treatment had a statistically significant effect on the soluble Ca, P and Mg. Even though immediately after sterilization soluble Na and Mg levels were not different from the untreated MCCs, after 24 hours from sterilization the levels of both diffusible minerals became significantly higher than in the soluble fraction of untreated samples. The levels of soluble Ca, P and Mg in the heat treated samples increased over 24 hours after the heat treatment (see Figure 5.3). In case of retorting,

the level of soluble Ca reverted to pre-treatment levels, while for the UHT treated samples the soluble levels remained lower than for the controls after 24h. The soluble Mg level reverted back to pre-treatment levels after 24 hours for both UHT and retorted samples.

The sterilization of R-MCCs led to a similar distribution of soluble/total minerals as for the MCCs for Ca, Na and K. The Ca/P molar ratios in the heat treated R-MCCs were similar to those calculated for the MCCs. Spray drying affected the soluble P and Mg levels after sterilization, as the levels of soluble P and Mg in the sterilized R-MCCs were significantly higher than in their MCC counterparts. Treatment and time after treatment had similar effects on the soluble mineral fraction of R-MCCs as for MCCs. The sterilized R-MCCs had significantly more soluble Ca, P and Mg, and a lower Ca/P ratio than sterilized MCCs after 24 hours of heat treatment, while the soluble Ca, P and Mg reverted to pre-treatment levels for both UHT and retorted samples (Figure 5.5).

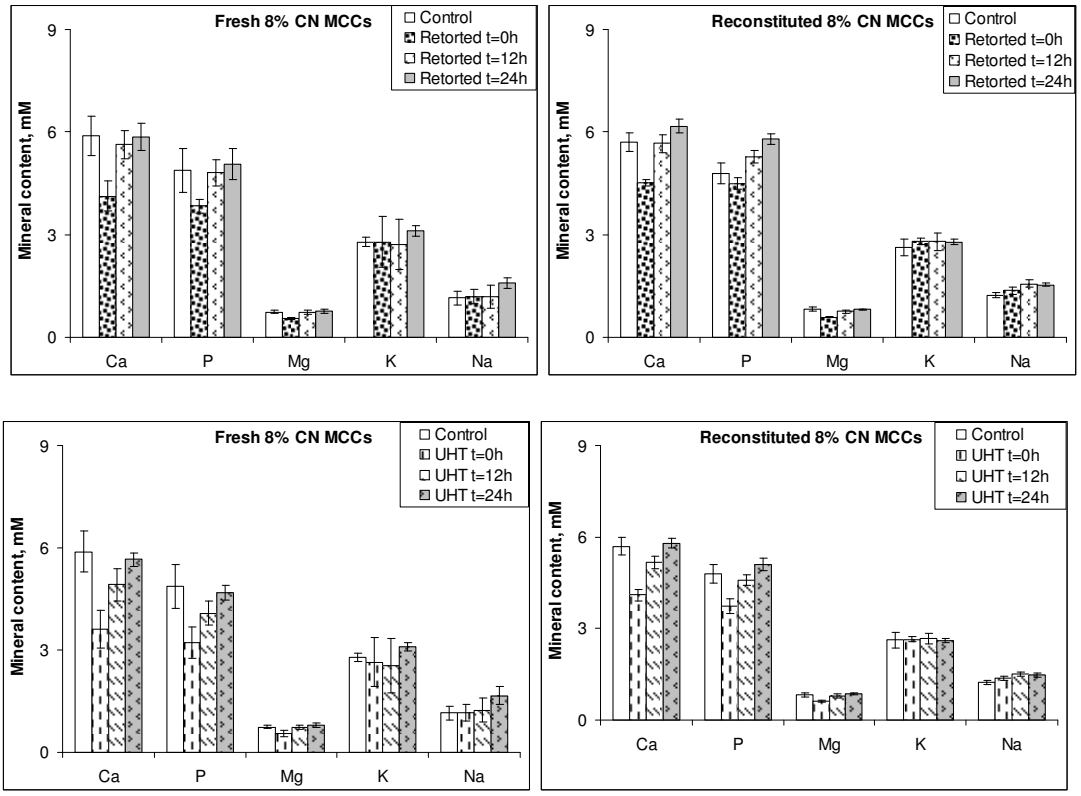

Figure 5.5 The time dependence of soluble mineral content of retorted and UHT treated MCCs and 8% R-MCCs.

pH

The pH of the micellar casein concentrates varied with casein content, with the lower casein concentration MCCs having a higher pH (Table 5.4). As discussed above, this was due to the pH of the dilution water. The pH of the RO water used for dilution was 7.65 (at 20ºC).

The pH values of untreated and UHT treated samples had similar values, while the retorted samples were characterized by a significantly lower pH as compared to the controls. The pH of the heat treated samples did not change significantly within 24 hours of processing.

Experimental replication had a significant effect on the pH of all MCCs (controls and heat treated), with the pH of the concentrates obtained in the third experimental replication being consistently lower than that for the previous two replications. The heat treatment and time after treatment had similar effects on pH for the R-MCCs as for the MCCs (Table 5.4).

Table 5.4 pH of micellar casein concentrates. Average of all three replications.

Casein concentration, %	**Product source**	**Control**	**UHT**	**Retorted**
5.0	MCC	7.17±0.06	7.17±0.03	7.02±0.08
7.5	MCC	7.1±0.06	7.12±0.03	6.96±0.10
8.0	MCC	7.11±0.08	7.12±0.05	6.93±0.13
	R-MCC	7.09±0.02	7.09±0.02	6.95±0.03
10.0	MCC	7.08±0.05	7.08±0.05	6.91±0.11

Zeta potential

Zeta potential (ζ) was used as a relative indicator of the colloidal stability of

the micellar casein concentrates studied in this work. The absolute value of ζ measured for MCCs was not affected by casein content (Figure 5.6). Heat treatment of the MCCs resulted in a significant decrease in the absolute values of ζ, but the ζ of the heat treated samples did not change within 24 hours after processing. Experimental replication only had a statistically significant effect on the ζ of retorted MCCs. MCCs obtained in the third experimental replication had significantly lower absolute ζ values after retorting ($-24.5 < \zeta < -17.2$ mV, at the different casein concentrations) than for the previous two replications ($-29.9 < \zeta < -25.5$ mV). The MCCs obtained in the third experimental replication had lower absolute values of ζ after retorting than after UHT treatment. For these MCCs, the absolute values of ζ increased after 12 and 24 hours after processing as compared to immediately after processing.

The drying and reconstitution process did not have an effect on the ζ of sterilized micellar casein concentrates. However, for untreated samples, the absolute values of ζ of R-MCCs were lower than for the MCCs (Figure 5.6), most likely because of a modified micellar surface charge as a result of the drying process. There was a significant effect of time after treatment in the case of 8% MCCs and R-MCCs that were sterilized, with higher absolute values of ζ measured 12 and 24 hours after the treatment as compared to the values measured immediately after sterilization.

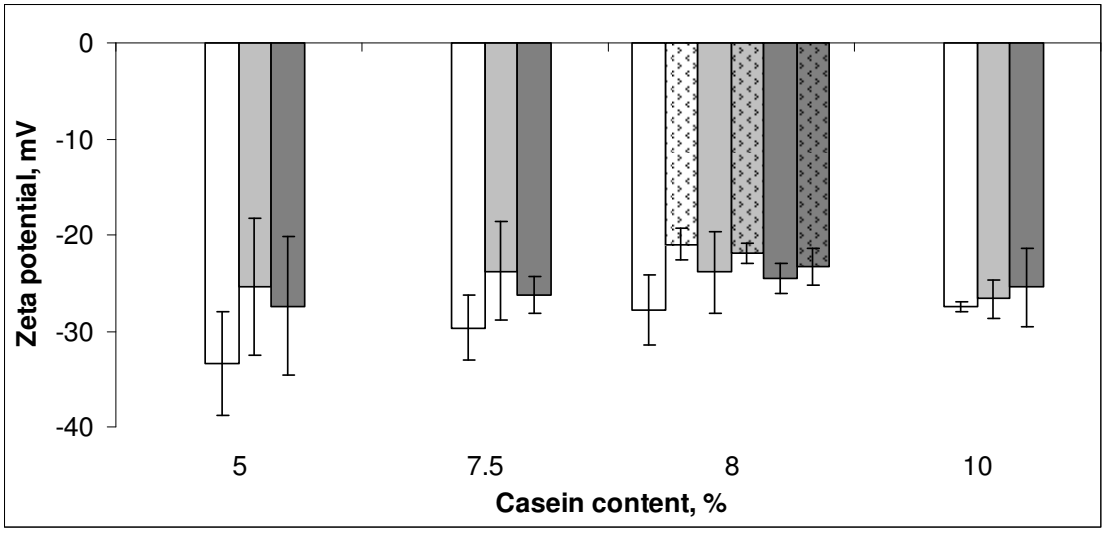

Figure 5.6 The zeta potential of untreated MCC (□), retorted MCC (▒) and UHT treated MCC (■), untreated R-MCC (▨), retorted R-MCC (▩) and UHT R-MCC (▩). The values represent averages of all three experimental replicates, and the error bars represent one standard deviation.

Particle size

The occurrence of particle aggregation in the micellar casein concentrates was evaluated by determining the effective diameter for each of the MCCs. Large, visible agglomerates formed in all UHT treated samples, which rendered the DLS method unusable for these samples.

The effective diameter measured in micellar casein concentrates was not affected by casein content (Figure 5.7a), while retorting led to a significant increase in particle sizes. The particle size remained constant within 24 h of the heat treatment.

Experimental replication had no significant effect on the effective diameter measured in the untreated MCCs, but the effective diameter of the MCCs obtained in the third experimental replication was consistently higher than that measured for the previous two replications. In order to illustrate how particle sizes have varied among

replications, data for each individual replication was shown in Figure 5.7. b-d. In case of the retorted MCCs obtained in the third experimental replication (Figure 5.7d), there was a linear increase in particle size with casein content (R^2=0.97).

For the 8% concentrates no difference in particle size between the heat treated MCCs and R-MCCs was observed, which indicated that drying did not affect aggregation trends in the sterilized concentrates. However, it must be noted that for untreated samples, the average diameter for R-MCCs was higher than for the MCCs (Figure 5.7a). The treatment and time after treatment had similar effects on particle size as for the MCCs.

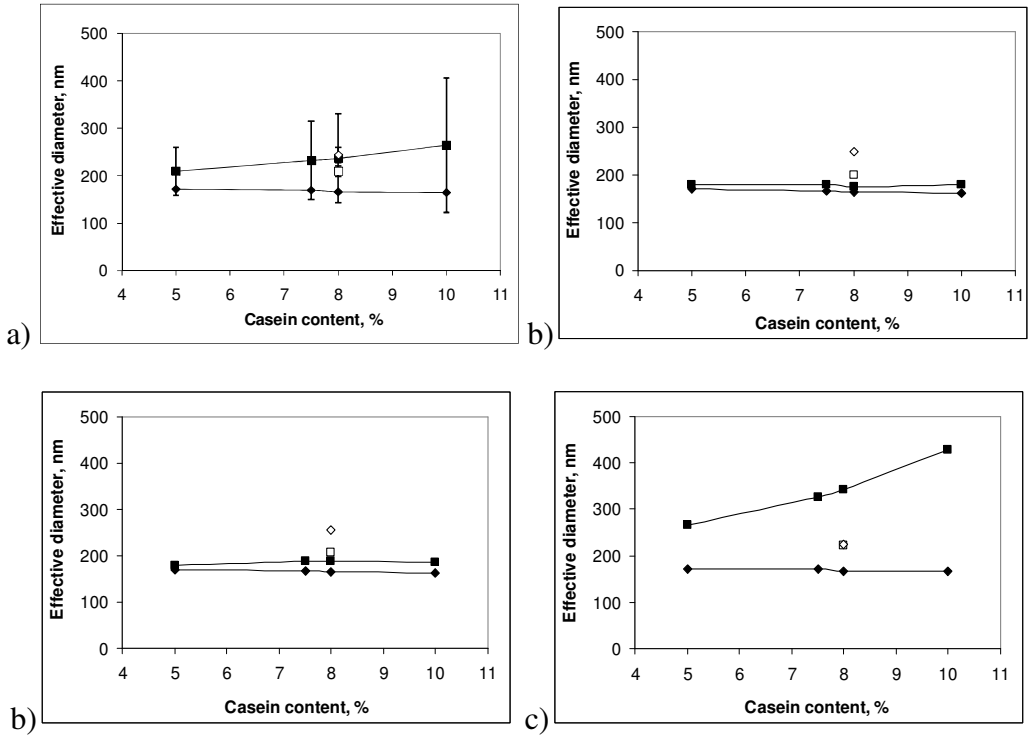

Figure 5.7 The particle size of untreated MCC (♦) and retorted MCC (■), untreated R-MCC (◊) and retorted R-MCC (□) a) average values for the three replications; b) values for experimental replication 1, c) values for experimental replication 2 and d) values for experimental replication 3.

Rheological properties of micellar casein concentrates

Viscosity

The apparent viscosity of the UHT treated samples was much higher than that of the untreated and retorted micellar casein concentrates (Figure 5.8a). The large variability in η_{100} of the UHT treated MCCs was likely due to the significant structural inhomogeneity of these samples. In order to illustrate how viscosity has varied among replications, data for each individual replication was shown in Figure 5.8 b-d. Viscosity was affected by both casein concentration and type of heat treatment. For each type of treatment, viscosity increased with casein concentration. When comparing the different treatments, retorted MCCs and R-MCCs had a significantly lower η_{100} than the untreated samples, with a ~50% reduction in the apparent viscosity induced by the heat treatment, while the viscosity of the UHT treated samples was significantly higher than of the controls (untreated samples), both for MCCs and R-MCCs.

For controls and the UHT treated concentrates viscosity did not change within 24 hours after processing, while for the retorted MCCs viscosity decreased significantly after 24 hours from the treatment.

Experimental replication had a significant effect on viscosity only for the sterilized MCCs. For retorted MCCs, samples produced in the 3rd experimental replication had significantly lower η_{100} than for the first two replications. In case of UHT treated MCCs, samples from the 2nd and 3rd experimental replications had significantly higher apparent viscosity than those obtained for the 1st replication.

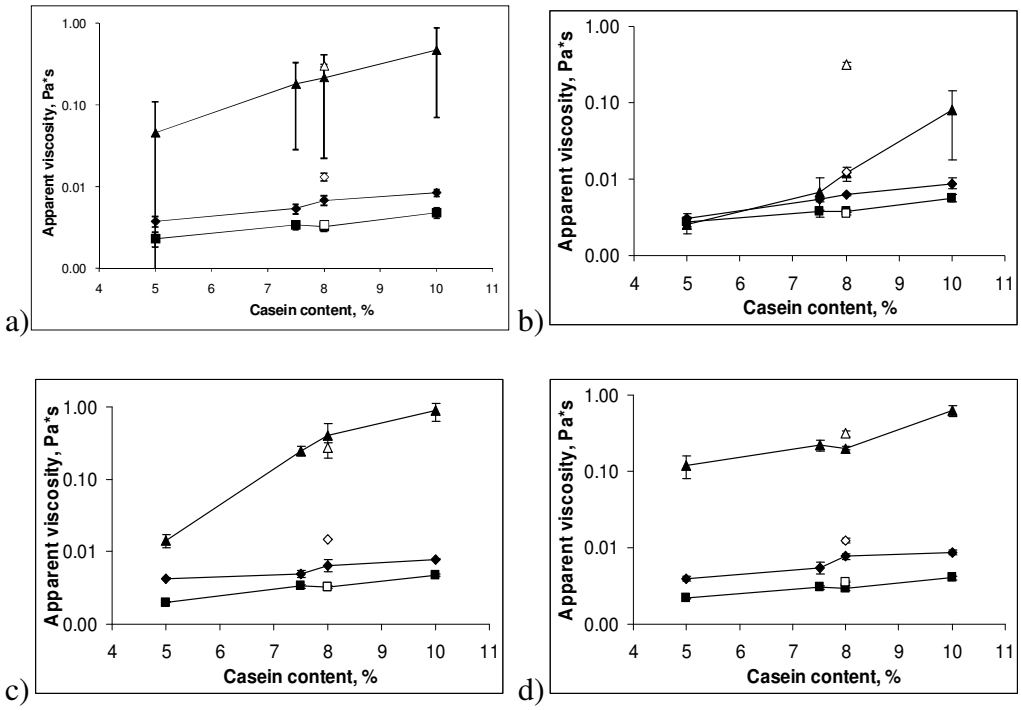

Figure 5.8 The apparent viscosity at 100 s^{-1} for untreated MCC (♦), UHT treated MCC (▲) and retorted MCC (■), untreated R-MCC (◊), UHT treated R-MCC (Δ) and retorted R-MCC (□) a) Average of all three experimental replicates; b) data from experimental replication 1, c) data from experimental replication 2 and d) data from experimental replication 3.

The apparent viscosity of untreated R-MCCs was significantly higher than η_{100} of the MCCs, at the same casein concentration. UHT treatment led to a two-fold increase in apparent viscosity in the R-MCCs as compared to the UHT-treated MCCs (Figure 5.8). The rheological data confirmed visual observations of UHT treated samples. In the case of retorted samples, there were no differences between the apparent viscosity of R-MCCs and MCCs. The UHT treated R-MCC from the first experimental replication had a significantly higher apparent viscosity than the UHT treated MCC.

Flow index

The UHT treated micellar casein concentrates were characterized by a rather irregular apparent viscosity vs. shear rate relationship, which did not allow for data analyses using the Herschel-Bulkley constitutive model. This was due to inhomogeneity of samples.

When untreated and retorted MCCs were compared, it was observed that retorting had a significant effect on the flow behavior of MCCs. Retorted MCCs had significantly higher flow indices than the untreated samples (Figure 5.9). Retorting seemed to induce a slight shear thickening behavior, at all casein levels. The flow behavior of the retorted samples did not change within 24 hours of processing. Experimental replication had a significant effect on the flow behavior of the untreated and retorted MCCs. Experimental replication did not have a significant effect on the untreated MCCs, but in the case of retorted MCCs samples obtained for the first replication were slightly less shear thickening than the samples obtained for replications 2 and 3.

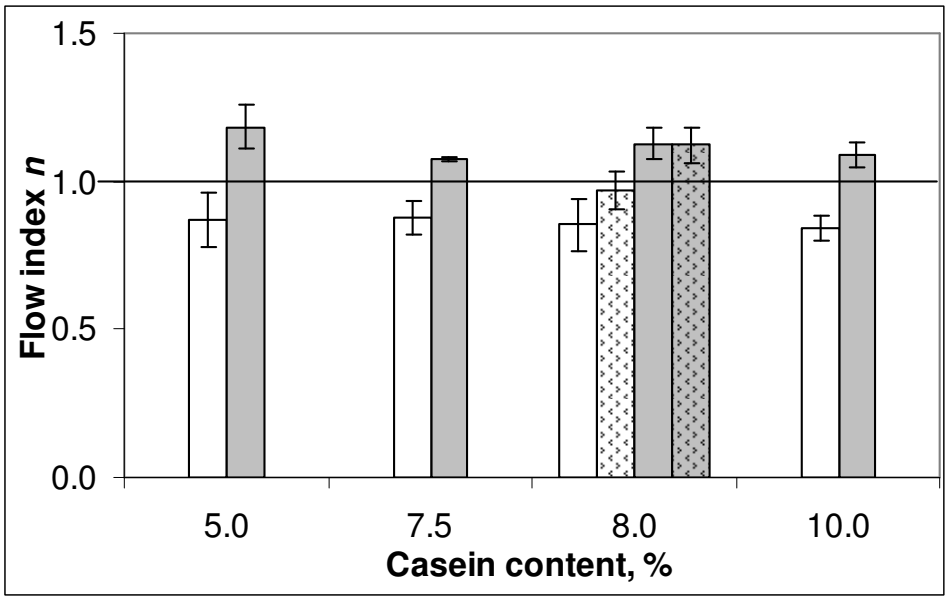

Figure 5.9 The flow index *n* of untreated MCC (□), untreated R-MCC (▨), retorted MCC (■) and retorted R-MCC (▩). The values represent averages of all three experimental replicates, and the error bars represent one standard deviation.

The sterilization of the R-MCCs led to similar values of the flow behavior index as for the MCCs (Figure 5.9). The retorted R-MCCs were characterized by a slight but significant shear thickening behavior, whereas the untreated R-MCCs were close to Newtonian. As for the MCCs, the flow behavior of the heat treated samples did not change within 24 hours of processing.

Yield stress

Most micellar casein concentrates in this study manifested yield stress, although the calculated yield stress values were rather low. The effects of casein content, sterilization and time after treatment on yield stress were similar with the effects on apparent viscosity. Since the physical causes for the two physical properties are similar, yield stress data will not be presented here, to avoid redundancy.

Dynamic rheological properties

Frequency sweeps were used to evaluate the viscoelastic characteristics of the MCCs and their behavior as a function of the time-scale. All untreated and retorted MCCs were characterized by predominantly liquid-like behavior (G'<G"), while most of the UHT treated samples were characterized by a predominantly solid-like behavior (G'>G"), which was indicative of structure formation. In the case of MCCs obtained for the 1st experimental replication, significant network formation was noticed only at C ≥ 8%, while for the 2nd experimental replication structure formation became significant at C ≥ 7.5%; in case of MCCs obtained in the 3rd replication, significant network formation was observed starting with C = 5%. A representative example of frequency sweep results is presented in Figure 5.10, which shows data for the UHT and retorted 10% MCCs obtained in the 3rd experimental replication. All R-MCCs were characterized by a solid-like behavior (G'>G").

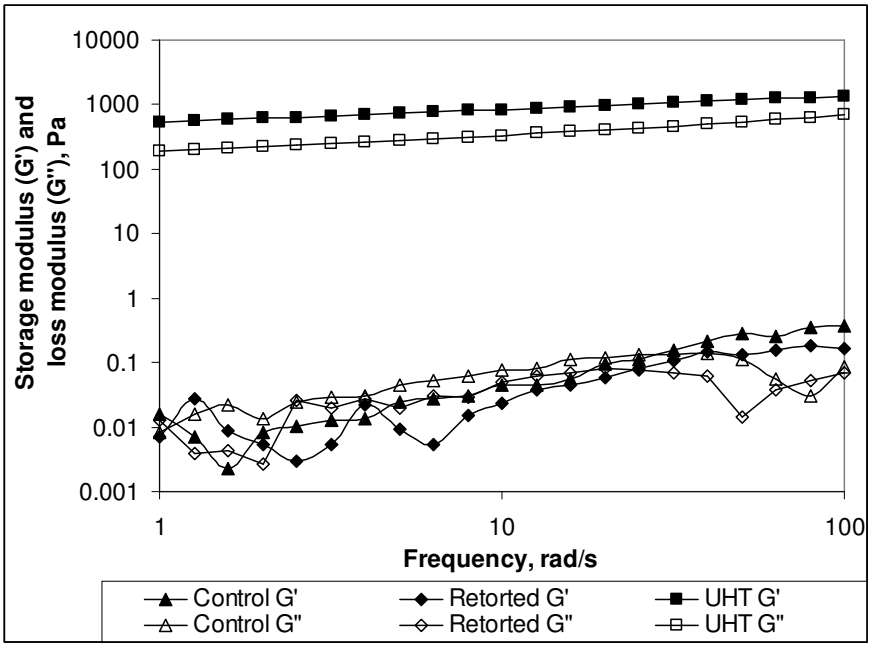

Figure 5.10 Example of a frequency sweep test. Data shown is a frequency sweep for the untreated (Control) and sterilized 10% MCCs obtained in the 3rd replication.

Discussion

The pH of the micellar casein concentrates varied with casein content, with higher pH values in the more dilute MCC (Table 5.4). Since these differences could not be avoided, they will be taken into consideration when explaining the observed results of this study. There was a significant decrease in pH for retorted as compared to the untreated MCCs, with an average pH drop of 0.15-0.2 units as a result of retorting. A drop in pH as a result of retorting has been reported before. For instance, in retorted milk with full lactose content, the pH drop caused by retorting was 0.2-0.3 units (Berg and van Boekel, 1994). Pyne and McHenry (1955) proposed three different causes for which the pH of milk decreases during extended high temperature heating 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. Since the MCCs used in this study contained very low concentrations of lactose, it is safe to assume that the contribution of the last two acidification mechanisms was more substantial. Dephosphorylation of casein can also explain the slight increase in soluble phosphorus reported for the retorted samples.

The pH of MCCs obtained in the 3rd experimental replication was consistently lower than for the previous two replications. This could have caused the significantly lower levels of total and soluble calcium, phosphorus and magnesium in the MCCs obtained in the 3rd replication, as a slightly acidic pH during filtration may have lead to solubilization of some of the micellar calcium and magnesium phosphate and their subsequent removal into the permeate fraction. This can explain the significantly lower overall Ca/P molar ratio, the higher soluble Ca/P ratio, and the higher instability to heat treatment of the casein micelles for the MCCs obtained in the 3rd replication.

The zeta potential of the sterilized MCCs became less negative than that of the

untreated samples. For the MCCs from the 3rd replicate the magnitude of the zeta potential was affected more by retorting than by UHT, with the zeta potential of the retorted samples reaching levels in the vicinity of the threshold of agglomeration of -5mV (Riddick, 1968). These observations support the hypothesis that the calcium phosphate that precipitated on the surface of the micelles as a results of heating interacted with the negative charges on the micelles' surface, reducing their zeta potential. Calcium phosphate precipitation might also be responsible for the less negative zeta potentials measured for the untreated RMCCs as compared to MCCs, as the drying process may lead to additional modification of the micelles' surface charge.

The lower soluble calcium and phosphorus contents in the sterilized MCCs immediately after the treatments seem to also support precipitation of calcium phosphate on the micelle's surface. A significant difference was however observed between the UHT and retorted samples, with significantly higher levels of soluble calcium and phosphorous for the retorted samples. This could be a consequence of the slightly lower pH levels in retorted samples, which could slightly increase the solubility of calcium phosphate. In case of the retorted MCCs, the soluble calcium reverted to pre-treatment levels 24 hours after sterilization, which did not happen for the UHT treated samples. This suggests some degree of irreversibility of the heat induced changes that occurred during the UHT treatment, at least over the 24h time frame evaluated in this study.

An interesting comparison can be made between calcium and magnesium, two minerals that are partially associated with the casein micelles, since the two sterilization processes affected them differently. Both the soluble Ca and Mg contents were higher in the untreated controls than in the freshly sterilized samples. The soluble Ca level in UHT treated MCCs was significantly lower than in the retorted samples, while there was no difference between the soluble Mg levels for UHT and retorted

concentrates. Research by Kaminogawa et al. (1977) and Aoki et al. (1987) demonstrated that calcium phosphate can form cross-links in artificial casein micelles systems, but magnesium phosphate does not have cross-linking ability. It is therefore reasonable to hypothesize that calcium phosphate may play an important role in the aggregation of casein micelles during the UHT treatment, due to its cross-linking ability. Mg phosphate does not have this ability, and subsequently the soluble Mg content reverts to pre-treatment level within 24 hours after the sterilization.

Additional support for the precipitation of calcium phosphate on the casein micelles' surface is represented by the fact that the viscosity of the retorted samples was lower than of the controls. For example, η_{100} of the retorted MCCs 50% lower as compared to the untreated MCCs, while the η_{100} of the retorted RMCCs was 75% lower than the untreated RMCCs. The heat induced precipitation of calcium phosphate on the surface of casein micelles could have caused the partial shielding of negative charges at the surface of the casein micelles and subsequently lowered repulsion among casein micelles, which caused a lower resistance to flow, thus a lower apparent viscosity of the retorted MCCs. Retorted MCCs from the 3rd replication, which had the least negative values of ζ, had also the lowest η_{100}. These findings are also in accordance with reports by Anema and Klostermeyer (1997) and Horne (1998).

It is also interesting to note that over the next 24 hours after sterilization the zeta potential of the MCCs did not significantly change, even though the soluble Ca, P, Mg and even K and Na levels seemed to increase. This suggests that the changes occurring at the surface of the casein micelles or aggregates were not reversible, while the mineral redistribution that seemed to occur within the 24 hours after sterilization was a slow exchange between the aqueous phase on one side and the casein micelles on the other side. There were however two exceptions to this overall trend: the sterilized MCCs obtained for the 3rd replication and the RMCCs, for which ζ became

more negative over time.

Both the UHT treated and retorted MCCs had significantly higher particle sizes than the untreated samples. Although quantitative particle size data for the UHT treated MCCs could not be obtained, aggregation was visible with the naked eye for these samples, and became more pronounced from the 1st to the 3rd experimental replication. There was a clear correlation between the lower zeta potentials measured for the 3rd replicate MCCs and their propensity for aggregation, especially in the retorted samples. This demonstrates that aggregation was induced by the two sterilization processes. Spray drying seemed to induce additional particle aggregation, as the average diameter for untreated R-MCCs was higher than for MCCs.

Retorting seemed to induce, at all casein levels, a slight shear thickening behavior. Shear thickening behavior has been previously reported for concentrated dispersions of charged rigid spheres (Shenoy et al., 2003), and was attributed to the formation of hydroclusters at shear rates where the hydrodynamic forces overcome the repulsive interparticle forces (Bender and Wagner, 1996). The decrease in the intensity of repulsion between casein micelles due to precipitation of calcium phosphate on the surface of the casein micelles during the heat treatments could allow the formation of hydroclusters (aggregates) under shear, thus justifying the shear thickening behavior.

A significant difference was however observed between the retorted and UHT treated samples. While a decrease in viscosity after heat treatment was observed for the retorted samples, in case of the UHT treated sample the opposite was observed. The apparent viscosity of UHT treated samples was more than 10 fold higher than for the untreated samples, which indicated structure formation took place during this treatment, this phenomenon being the most pronounced for the 3rd replication. Similar observations were made from the dynamic rheology data, which showed increasingly pronounced structure formation after UHT treatment took place from the 1st to the 3rd

replicate; the UHT treated MCCs from the 3rd replication showed solid-like behavior at a casein content as low as 5%.

An attempt to explain observed differences among replications. The effect of membrane processing time on the mineral profile of MCCs

The reasons for the observed differences among experimental replications could reside in compositional differences, differences in processing, or both.

In order to evaluate the effect of processing time on the behavior of MCC, the duration of MCC production was evaluated for each processing replication. Data from two preliminary trials was also considered, for comparison purposes. Processing time was considered very important, since the membrane separation of the MCCs was performed at 50ºC. At this temperature, the solubility of colloidal calcium was proven to decrease (Rao et al., 1994), and some of the colloidal calcium may precipitate as calcium phosphate on the surface of casein micelles (Wahlgren et al., 1990).

Different starting amounts of pasteurized skim milk were used in each trial, leading to significant differences in processing time. The processing times for the three experimental replications used in this study were as follows: 983 min for the 1st replication, 992 min for the 2nd replication and 1034 min for the 3rd replication. The preliminary trials did not include an initial UF stage, while in the case of experimental processing an UF stage was added in order to decrease the lactose content of MCCs before MF. No aggregation or destabilization of micellar casein concentrates as a result of the UHT treatment was noticed for the two preliminary processing trials, which were considerably shorter than the 3 experimental runs: 601 min for the 1st preliminary trial and 450 min for the 2nd preliminary trial.

In terms of compositional differences, special attention was given to the mineral content, particularly calcium. The MCCs had slightly different casein contents

for each trial, therefore a conversion from total mineral content of the different MCCs, to mineral concentration per gram of casein was necessary (Figure 5.11).

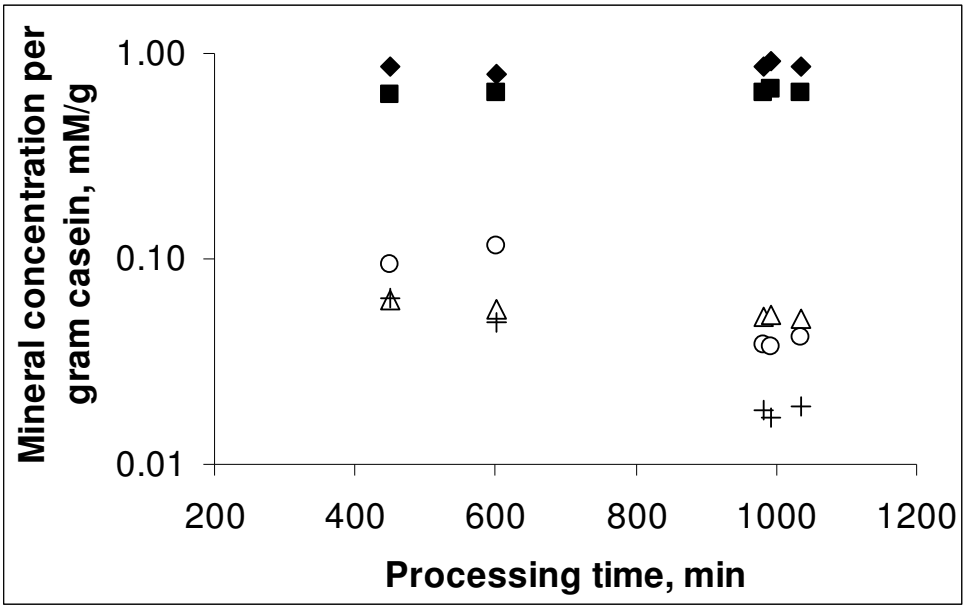

Figure 5.11 The mineral content as a function of casein content for calcium (♦), phosphorus (■), magnesium (Δ), potassium (○) and sodium (+), plotted vs. processing time at 50ºC.

The different minerals were affected differently by processing time. The highly diffusible potassium and sodium decreased significantly and linearly as a function of processing time (R^2=0.83 and 0.99, respectively), because longer processing times led to a more significant removal of these minerals from the MCCs. The calcium load was significantly affected by processing time, higher concentrations being recorded after longer processing times. The increased calcium concentration per gram of casein as a result of longer processing times can be explained by precipitation of calcium phosphate on the surface of the casein micelles. The increased calcium load in the MCCs due to longer processing time may be critical in explaining the extensive aggregation during the UHT treatment. The significantly lower calcium load values

for the MCCs produced in the preliminary trials may explain why destabilization was not observed during the UHT treatment of those casein concentrates.

An exception from the trend of increasing calcium load in the MCC with increasing processing time was observed for the 3rd replication which, despite being characterized by the longest processing time, had a lower calcium load than the other two replications. The calcium loads were: 0.87 mM/g casein for the 1st replicate, 0.92 mM/g casein for the 2nd replicate and 0.86 mM/g casein for the 3rd replicate. The reason for this behavior could be the lower pH values during the last two MF stages in the 3rd replication as compared to the previous two replications (Table 5.5). The lower pH may have promoted some solubilization of micellar calcium during the last 2 MF stages. Migration of calcium out of the micelle on one hand lowered the calcium load of the MCC from the 3rd replicate but also, more importantly, destabilized the casein micelles in this casein retentate. This may have rendered the MCCs from the 3rd replication more prone to destabilization during the subsequent heat treatment.

Table 5.5 pH values measured in the intermediate stages of the MCC manufacturing process.

Experimental replication / **Material**	**1st replication**	**2nd replication**	**3rd replication**
MF stage 1 retentate	6.67	6.71	6.71
MF stage 2 retentate	6.86	6.87	6.86
MF stage 3 retentate	6.95	7.00	6.73
MF stage 4 retentate	6.81	6.83	6.69

Proposed mechanisms of aggregation in the heat treated MCCs

Sterilization treatments have led to redistribution of minerals (Ca, P and Mg) between the soluble phase and the insoluble phase. Different forms of insoluble calcium phosphate most likely formed as a result of the two different sterilization processes, due to the different heat treatment temperatures (O'Connell and Fox, 2003). The different Ca/P molar ratios in the soluble phase probably correspond to different insoluble forms of $Ca_x(PO_4)_y$ formed in the MCCs subjected to the two types of sterilization treatments.

It has been proven before that when milk is heated above 120ºC, micellar casein stability decreases due to the dissociation of micellar κ-casein (Singh and Creamer, 1991; Singh and Latham, 1993). The κ-casein-depleted micelles become more sensitive to calcium and calcium phosphate, and heat induced aggregation is essentially induced by calcium bridging (Singh, 2004). Another important fact is that significantly more κ-casein dissociation was observed to occur from casein micelles in milk heated at 140°C at pH of 6.9-7.1 than at lower pH values (Singh and Latham, 1993; Anema and Klostermeyer, 1997). In this study, all MCCs had pH >7.0. Therefore, it can be assumed that extensive κ-casein dissociation from the micelles occurred in case of the UHT treatments, as a result of the high temperature. This resulted in κ-casein-depleted, highly unstable micelles. The heat induced precipitation of calcium phosphate led to significant aggregation in these MCCs (Figure 5.12a). An additional argument for the UHT destabilization mechanism proposed above can be made by examining closely the effect of UHT treatments on the R-MCCs: on average, the UHT-treated R-MCCs were characterized by an apparent viscosity that was twice higher than values recorded for their UHT-treated MCC counterparts.

During retorting of the MCCs, due to the lower treatment temperature as compared to the UHT treatment, less κ-casein dissociation is expected to have

occurred. Additionally, less calcium phosphate became insoluble as a result of retorting as compared to the UHT treatment, possibly due to a lower reaction rate. Consequently, only limited aggregation was induced in the retorted MCCs as compared to the UHT treatment, mainly via neutralization of negative charges at the surface of casein micelles (Figure 5.12b).

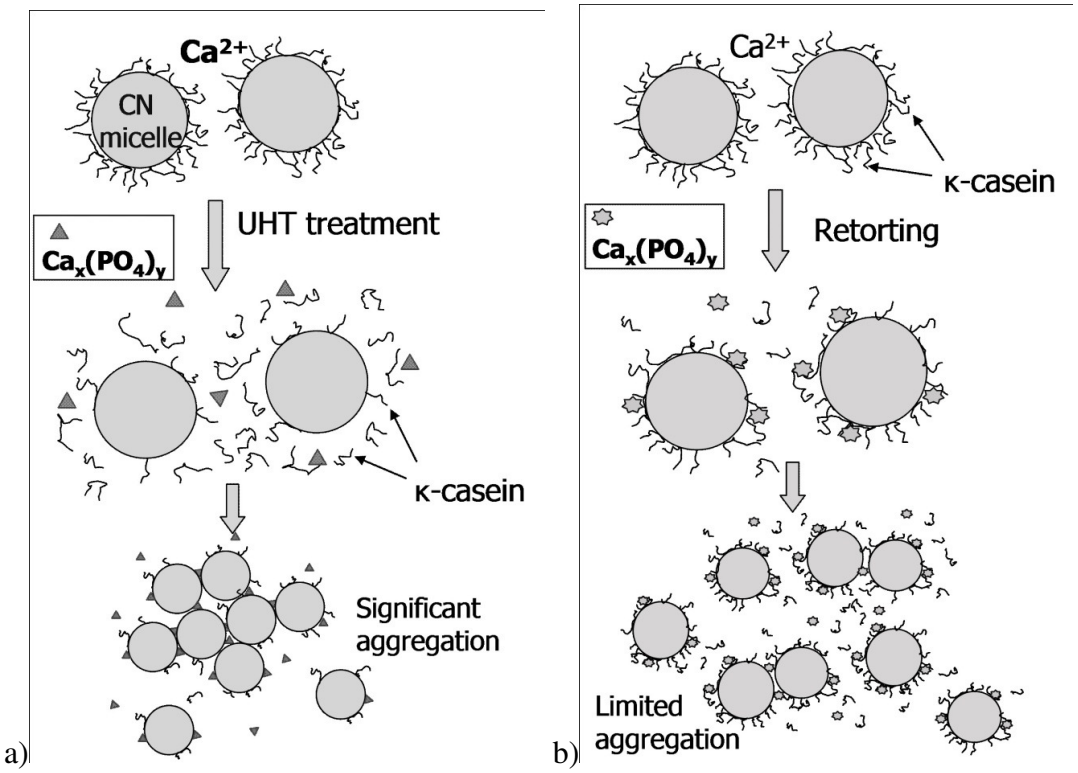

Figure 5.12 Proposed mechanisms of destabilization during a) UHT treatment; b) retorting of micellar casein concentrates.

Conclusions

Commercial sterilization affected the stability, viscosity and flow behavior of the micellar casein concentrates obtained by membrane separation. Most of the changes observed were related to the re-distribution of minerals (mainly calcium and phosphorus) at the micelle level as a result of heat treatments, more specifically the

loss in solubility of calcium phosphate. The calcium load (calcium concentration per gram of casein) was identified as a critical characteristic of micellar casein concentrates obtained by membrane separation, especially in the case of UHT sterilization. Additional mechanisms such as κ-casein dissociation may have a significant role in this behavior, however a separate study would be required to establish this as a fact.

Overall, the results of this study provide useful information about the heating behavior and physical properties of micellar casein concentrates obtained by membrane separation. Retorting of micellar casein concentrates seemed to result in a more stable product than UHT treatment. The drying and reconstitution of micellar casein concentrates resulted in products that were significantly more unstable when UHT treated than the concentrates that had not been subjected to drying prior to the heat treatment. The use of UHT treatments for sterilization of MCCs obtained by membrane separation in commercial applications may require additional stabilization.

Acknowledgments

The authors thank NY State Milk Promotion Board for funding of this research. The guidance and technical assistance of Dr. D.M. Barbano, Mark Newbold, Emily Hurt, Michael Adams and Cynthia L. Lopez-Pena from the Department of Food Science at Cornell University (Ithaca, NY), Isabel Doehner from Technische Universität Berlin, Tom Gibson from the Cornell University/NYSAES (Geneva, NY) and the Cornell Dairy Plant staff was greatly appreciated.

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CHAPTER 6

CONCLUSIONS

Casein micelles are significantly affected by environmental factors in complex ways. The medium in which casein micelles are dispersed is very important for their stability. The native structure of micelles can undergo changes in response to factors such as: variations in pH, temperature and ionic equilibrium of their environment; this may result in mineral redistribution, variation in degree of hydration, changes in the bonding forces that insure the casein micelle structure and, in some cases, dissociation and aggregation phenomena.

The first study set out to compare the effects of three relevant solvents on the casein micelle size. Its main deliverable was to identify a suitable solvent for measuring particle sizes of casein micelles using dynamic light scattering. DLS measurements require significant dilution of milk samples, and choice of solvent can affect the analysis results. These solvents were water, simulated milk ultrafiltrate (a lactose-free artificial milk serum) and UF permeate (obtained by membrane separation from pasteurized milk). Water was not a proper environment for suspending casein micelles, as it led to significant hydration and swelling of the micelles. Simulated milk ultrafiltrate was highly sensitive to temperature variations, exhibiting significant precipitation of amorphous calcium phosphate at 50ºC. The most suitable dispersing medium for casein micelles was UF permeate, which has a very close chemical composition to that of the milk serum.

The use of UF permeate allowed the investigation of the effect of temperature on the casein micelle size. The casein micelles decreased in size as temperature increased, due to temperature-dependence of hydrophobic interactions, which play a major role in casein micelle structure. For example, the measured micelle size at 50ºC

was significantly lower than at 20ºC. At 6ºC, the hydrophobic bonds became weaker, causing the micelle structure to become looser and more porous, which was reflected in an increased size.

These well-documented effects of temperature on casein micelle structure served as a validation step for the choice of UF permeate as an ideal solvent for a DLS protocol for measuring particle size of casein micelles.

Casein can be used in food applications in form of several ingredients. Recent developments in membrane filtration allowed the large scale production of micellar casein concentrates, in which casein is closer to its native state than in all other casein ingredients.

One of the possible uses of micellar casein is the manufacture of dairy-soy blends. Soy proteins are well known for their health and functional benefits, but the sensory characteristics of soy based products are not acceptable to many consumers. One way to enhance the acceptability of soy proteins could be to incorporate them in dairy products. While the interactions between soy proteins and milk serum proteins have been previously studied, the interactions between soy proteins and casein have not yet been investigated.

One of the objectives of this work was to evaluate the effect of heat treatment on the properties of micellar casein – soy protein blends, and the nature of the heat induced interactions between soy proteins and micellar casein.

Micellar casein (MCN)-soy protein (SP) mixtures (1:1 ratio) were characterized by complex behaviors, highly dependent on total protein content and heat treatment temperature. MCN-SP mixtures had a rheological behavior that was closer to that of soy proteins than of micellar casein. They behaved like a soy protein continuum, with casein micelles acting as filler. The structure and rheological

behavior of the mixtures was controlled by the degree of denaturation and concentration of soy proteins. Heat treating MCN-SP mixtures at temperatures above 80ºC led to partial denaturation and unfolding of soy protein (glycinin) molecules. In MCN-SP mixtures with a protein content lower than 12.5% (soy protein <6.3%), local phase separation occurred between the spherical casein micelles and the smaller, more flexible unfolded SP molecules. From a practical point of view, these results are particularly interesting since through controlled denaturation of soy proteins it would be possible to develop beverages with a high protein concentration, relatively low shear viscosity and almost Newtonian flow behavior.

At higher protein content (15%), mixtures treated at temperatures above 80ºC displayed protein aggregation and incipient network formation via protein-protein interactions, which were identified as mainly disulfide bonding. There was no evidence of cross-linking between soy proteins and casein micelles. The disulfide bonds seemed to occur exclusively between soy protein molecules.

The wide range of rheological characteristics displayed by the heat treated micellar casein – soy protein mixtures has potential practical applications in the development of food products with unique textural properties and a healthy image.

Significant insights into the effect of heat treatments on micellar casein systems were gained in the second study, in which the effects of commercial sterilization treatments on the physical properties of micellar casein concentrates of different casein concentrations were evaluated. The results of the study indicated that the mineral fraction of casein micelles is affected by heat treatments, which likely plays an important role in the stability of heat treated micellar casein concentrates.

Micellar casein concentrates (MCC) produced in the Cornell Food Science Pilot Plant were subjected to continuous-flow sterilization (UHT treatment) and batch

retorting, which were designed to achieve the same microbial inactivation effect. Sterilization affected the stability, viscosity and flow behavior of the micellar casein concentrates. Retorting of micellar casein concentrates seemed to result in a more stable product than UHT treatment. The effect of drying and reconstitution of micellar casein concentrates (R-MCCs) on the sterilization behavior was also studied. The R-MCCs were significantly more unstable to UHT sterilization than the MCCs.

Most of the observed changes in physical properties of the micellar casein concentrates were related to the re-distribution of minerals - mainly loss in solubility of calcium phosphate - at the micelle level as a result of heat treatments. Additional mechanisms such as κ-casein dissociation may also play a significant role in the changes induced by sterilization. However, more research is needed to establish this as a fact.

The calcium concentration per gram of casein (calcium load) was identified as a critical characteristic of micellar casein concentrates obtained by membrane separation; surpassing a critical calcium load may lead to lower heat stability, especially during UHT sterilization.

Micellar casein concentrates obtained by membrane separation are particularly attractive for shelf stable high protein beverages. The data generated in this study could be particularly useful in developing such applications. While retorting results in low viscosity, homogeneous MCCs, the use of continuous flow UHT sterilization for commercial applications may require additional stabilization.

CHAPTER 7

SUGGESTIONS FOR FUTURE WORK

Significant aggregation and structure formation was observed when micellar casein concentrates obtained by membrane separation were UHT treated. Even though the loss in solubility of calcium phosphate as a result of sterilization was documented and it was considered to play an important role in casein micelles' decreased stability, a clear understanding of the forms of calcium phosphate that occur as a result of sterilization was not achieved. A clear characterization of these insoluble forms of calcium phosphate is necessary.

An important role in the UHT-destabilization mechanism may have been played by κ-casein dissociation. A new study that would quantify the κ-casein fraction in the serum phase after the UHT treatment would be necessary in order to clearly establish the role of κ-casein dissociation within the mechanism of UHT destabilization for casein micelles.

Another important parameter that may have impacted the low UHT treatment stability of MCCs was the pH level, which was shown to be an important factor for the extent of κ-casein dissociation in UHT-treated skim milk. Understanding the effect of decreasing the pH of micellar casein concentrates to the levels of pH in milk (pH=6.7) before the UHT treatments would benefit greatly the understanding of the UHT destabilization mechanism. This could prove to be an accessible means of improving UHT stability of MCCs. Additional stabilization mechanisms for MCCs which will be UHT treated could be using stabilizers such as gums or exo-polysaccharides.

Another possible stabilization method for the UHT treatment of MCCs could be based on the work presented in Chapter 4 of this dissertation. It was shown that the heat treatment of micellar casein-soy protein mixtures (1:1 ratio) at temperatures

above the denaturation point of glycinin (90-95ºC) induced aggregation and gelling via disulfide bonding only when soy protein content exceeded a critical concentration (6.6% SP). Even though glycinin contains at least 2 free sulfhydryl groups per subunit, and κ-casein contains also 2 free SH groups per molecule, there was no evidence of cross-linking between soy proteins and casein micelles. The disulfide bonds seemed to occur exclusively between soy protein molecules.

However, the UHT treatment of micellar casein concentrates may induce significant κ-casein dissociation. This may improve the accessibility of κ-casein's free sulfhydryl groups and allow disulfide bonds to occur with glycinin molecules. New protein complexes may form which may lead to improved stability of UHT treated micellar casein concentrates obtained by membrane separation.