Colloidal and Rennet Coagulation Properties of Concentrated Casein Micelles with Altered Mineral Equilibrium

by

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ABSTRACT

EFFECT OF CHANGES OF MINERALS ON THE PHYSICO-CHEMICAL PROPERTIES OF CASEIN MICELLES IN CONCENTRATED MILK

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The changes in mineral equilibrium in milk affect the structure of casein micelles, and as a consequence, their processing functionality. In this thesis, the changes in the physico-chemical properties of casein micelles were measured after NaCl addition or pH-modification as a function of concentration. The addition of NaCl led to the dissolution of colloidal calcium phosphate and the decrease of the surface charge. The light scattering properties were also affected as a function of concentration. The addition NaCl retarded rennet induced gelation, indicating a change in the surface properties of the micelles, and the inhibition was less evident at high casein volume fractions.

The changes of the serum composition, rheological and the light scattering properties of the micelles were also studied as a function of pH before concentration. The pH modified concentrates were also studied for their rennet-induced coagulation behaviour. Acidification leads to the release of colloidal calcium phosphate, and a decrease of radius. After re-equilibration, casein dissociation occurs, especially for samples previously adjusted to pH<6.0. It was also determined that the re-equilibration temperature has no influence on the dissociation of the caseins. After concentration, more
caseins are released to the serum phase for the samples with lower pH, especially at protein concentration >6%. The voluminosity of casein micelles decreased with reduced pH, and a maximum $\phi_{\text{max}}$ value of 0.9 was found at pH 6.0. Acidification significantly decreased rennet gelation and a slight increase in the gelation time was found after concentration. The mixture of these modified and concentrated casein micelles with raw milk facilitated the gelation process and the elastic modulus of the final gels was much higher. However, when the mixtures were equilibrated against raw milk, no gelation occurred, due to the loss of colloidal calcium and micellar dissociation in the serum phase. These results clearly demonstrate that processing history of milk strongly affects the structure and the functionality of casein micelles. This project helps us better understand the changes of structure-function of casein micelles and the role of minerals during the processing.
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Values are means of two separate experiments. Values with a different superscript letter are significantly different (p<0.05).
LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

CCP: Colloidal calcium phosphate

CMP: Caseinmacropeptide

CN: Casein

DLS: Dynamic Light Scattering

DWS: Diffusing Wave Spectroscopy

GDL: Glucono-δ-lactone

HPLC: High Performance Liquid Chromatography

IC: Ion Chromatography

MSD: Mean squared Displacement

OS: Osmotic stressing

PEG: Polyethylene glycol

RCT: Rennet Coagulation Time

SDS-PAGE: Sodium Dodecyl Sulfate-Poly Acrylamide Electrophoresis

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid

UF: Ultrafiltration
CHAPTER 1

GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Bovine milk contains around 32 g/L of protein, 80% of which are caseins. They exist mainly in a supramolecular assembly composed of four types of caseins: \( \alpha_{S1}, \alpha_{S2}, \beta \) and \( \kappa \)-casein, in an approximate 4:1:3.5:1.5 ratio. Caseins are a family of calcium binding phosphoproteins. In native conditions, they can combine with calcium phosphate, assemble into a stable, highly hydrated and polydispersed structure called casein micelles (Dalgleish and Corredig 2012). In bovine milk, casein micelles are polydisperse in size with an average diameter of about 150 nm and contain calcium phosphate bridging between the proteins (De Kruif 1999). The structure of the casein micelles has not been fully elucidated, especially in regards to the changes occurring during processing. In a matter of fact, the structure of casein micelles is very dynamic as the proteins adapt their structures to changes in environmental conditions. Thus, the structure of casein micelles, and, as a consequence, the reactivity and processing behaviour can be modified by chemical, biochemical, or physical methods.

The understanding of the structure of casein micelles and the changes during processing is very important as they are critical to many processing operations, such as cheese manufacture and stability of reconstituted milk. However, the internal structure of casein micelles is still debatable and holds many challenges to fully explain the changes in the structure during processing, especially after the process of concentration. Models for the structure of casein micelles have been proposed over the years. Among all these models, a modified nanocluster model seems to accommodate all the behaviors of casein
micelles in response to renneting or acid induced gelation, changes in pH, temperature, or concentration (Holt et al. 2003; Bouchoux et al. 2010; Dalgleish 2011; De Kruif et al. 2012). In this model, phosphorylated caseins, including the αs- and β-caseins, can interact with the calcium phosphate nanoclusters via the phosphate centers (Holt 1998; Holt 2004) and grow into the micellar particles. κ-casein doesn’t have phosphate centers, it predominately presents on the surface of casein micelles, playing a size limiting role (De Kruif and Zhulina 1996).

Minerals play important roles in the structure of casein micelles, especially calcium and phosphate. They are present mainly in the form of colloidal calcium phosphate (CCP) and are linked to the serine residues of casein proteins (Dalgleish and Corredig 2012). Besides of CCP, calcium and phosphate are also present in the serum phase. This fraction is often referred to as soluble calcium and phosphate or diffusible calcium and phosphate, the latter being the ions not associated to the proteins in the serum (Zhao and Corredig 2015).

The changes in the mineral equilibrium between colloidal phase and serum/diffusible phase play a crucial role in the stability of casein micelles, which further influence the overall processing properties of milk (Gaucheron 2005). For example, addition of sodium chloride is reported to change the mineral equilibrium between the serum and colloidal phase, causing an increase of the non-sedimentable calcium and magnesium (Gaucheron et al. 2000). There are conflicting reports on the effect of NaCl on the non-sedimentable phosphate. While some researchers report that NaCl addition has little effect on the non-sedimentable phosphorus (Le Graët and Gaucheron 1999; Gaucheron et al. 2000; Huppertz and Fox 2006a), others have demonstrated that NaCl
significantly increased phosphorous concentration in ultrafiltrate of casein micelles dispersions (Famelart et al. 1999; Karlsson et al. 2007). The discrepancy may be due to the processing history of the milk. The addition of NaCl reduces the pH of milk (Grufferty and Fox 1985; Huppertz and Fox 2006a) and increases the hydration and voluminosity of casein micelles (Grufferty and Fox 1985; Van Hooydonk et al. 1986). The rennet gelation of casein micelles is retarded after the addition of NaCl and this is ascribed to the competition between Na\(^+\) and Ca\(^{2+}\) for the sites on the para-caseins (Grufferty and Fox 1985; Awad 2007). Little has been reported on the effect of NaCl addition on the properties of concentrated milk. Further research about the NaCl on the colloidal and gelation properties of concentrated milk is necessary, especially to understand the changes occurring during processing.

One of the most commonly encountered methods to affect the mineral equilibrium and the protein charges in dairy processing is the modification of pH. During acidification from pH 6.6 to 5.3, colloidal calcium phosphate, magnesium and citrate progressively dissociate from the interior of casein micelles (Jacob et al. 2011). The effect of loss of colloidal calcium phosphate nanoclusters on the structure of the micelles has been shown by SAXS (Marchin et al. 2007). At about pH 5.2, all the inorganic phosphate is solubilized and most of the remaining calcium ions are solubilized when pH 4.6 is reached (Le Graët and Gaucheron 1999). The negative charges on the surface of casein micelles decrease and caseins dissociate from the casein micelles. It has been reported that casein dissociation is temperature-dependent, whereas mineral solubilisation is similar at 20 or 30°C (Dalgleish and Law 1989). In skim milk, at low temperatures (4°C), a significant quantity of casein dissociates from the micelles even at the natural pH.
of the milk (about pH 6.7), while at 30°C, only limited dissociation occurs even when the pH is reduced (Rose 1968; Law and Leaver 1998). Casein and mineral dissociation as a function of concentration and temperature has yet to be reported. On the other hand, alkalization has been reported to lead to partial dissociation of casein micelles and precipitation of calcium phosphate onto casein micelles, and these changes seem to be only partly reversible upon re-equilibration (Vaia et al. 2006). Nevertheless, whether or how these changes can influence the gel formation is still uncertain. In addition, it is still unclear whether these changes of casein micelle structure are reversible once the pH of milk is recovered.

In recent years, scientists have increased their interest in the changes occurring to the structure and physical properties of casein micelles in concentrated milk prepared using membrane filtration (i.e. ultrafiltration) (Karlsson et al. 2005; Karlsson et al. 2007; Li and Corredig 2014), evaporation (Védez-Ruiz and Barbosa-Cánovas 1997; Liu et al. 2012) or powder reconstitution (Alexander et al. 2002; Dahbi et al. 2010). With an increase of casein concentration, the average distance between casein micelles decreases and the micelles tend to interact with each other more frequently. The viscosity of milk increases with concentration in a non-linear fashion, with a change from Newtonian to non-Newtonian behaviour at a volume fraction of 0.4 (Nair et al. 2014). Recent research confirmed these results, using osmotic stressing techniques to concentrate the milk (Bouchoux et al. 2009; Nair et al. 2014). Both light scattering (Dahbi et al. 2010) and rheology (Nair et al. 2014) data on casein micelles behaviour with concentration can be well modeled after the behaviour of colloidal hard-spheres. No changes in the average size of casein micelles can be found up to 20% protein concentration, and the viscosity
behaviour as a function of concentration fits well with the Mendoza model, which takes into account the hydrodynamic interactions between the colloidal particles (Mendoza and Santamaría-Holek 2009). It has also been reported that modified casein micelles heated at different pH values (6.4, 6.7, 7.0), show relative viscosities that can be modeled after hard-sphere theory using the Mendoza equation, assuming no changes in voluminosity (Nair et al. 2013). However, whether pH-modified casein micelles will have similar behaviour with concentration is still unknown. Further research is necessary on pH-modified casein micelles, as this will help better understand the influence of processing history on the structure-function of casein micelles, especially during concentration.

It is known that the κ-casein presents on the surface of casein micelles and causes the micelle to be stable against aggregation. Once the rennet is added, it will specifically hydrolyze the κ-casein at the bond 105-106 and liberate the C-terminal macropeptide (De Kruif 1992). When at least 85%-90% of the casein-macropeptide is released into solution, the aggregation of casein micelles happens and the rennet gel forms (Dalgleish 1979a; Dalgleish 1979b; Sandra et al. 2007). The rennet coagulation of milk is a very important aspect in the dairy industry and has been intensively investigated over many years. It is generally recognized that the hydrolysis of κ-casein leads to a decrease in the density of hairy layer and hence to a decrease of steric stabilization (Mellema et al. 1999). One interesting finding is that concentrated milk showed a similar coagulation time to unconcentrated milk (Dalgleish 1979a; Sandra et al. 2011).

Pre-acidification of milk has been shown to have a significant influence on the rennet coagulation properties. A mild pH reduction (to pH 6.3) can improve the rennet coagulation properties of milk, and this effect is reinforced when milk acidification
occurs at lower temperature (25°C instead of 35°C) (Lucey et al. 1996; Law and Leaver 1998; Renault et al. 2000). Acidification of milk, followed by neutralization, results in improved rennet properties, probably due to an elevated Ca²⁺ activity in the serum (Lucey et al. 1996). However, the ionic equilibrium and the role played by the changes in composition of the soluble fraction (especially with the presence of soluble caseins) are not fully understood. Hence, further investigations on the effect of partial acidification on casein micelle structure, and above all as a function of concentration on the formation of gels are needed.

In the present research, two methods were adopted to affect ionic equilibrium in milk: addition of NaCl or acidification. The effect of NaCl on the properties of concentrated milk was investigated first. Two different ways were used to concentrate the milk, osmotic stressing (chapter 3) and ultrafiltration (chapter 4). Compared to ultrafiltration, a membrane separation method, osmotic stressing can be considered non-invasive, as shear or mixing forces are not applied. This technique has been successfully employed to concentrate the milk protein in previous research (Farrer and Lips 1999; Bouchoux et al. 2009a; Bouchoux et al. 2009b; Famelart et al. 2009). After concentration, the effect of NaCl addition on the composition of the serum phase, the ionic equilibrium, and rennet coagulation behaviour of the concentrated milk were investigated.

Osmotic stressing was employed to concentrate the pH-adjusted milk, while maintaining the serum composition as close as possible to that of original samples. In this part of the thesis, the milk was pre-acidified and then concentrated. The colloidal behaviour and rennet gelation properties of the suspensions were then investigated. As previous reports have suggested that casein dissociation is temperature-dependent
(Dalgleish and Law 1988), so in chapter 5, a preliminary study was conducted to test the hypothesis that re-equilibration by dialysis at different temperatures may affect the dissociation behaviour of casein micelles with concentration. In chapter 6, the pH-modified milk was then concentrated to different concentrations using osmotic stressing and then the colloidal properties of the concentrated samples were studied, using rheology and light scattering. Finally in chapter 7, the rennet gelation of concentrated samples was compared with those of unconcentrated samples. To carry out a well-controlled experiment, 3x concentrated samples were obtained, and added back to skim milk at a ratio of 1:1 and the effect of pH-modified casein micelles on the rennet properties of raw milk was investigated. As differences in coagulation can be attributed to differences in the serum phase, all samples were also dialyzed against original milk, in an attempt to have similar serum concentrations.

The overall objective of this research was to extend our understanding about the changes in the structure-function of casein micelles as a function of modification to the mineral equilibrium. By a better understanding of the physico-chemical changes occurring to the casein micelles with changes in mineral equilibrium and processing, it will be possible to better predict their behaviour during dairy processing, when concentrated milk is modified and used as an ingredient. The work was divided in the following separate objectives:

1. Investigate the effect of NaCl addition on the physicochemical properties of untreated milk and concentrated milk using Osmotic stressing.
2. Study the changes in the colloidal and rennet coagulation behavior of casein micelles as a function of NaCl addition in concentrated milk by ultrafiltration.
3. Investigate the influence of dialysis temperature (4 or 22 °C) on the dissociation behavior of pH-modified casein micelles during the re-equilibration process.

4. Characterizing the effect of partial acidification on the physicochemical behavior and particle dynamics of casein micelles as a function of concentration.

5. Investigate the rennet gelation behavior of casein micelles as a function of pH-modification and concentration and understanding how will these acidified casein micelles influence the coagulation behavior of raw milk when used as food ingredient
CHAPTER 2
LITERATURE REVIEW AND METHODOLOGY

The proteins of bovine milk have been studied intensively since the end of the 19th Century, when Mulder, a Dutch chemist, devised the first acid precipitation methods for separation of casein. Since then, increased interest has been devoted to the composition and functionality of milk proteins. Bovine milk contains about 3.2 g proteins per 100 ml. The proteins are distinguished into two major groups according to their solubility at pH 4.6 at >8℃ (Fox 1992). The caseins are the major proteins in milk, constituting about 80% of total, and they are insoluble at their isoelectric point (pH 4.6), and are present as colloidal particles. On the other hand, about 20% remain soluble at this pH in the serum or whey, about 15% being whey proteins with the remainder being non-protein nitrogen (Fox 1992).

Caseins are calcium binding phosphoproteins. In milk, they occur as larger aggregates, referred to as casein micelles, which can be separated from the whey protein using ultracentrifugation (Fox and Mcsweeney 2003). Casein micelles are heat resistant and can form gels by acidification or using a specific enzyme (chymosin). Caseins have important nutritional functions, including being a significant transporter of calcium and phosphate. The casein micelles can be regarded as being composed of rheomorphic proteins with a polydisperse size range, from 60 to 500 nm in diameter (De Kruif 1998). The dry matter of casein micelles contains about 94% proteins, including α_{S1}, α_{S2}, β and κ-casein, 6% salts, and low molecular weight components, with the majority being referred to as colloidal calcium phosphate, consisting mainly of calcium and phosphate with small amount of magnesium and citrate and trace amounts of other species.
The composition of micelles affects their size. Previous studies have shown that smaller micelles are relatively rich in κ-casein and relatively depleted in β-casein, while the contents of αs caseins are independent of size (Dalgleish and Law 1989; Marchin et al. 2007). Casein micelles are highly hydrated, holding about 4 g H₂O per g of protein (Walstra 1979). They occupy 10% of the total volume fraction of milk, which indicate that they are very close to each other (Tuinier and De Kruif 2002).

Over the last 50 years, several models about the structure of casein micelles have been proposed and several reviews have been published, giving somewhat divergent views (see for example, Garnier and Ribadeau Dumas 1970; Farrell 1973; Slattery 1976; Horne 2002; Holt et al. 2003; Phoebe 2007; Horne 2009; Dalgleish 2011; Dalgleish and Corredig 2012; Holt 2013). All these models interpret the casein micelle behavior under different physicochemical conditions with the existing experimental evidence. However, there is still some disagreement on the stability and integrity of the casein micelles under complex processing conditions. It is generally recognized that the κ-caseins are present on the surface of casein micelles, limiting the growth of casein micelles and providing steric and electrostatic repulsion (Dalgleish and Law 1988; De Kruif and Zhulina 1996; De Kruif 1999).

2.1 The structure of casein micelles

A model of the structure of casein micelles has gradually developed in the last 50 years. From the relatively simple coat-core models of αs- and β-caseins surrounded by a layer of κ-casein (Nöbel and Waugh 1965a; Nöbel and Waugh 1965b) to two more advanced models, namely the submicellar and the nanocluster models. The submicellar
model was derived from the observation that proteins in sodium caseinate form small aggregates via non-covalent interactions when they are dispersed in aqueous media (Waugh et al. 1970). In this model, the internal structure of the casein micelle was viewed as uniform, and composed by subunits having similar composition. It was proposed that the polar regions of $\alpha_{\text{s1}}$, $\alpha_{\text{s2}}$ and $\beta$-caseins are oriented toward the outside of the submicelles to reduce electrostatic repulsion between neighboring charged groups. These subunits, also often referred to as submicelles, are held together by calcium phosphate bridges between serine residues. However, within the complex, $\kappa$-casein plays a size limiting role, as its concentration is linked to the average size of the casein micelles.

The presence of $\kappa$-casein on the surface of the micelles also explains rennet-induced casein micelles aggregation. Once the structural role of $\kappa$-casein became clear, a new internal structure was developed, highlighting that two types of submicelles needed to be present within a micelle (Slattery and Evard 1973): $\kappa$-casein rich and $\kappa$-casein poor submicelles, hence explaining the presence of this protein mainly on the surface of the protein particles. But this model falls short in the lack of experimental evidence for the existence of 2 different submicelles; moreover, the cooling induced $\beta$-casein dissociation can also not be well explained. More recent studies have suggested that the internal structure of the micelle presents more or less hydrated regions (Dalgleish and Corredig 2012). In addition, microstructural studies highlighted heterogeneities at the surface of the micelles, with tubular structures of 10-20 nm protruding in the serum phase (McMahon and McManus 1998; Dalgleish et al. 2004; Trinh et al. 2007).

The nanocluster model derives from the idea that one of the main biological functions of caseins is the control of calcium precipitation in the mammary gland and the
delivery of calcium phosphate to the neonate (Holt 2013). The phosphopeptides derived from β-casein are capable of stabilizing calcium phosphate in solution at concentrations above saturation, indicating that the phosphopeptides are able to bind to the small domains of calcium phosphate, termed nanoclusters, present within the micelles in form of clusters of a few nanometers in size (Little and Holt 2004).

The most recent nanocluster model of the casein micelle is based on the fitting of x-ray and neutron scattering data for a micelle of radius 108 nm, with a mass of 7.2 x 10^8 Da and 830 clusters, with a mean spacing of 18 nm, acting as scattering points (Holt et al. 2003; De Kruif et al. 2012). The phosphoserine residues of the casein proteins surround the nanoclusters, forming an outer layer of phosphate groups. The interactions between these amorphous nanoclusters and the proteins cement the inner structure of the casein micelles. The main limitation of this model is the role played by β-casein and water on causing the micelles to have high voluminosity values (>3.5 mL/g) (Nöbel et al. 2012; Dalgleish and Corredig, 2012). In addition, this model has been criticized for overlooking the important role played by the hydrophobic interactions in determining the self-assembly of the caseins within the micelles (Horne 2006).

The dual binding model (Horne 1998; Horne 2002; Horne 2009) focuses on the molecular interactions causing the caseins to self-assemble in a micellar structure, namely the associations through hydrophobic protein moieties and the bridges formed by calcium phosphate nanoclusters with serine amino acids. In this model, casein micelle integrity is explained by the equilibrium between attractive and repulsive forces. Furthermore, the dual binding model also describes clearly the presence of κ-casein on the surface of the casein micelles, as it functions as a chain terminator of the growth during the micelle

Although these proposed models interpret the existing experimental evidence within the context of the casein micelle behaviour under different environment conditions, they still are limited at explaining in detail the changes occurring to the structure of casein micelles during processing. First of all, none of these models explains the peculiar role of β-casein in the structure, which is released upon cooling, and its reversible integration in the structure upon rewarming (Creamer et al. 1977). Second, all the models do not describe the role played by the high amount of water present in the interior of casein micelles. It is known that the hydrophobic moieties of the caseins are important in caseins self-assembly; but the role of hydrogen bonding and the location of the water molecules has yet to be described in detail. It has been suggested that the casein micelles are to some extent porous, as β-casein and other large molecules can penetrate the micelles (Creamer et al. 1977; Colsenet et al. 2005; Le Feunteun and Mariette 2007).

A recent model (Dalgleish 2011) suggested that the internal structure of casein micelles is composed of clusters of casein proteins linked by colloidal calcium phosphate via phosphoserine centers and the interior of the assembly is heterogeneous, containing more dense and less dense protein regions described as “water channels” (McMahon and McManus 1998; Dalgleish 2011). The presence of these water channels provides pathways through which small molecules can diffuse and these water channels can be stabilized by β-casein interacting with the hydrophobic portions of the nanoclusters. This model is further supported by the recent interpretation of SAXS data (Bouchoux et al. 2010), claiming that the micelle contains hard regions of protein/calcium phosphate within a highly hydrated sponge-like structure. This modified nanocluster model explains
well the high voluminosity of casein micelles and the role of the β-casein within the micelles. The model also explains the behavior of casein micelles during processes such as renneting, changes in temperature, pH, ethanol and urea addition, removal of calcium phosphate by chelating molecules and concentration.

2.2 Effect of NaCl on the structure of casein micelles

NaCl is often added to modify milk protein functionality in milk, namely, solubility, gelation or colloidal stability. For example, in the production of milk protein concentrate (MPC), the addition of NaCl during diafiltration improves their solubility (Mao et al. 2012; Sikand et al. 2013). The addition of NaCl changes the mineral equilibrium between the serum and colloidal phase of milk due to the exchange between Na$^+$ and Ca$^{2+}$. The level of non sedimentable calcium and magnesium increases significantly after the addition of NaCl (Grufferty and Fox 1985; Gaucheron et al. 2000). The release of colloidal calcium phosphate loosens the internal structure of casein micelles, leading to the increase of non sedimentable proteins (Mao et al. 2012). In addition, the pH of milk decreases (Grufferty and Fox 1985; Huppertz and Fox 2006a) and the hydration and voluminosity of casein micelles increases (Grufferty and Fox 1985; Van Hooydonk, et al. 1986a). The negative charges on the surface of casein micelles decrease after the addition of NaCl, with no changes in the size (Huppertz and Fox 2006a). The addition of NaCl affects the processing functionality. The maximum heat coagulation time increases and shifts to a higher pH value after adding 50-100 mmol.L$^{-1}$ NaCl for both unconcentrated and 2x concentrated milk (Huppertz and Fox 2006a;
Huppertz and Fox 2006b). However, there is a lack of data regarding the effect of NaCl on the properties of casein micelles, especially in concentrated milk.

2.3 Effect of acidification on the structure of casein micelles

Acidified milk products are one of the oldest and most popular foods among the world. A wide variety of acidified milk products are produced, such as yogurt, acid milk drinks or fresh acid cheese. Generally, milk can be acidified by lactic acid bacteria, which ferment lactose to lactic acid, the direct addition of acids, such as HCl, or by the use of glucono-delta-lactone (GDL), which can be hydrolysed in solution to gluconic acid. During acidification many of the physico-chemical properties of the casein micelles undergo considerable changes, while the colloidal calcium phosphate gradually dissociates from casein micelles (Jacob et al. 2011), the net charge of casein micelles decreases and caseins are released in the serum phase (Renault et al. 2000). Casein dissociation is temperature-dependent, whereas mineral solubilisation is similar at 20 or 30°C (Dalgleish and Law 1988; Dalgleish and Law 1989). The extent of mineral solubilisation increases markedly below pH 5.6 and is almost complete at around pH 5.0. All of the inorganic phosphate is solubilised at pH 5.2, and most of the remaining calcium ions are solubilised when pH 4.6 is reached (Le Graët and Gaucheron 1999). At low temperatures (4°C), a significant quantity of casein separates from micelles even at the natural pH of the milk (about pH 6.7), while at 30°C, only a limited dissociation occurs even when the pH is reduced (Rose 1968; Law and Leaver 1998). It is unknown if such dissociation is reversible. In a recent study, acidification was followed by neutralization, using dialysis of the partly acidified milk with milk ultrafiltrate. It was shown that
acidification, followed by neutralization (re-equilibration) causes demineralization and disruption of casein micelles. All the re-equilibration treatments (dialysis) were conducted at 4°C, and all the samples were freeze dried and reconstituted (Silva et al. 2013). As casein dissociation is temperature-dependent, the dialysis at cold temperatures may have an influence on the dissociation behaviour of casein micelles. On the surface of micelles, the decrease in the κ-casein’s charge results in the collapse of the κ-casein layer and a decrease in the stability of the micelles, as the intra- and inter-chain interactions are no longer sufficient to keep the protein fraction extended in solution (De Kruif 1999). As a result, the micelles can diffuse closer to each other, and aggregation occurs, leading to a macroscopic sol-gel transition. In unheated milk, the aggregation occurs very close to the isoelectric point of caseins, at a pH of around 4.8 (Cassandra Rodriguez and Dalgleish 2006).

When the milk is heated at a temperature above 70°C, the whey proteins denature and associate with casein micelles through hydrophobic interactions and intermolecular disulphide bonds. The pH at which the milk is heated has considerable effects on the distribution of the whey protein complexes between the surface of casein micelles and serum. Heating at an acidic pH results in the attachment of most whey proteins to the surface of casein micelles; on the other hand, heating at an alkaline pH results in the formation of colloidally stable whey protein aggregates in the serum (Vasbinder and De Kruif 2003). The changes in the average hydrodynamic radius of casein micelles when heated at different pH are due to the association of the denatured whey proteins with the casein micelles (Anema and Li 2003). Heat treatment improves the texture of acid induced gels, the gels made from heated milk have a higher pH at gelation and are
considerably firmer than those from unheated milk (Lucey et al. 1997; Lucey et al. 1998). Upon heating, the denatured whey proteins associate with the casein micelles appearing as appendages or filaments on the surface of casein micelle in electron micrographs (Davies et al. 1978; Mottar et al. 1989). When acidified, the denatured whey proteins associated with the caseins tend to aggregate, as the repulsive charge on the surface of protein is decreased. Heated milk has higher gelation pH, about 5.3, the isoelectric pH of the major whey proteins (Kinsella and Whitehead 1989). Cross-linking of aggregating casein micelles occurs via denatured whey proteins associated with casein micelles. These soluble heat induced aggregates of denatured whey proteins associated with caseins act as bridging material in the protein gel. This increase in the number and strength of bonds between particles causes the formation of gels with a higher elastic modulus (Lucey et al. 1997; Lucey et al. 1998).

On the other hand, an increase of milk pH to about 7.5, has been reported to lead to partial dissociation of casein micelles and precipitation of calcium phosphate onto casein micelles, and these changes seem to be only partly reversible upon re-equilibration (Vaia et al. 2006).

2.4 Casein micelles and concentration

2.4.1 Concentration methods

In the dairy industry, concentration of milk is a very important unit operation. Many different technologies have been employed to concentrate the casein micelles, such as evaporation and membrane technology. During evaporation water is removed preferentially from the serum and all the components remain in final product. Heating
leads to interactions between whey proteins and micelles, and there is an increase in the amount of colloidal calcium and phosphate recovered (Le Graët and Brulé 1982; Liu et al. 2012). The equilibrium between the mineral ions and the proteins changes and the functionality of casein micelles is altered.

Unlike evaporation, membrane concentration can selectively retain different components of the feed depending on the size and selectivity of the membrane without modification to the serum composition. The most frequently used membrane processes in the dairy industry are microfiltration, ultrafiltration, diafiltration, nanofiltration and reverse osmosis. It is understood that only small changes occur to the micelles during membrane concentration and the amount of colloidal calcium phosphate remains constant unless there is an extensive addition of water (during diafiltration) or the pH changes (Singh 2007; Ferrer et al. 2011).

In cross flow filtration the feed flows tangentially to the surface of the membrane, preventing the solids from residing on the membrane. During this process the hydrodynamic radius of casein micelles does not change greatly (Singh 2007; Ferrer et al. 2011). However, a layer of compressed micelles is found at the surface of membrane during membrane filtration (Pignon et al. 2004; Jimenezlopez et al. 2008). It has been hypothesized that casein micelles may be irreversibly changed during membrane filtration, with consequences on functionality (Havea 2006; Ferrer et al. 2011).

As both evaporation and membrane filtration have irreversible influence on the properties of micelles, an alternative concentration method, osmotic stress, can also be used to obtain concentrated protein suspensions without temperature or shear applied. During the osmotic stress process, the water moves across a selectively permeable
membrane from an area with high water potential (low solute concentration) to an area with lower water potential (high solute concentration). The net pressure needed to prevent the net movement of solvent is defined as the osmotic stress. The osmotic pressure is a function of concentration and can be expressed by the following equation (Flory 1953).

\[ \pi = RT \left[ \frac{1}{M_n} c + \left( \frac{1}{2} - \kappa \right) \left( \frac{v_s^2}{V_s} \right) c^2 + \left( \frac{v_s^3}{3V_s} \right) c^3 \right] \]  

[Eq.2.1]

Where \( \pi \) is the osmotic stress, \( c \) is the concentration of the polymer, \( M_n \) is the average molecular weight, \( \kappa \) is the Flory- Huggins interaction parameter which is the strength of the interactions between the polymer and the solvent, \( v \) is the specific volume of the polymer, \( V_s \) is the molar volume of the solvent, \( R \) is the gas constant and \( T \) is absolute temperature.

Farrer and Lips (1999) first employed the method of osmotic stress to concentrate sodium caseinate dispersions, by equilibrium dialysis against dextran solutions of the known osmolarity. The concentrations that can be reached are higher than close packing concentrations. This method, using polyethylene glycol (PEG) as a stressing polymer and dissolved in the UF permeate, was further applied to maintain the chemical potential of all ions identical to their values in milk (Bouchoux et al. 2009a; Bouchoux et al. 2009b). The interactions in model dispersions of casein micelles over a wide range of casein concentrations have been studied using this method, reaching concentrations from 20 to 500 g. L\(^{-1}\). The study allowed the identification of three compression regimes in milk as a function of volume fraction of the casein micelles: (1) a dilute regime in which the casein
micelles are well separated and do not interact, (2) a transition regime in which the behaviour of the dispersion changes from liquid-like to solid-like as a result of stronger interactions between micelles, and (3) a concentrated regime in which the dispersions behave as condensed matter made of micelles that are in direct contact with their neighbours.

2.4.2 Concentration behaviour of casein micelles

During the concentration process, the water is removed; the particles become closer and interact more frequently with each other due to the increased volume fraction. The presence of κ-caseins on the surface provides steric and electrostatic repulsion and this keeps casein micelles away from each other maintaining colloidal stability (De Kruif and Zhulina 1996). Thus, the micelles cannot approach closely and still behave as hard spheres at normal concentrations of milk (Beenakker and Mazur 1984; De Kruif 1999; Alexander et al. 2002). Previous research showed that the viscosity of casein micelles increases with increasing concentration in a non-linear fashion, and a transition occurs from Newtonian fluid to non-Newtonian fluid at a volume fraction around 0.4 (Bouchoux et al. 2010; Nair et al. 2014). No changes in the average hydrodynamic radius were found when the concentrated milk was rediluted, indicating that there was no aggregation at high volume fraction (Nair et al. 2014). Moreover, both rheological and light scattering parameters as a function of concentration show that casein micelles behave as typical hard-sphere (Dahbi et al. 2010; Ferrer et al. 2011; Nair et al. 2014). However, at concentrations higher than a volume fraction of 0.65 (about 178 g.L\(^{-1}\) of protein), the system is structurally arrested and is not able to flow anymore (Dahbi et al. 2010). At these concentrations, the hairy layers overlaps and compress against each other. Thus, at
the critical concentrations, the elastic modulus increases rapidly and close packing occurs. Further compression results in the expulsion of the interior water and some micellar fusion might happen at this stage, possibly due to the distortion of the κ-casein layer. The interior parts of the micelles will then come into contact and interact with each other (Bouchoux et al. 2010; Dahbi et al. 2010). In this case, redilution will not result in casein micelles with normal properties. It is important to note that much of the available research results were obtained using reconstituted skim milk powder as material; although reconstituted casein micelles are quite close to their native state, there may be slight differences in their behaviour compared to native casein micelles.

To further investigate the concentration of casein micelles, Nair et al. (2014) investigated the concentration behaviour of raw skim milk, concentrating the casein suspensions using osmotic stress. It was found that the rheological behaviour of concentrated casein micelles suspensions fits well with the Mendoza model for solid sphere suspensions (Mendoza and Santamaría-Holek 2009), which takes into account the hydrodynamic interactions between colloidal particles, with a predicted critical packing volume fraction around 0.8, in agreement with previous research (Karlsson et al. 2005). The results obtained using diffusing wave spectroscopy (DWS) suggested that casein micelles behave as hard spheres with a characteristic free diffusing Brownian motion up to a volume fraction of 0.3, while show restricted motion at higher concentrations. The diffusivity of the casein micelles is consistent with that reported for hard-spheres of a similar size, up to a volume fraction of 0.45 (Nair et al. 2014).

The casein micelles were also modified by heating at different pH (6.4, 6.7 and 7.1) and then concentrated by osmotic stress. The rheological properties of milk
concentrated after heating did not seem to be very different between heated and unheated milk. Turbidity values of milk heated at 6.4 were higher than that of normal pH, and this difference was attributed to the association of denatured whey proteins with caseins on the surface of the micelles (Nair et al. 2013) However, it is unknown whether other mild modification of the structure of casein micelles, such as partial acidification, renneting, or cross-linking of the internal structure, may influence the hard sphere behaviours of casein micelles.

2.5 Rennet gelation

Milk coagulation is a key step in cheese making. It is estimated that for about 75% of all cheeses, the coagulation is achieved by adding a small amount of rennet or other milk clotting enzyme. Rennet is an extract from calf’s stomach and contains a specific enzyme, chymosin, which hydrolyzes κ-casein molecules at a specific position and releases the C-terminal region of the κ-casein [caseinomacropeptide (CMP)]. When rennet is added to the milk, after a lag phase, the casein micelles aggregate and the aggregation leads to the formation of a gel. Numerous changes occur during this process and it is convenient to divide this process into two overlapped stages (Dalgleish 1979a). The primary stage is the enzymatic cleavage of κ-casein. Once a sufficient amount of protein has been hydrolyzed, aggregation occurs. In untreated skim milk at native pH, the second stage of the reaction occurs when at least 85%-90% of the casein-macropeptide is released into solution (Sandra et al. 2007).
2.5.1 Principles of rennet gelation

It is generally recognized that the \( \kappa \)-casein is situated at the surface of casein micelles and the macropeptide portion protrude from the surface providing a steric layer (De Kruif and Zhulina 1996). The critical activity of chymosin is to hydrolyze the Phe_{105}-Met_{106} peptide bond, resulting into the formation of para-\( \kappa \)-casein (residues 1-105), which is hydrophobic, and caseinomacropeptide (CMP) (residues 106-169), which is very hydrophilic. The most common method to determine the extent of proteolysis has been to isolate and quantify CMP (Van Hooydonk and Olieman 1982).

During the primary stage of the reaction, the diffusion coefficient of the micelles increases due to the gradual removal of the CMP. The apparent radius of the casein micelles decreases initially by approximately 10 nm, before increasing once the CMP removal reaches > 80% (Sandra et al. 2007). It was also found that the milk viscosity decreases until about 85% of the \( \kappa \)-casein is hydrolyzed (Tuinier and De Kruif 2002). At this point, the steric repulsion provided by the remaining \( \kappa \)-casein is not enough to keep the micelles apart, they begin to aggregate with each other and eventually form a gel (Dalgleish 1979a; Kethireddipalli et al. 2010). Although the internal structure of casein micelles are not important in the initial stages of rennet aggregation, they become progressively more critical when interparticle rearrangements occur and the micelles begin to fuse (Dalgleish and Corredig 2012). A partial loss of colloidal calcium may reduce the charge interactions, resulting in the formation of a weaker, more flexible casein network (Choi et al. 2007).
2.5.2 Effect of NaCl on the rennet gelation

Addition of NaCl retards the rennet coagulation of milk, and decreases the gel firmness (Famelart et al. 1999; Sbodio et al. 2006; Awad 2007). Although it is reported that the NaCl was able to decrease the enzymatic rate greatly, the underlying mechanism has not been fully elucidated (Gatti and Pires 1994). The effect of NaCl on renneting is probably due to the increased ionic strength, which leads to the screening of negative charge on both enzyme and substrate and thus decreases the enzymatic rate (Dalgleish 1992). So far, few studies have been conducted on the influence of salt addition on concentrated milk and further research is necessary.

2.5.3 Effect of acidification and concentration on the rennet gelation

As the pH of the milk decreases, the negative charges provided by the κ-casein are reduced and this leads to the partial collapse of κ-casein (De Kruif 1999). So when rennet is added to milk at a lower pH, the gelation occurs earlier, at a lower degree of κ-casein breakdown (Van Hooydonk, et al. 1986). The rennet coagulation properties of milk significantly improve at pH 6.3, this effect is reinforced when milk acidification occurs at lower temperature (25°C instead of 35°C) (Law and Leaver 1998; Renault et al. 2000). Acidification of milk to low pH values, followed by neutralization, results in improved renneting properties and a reduction of rennet coagulation time, probably due to an elevated Ca$^{2+}$ activity in the serum (Lucey et al. 1996). However, further dialysis of this reformed milk against raw milk results in a reduction of renneting properties, possibly due to its reduced CCP content or structure changes in the micelles caused by removal of CCP. This research indicated that both Ca$^{2+}$ and CCP play important roles in
the formation of rennet gels (Lucey et al. 1996). A decrease in pH also improves the binding of chymosin to the casein micelles. It was shown that only about 31% of the rennet added to milk remains in the curd after renneting at pH 6.6 and this number increases to 86% when the pH of milk is 5.2 (Holmes et al. 1977; De Roos et al. 1995).

Dilution of milk with water increases the rennet coagulation time and decreases gel stiffness (Dalgleish 1980). On the other hand, concentrated milk samples form stiffer gels due to an increased number of bonds in the network (Waungana et al. 1999; Sandra et al., 2011). However, the effect of concentration on coagulation time is still under debate. Some researchers have reported that concentrated milk has a shorter coagulation time and higher gel firming rate than non-concentrated milk (Garnot and Christian 1980; Waungana et al. 1999) while others (Dalgleish 1980; Sandra et al. 2011) reported that concentration has no significant effect on the release of caseinomacropetide and coagulation time when same amount of rennet is added. Lucisano et al. (1985) determined the clotting time of UF concentrates for milk acidified to pH 6.5 and found a sharp reduction in clotting time as the protein concentration increased from 3 to 11% with a constant amount of rennet. However, other research found that adjustment of milk to pH 6.5 results an increase in gelation time with the increase in protein concentration (Waungana et al. 1999). In a recent research, the rennet coagulation of highly concentrated milk (19% casein) and regular milk at pH 5.8 was investigated, and it was found that when the same amount of rennet is added to the UF retentate and the skim milk, the concentrated milk has longer coagulation time and higher gel firming rate than non-concentrated milk (Karlsson et al. 2007). Hence, more research needs to be carried out to understand the effect of pH adjustment and concentration on the rennet induced
aggregation behaviour of casein micelles. Furthermore, the effect of the addition of pH-modified and concentrated casein micelles as food ingredients in milk is unknown, and it may be of great interest in the development of new cheese making processes.

2.6 Methodologies

2.6.1 Protein analysis

2.6.1.1 Dumas method

The Dumas (combustion) method is an attractive alternative to Kjeldahl method and is now approved for the determination of crude protein in many food categories. For dairy products, the Dumas technique gives higher total nitrogen values than Kjeldahl method and good repeatability (King-Brink and Sebranek 1995; Wiles et al. 1998). It can measure nitrogen gas within 3-7 min by the thermal conductivity after the complete combustion of the food products. The principles about the Dumas combustion method were first established by Jean-Baptiste Dumas in 1831. The sample is combusted at high temperatures (700-1020°C) in the presence of oxygen. All carbon in the sample is converted to carbon dioxide during the flush combustion process. On the other hand, nitrogen-containing ingredients are converted to N₂ and nitrogen oxides, which are passed by a copper reduction column at a high temperature (600°C) and then reduced to nitrogen. This leads to the release of carbon dioxide, water and nitrogen. The gases are then passed over special columns that absorb the carbon dioxide and water. The total nitrogen released is carried by pure helium and quantified by gas chromatography using a thermal conductivity detector at the end. The instrument must first be calibrated by analyzing a material that is pure and has a known nitrogen concentration. In this research,
EDTA (ethylenediamine tetra acetate) is used as the calibration standard and the total protein is determined using a Leco FP-528 (Leco Corp., St. Joseph, MI, USA). A conversion factor of 6.38 was used to calculate the protein content.

2.6.1.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE is a technique commonly used to determine the molecular weight of proteins. It has been successfully used to separate the four bovine caseins in the presence of reducing agent, giving four corresponding bands, in the order of increasing mobility, \( \alpha_{s1} \), \( \alpha_{s2} \), \( \beta \)- and \( \kappa \)-caseins (Creamer and Richardson 1984). SDS-PAGE is a technique that separates proteins based on their electrophoretic mobility and identifies them based on molecular weight or size. Electrophoresis refers to the study of the movement of charged molecules in an electric field. The mobility of a substance is influenced by both size and charge. If the samples are treated so that they have a uniform charge, electrophoretic mobility then depends primarily on size. In a Polyacrylamide Gel electrophoresis (PAGE), SDS is added to disrupt the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain. The SDS is an anionic detergent that can bind strongly to proteins, mainly through hydrophobic interactions. The amount of SDS bound is usually added approximately proportional to the weight of protein, about 1.4 g SDS/g protein (Reynolds and Tanford 1970). Thus, all the proteins have identical charge per unit mass and the indigenous net charge on the protein at any pH is thus negligible. Usually, a reducing agent, namely mercaptoethanol or dithiothreitol, is used to disrupt covalent disulphide bonds between/within proteins. The heating is also employed to elongate the structure of proteins and help the binding with SDS. Under
these conditions, the protein is reduced to its primary structure and fully charged, allowing an electrophoretic separation of proteins by size.

There are two types of different gels in an electrophoretic gel, the resolving gel and the stacking gel. The stacking gel locates at the top where the sample is loaded and the polypeptides are concentrated. On the other hand, the resolving gel locates at the bottom, where the polypeptides migrate to different extents depending on their size. The gel matrix is prepared with polyacrylamide and with N,N'-methylene-bis-acrylamide, which was used as a crosslinker. The ammonium persulfate and TEMED are added to initiate polymerization. The polymerization occurs by opening the double bond of acrylamide; CH$_2$=CHCONH$_2$. The polymerization reaction creates a gel due to the incorporation of bisacrylamide (CH$_2$=CHCONH)$_2$CH$_2$, which can form cross-links between two acrylamide molecules. Ammonium persulfate is able to produce free radical SO$_4^-$ ions, which react with the acrylamide molecules and initiate their polymerization. TEMED, (CH$_3$)$_2$NCH$_2$CH$_2$N(CH$_3$)$_2$, behaves as a catalyst, accelerating the decay of the ammonium persulfate.

The pore size of the gel is chosen based on the molecular mass of the proteins to be separated and is varied by altering the concentration of acrylamide in solution. The stacking gel has a low acrylamide concentration, which ensures that the protein sample is concentrated at the front, and the run is homogeneous between samples in the same gel. On the other hand, the resolving gel has higher acrylamide concentration which makes the protein molecules slowly travel in the gel and allow a good separation of proteins. Proteins are normally separated on resolving gels that contain 4–15% acrylamide. Lower percentage gels are better for separating proteins with high molecular weight, while much
higher percentages are needed to separate small proteins. It is reported that resolving gel with acrylamide concentrations of 15% can be used to separate proteins with molecular mass below 50 kDa. Proteins with high molecular weight greater than 500 kDa are often separated on gels with acrylamide concentrations below 7% (Smith 2010).

As proteins are mostly colorless, the progress through the gel during electrophoresis is very hard to follow. Anionic dyes of a known electrophoretic mobility are therefore included in the sample buffer. A very common tracking dye is bromophenol blue. This dye is a small molecule that migrates ahead of the proteins and is used to monitor the progress of a separation. Once the samples are loaded to the wells of the stacking gel, an electric field is applied across the gel, causing the negatively charged proteins to migrate across the gel from the negative electrode (the cathode) towards the positive electrode (the anode). After the electrophoresis run, the bands on the gels are stained by using a protein stain, usually Coomassie blue, allowing visualization of the separated proteins. Once stained, by scanning densitometry of a single gel, the absolute amounts of the main proteins present can be quantified (Smith 2010).

2.6.2 Light scattering

2.6.2.1 Dynamic light scattering

Dynamic light scattering measures the Brownian motion of the particle scattering in a sample, as it relates to their size. A monochromatic light source, usually a laser, is employed to illuminate the particles and the intensity fluctuations in the scattered light are then collected by a photomultiplier. According to Rayleigh scattering, when small particles are illuminated by the laser, the particles will scatter the light in all directions. The resulting image, which is known as a speckle pattern, is projected onto a screen. The
scattering intensity at a given angle fluctuates over short scales due to the fact that the small molecules are undergoing Brownian motion. The Brownian motion is the movement of particles due to random collision with the molecules of the liquid that surrounds the particle. As indicated by the Stokes-Einstein Equation,

\[ D = \frac{kT}{6\pi\eta R} \]  \hspace{1cm} \text{[Eq. 2.2]}  

where \( D \) the translational diffusion coefficient, \( R \) is the hydrodynamic radius, \( k \) is Boltzmann’s constant, \( T \) the absolute temperature and \( \eta \) is the viscosity of the medium. An important feature of the Brownian motion for DLS is that small particles move faster than large particles. It is important to state that the diffusion coefficient will not only depend on the size of the particle but also by other factors such as the viscosity of the medium, the surface structure and shape of the particle. The basic principle of DLS is to measure the fluctuation in scattering intensity and use this to calculate the diffusion coefficient and hence the apparent radius of particles within the sample. This is achieved using a digital correlator, which measures the degree of similarity between signals over a period of time.

It is easy to understand that the intensity signal of a particular area of the speckle pattern at one point in time (say \( t \)) is very similar to the intensity of the signal a very short time later (\( t+\delta t \)), or we can say the two signals are very strongly correlated. If we compare the original signal a little further ahead of time (\( t+2\delta t \)) there would be still reasonable comparison between the two signals, but it will not be as close as (\( t+\delta t \)). In this way, the correlation function decreases with time and there will be no longer a correlation at a much later time, as the particles are moving in random directions.
With DLS we are dealing with short time scales. In a typical speckle pattern it takes only in the order of 1 to 10 ms for the correlation to reduce to zero. If we continue to measure the correlation with time, a typical correlation function from 1 to 0 against time can be obtained. As the rate of decay for the correlation function of smaller particles is much faster than the large particles, a number of autocorrelation function graphs can be obtained. Then all the autocorrelation functions are fitted to the following equation:

\[ g(1)(t) = e^{-(t/\tau)} \]  
[Eq.2.3]

where \( \tau \) is the characteristic decay time of the intensity autocorrelation function and defined by the equation:

\[ \tau = \frac{1}{(Dq^2)} \]  
[Eq.2.4]

Where D is the diffusion coefficient and q is the scattering vector:

\[ q = \frac{4\pi\eta}{\lambda} \sin \frac{\theta}{2} \]  
[Eq.2.5]

\( \theta \) is the scattering angle, \( \eta \) the refractive index of the medium, and \( \lambda \) the wavelength of the laser. The D value can then be determined based on the fitting and then the size of the particles can be determined by the Stokes-Einstein equation. A number of sizes were calculated based on the different characteristic decay times to produce a size distribution.

2.6.2.2 Diffusing wave spectroscopy

One limit of the traditional dynamic light scattering is that the sample needs to be extensively diluted to achieve single scattering, which is necessary for the size
measurement. It cannot be used to investigate concentrated suspensions and the dilution might change the “true” behaviour of the colloidal particles. In recent years, an extension technique of the traditional dynamic light scattering, diffusing wave spectroscopy (DWS) has been successfully used to study the colloidal properties of the concentrated suspensions, such as aggregation behaviour and phase separation (Weitz et al. 1993; Sandra et al. 2007). DWS is defined as a light scattering technique that permits the investigation of the interparticle interactions \textit{in situ}, avoiding the necessity of extensive dilution. It relies on many scattering events happening when the light passes through a colloidal dispersion. The light propagation through the sample is assumed to happen in a diffusive way. Similarly to dynamic light scattering, DWS measures the intensity fluctuations of the transmitted scattered light caused by the Brownian motion of colloidal particles.

In this research, a transmission DWS is used with a solid-state laser light with a wavelength of 532nm and a power of 100mW. According to Weitz et al. (1993), the detected correlation function can be mathematically described by the following equation:

$$g(t) \approx \frac{\left(L^{(\tau+4/3)}(\tau)\right)^{1/2}}{\left(1+\frac{6\tau}{L}\right)^{1/2}} \left[\frac{L^2}{L^2(\tau)^{1/2}} + \frac{6\tau}{L} \cos h\left[\frac{L^2}{L^2(\tau)^{1/2}}\right]\right]^{1/2}$$  \hspace{1cm} [Eq. 2.6]

where $L$ is the sample thickness and $\tau$ the decay time. The $l^*$ is known as the photon transport mean free path and can be defined as the distance that a photon must travel for randomization of the photon path over many scattering events. This equation holds true when $t << \tau$ and $L/l^* > 10$. 

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To be able to use the above equation to obtain dynamic information on the milk samples, it is first essential to determine the parameter $l^*$. Transmission measurements can give the value of $l^*$ according to the relation:

$$\frac{I}{I_0} = \left(\frac{6l^*}{\pi} \right)^{-1/2} \left(1 + \frac{4l^*}{3\pi}\right)$$  \hspace{1cm} \text{[Eq. 2.7]}

where $I$ and $I_0$ are the initial and transmitted laser intensity. Since we cannot measure the value of $I_0$ directly, the scattering of a known colloid is used to calibrate the system (Alexander et al. 2002). For this reason, an autocorrelation function for a reference sample made up of 269 nm diameter latex spheres (Portland Duke Scientific, Cat no 3269A, Palo Alto, CA, USA) was obtained under conditions where interparticle interaction influences are negligible. As this is a well-defined, monodisperse system with known values of the diffusion coefficient $D$, the characteristic decay time can be calculated by the equation $\tau = 1/q^2D$, where $q$ is the wave vector ($q = 2\pi n/\lambda$, where $n$ is the refractive index of the continuous phase and $\lambda$ is the wavelength). The value of $l^*$ for the latex could be obtained directly from the measured autocorrelation function using equation 2.5. A transmission experiment on the latex then allowed the calculation of $I_0$ according to Equation 2.7. Measurement of the transmitted light intensity of the unknown sample, together with the calculated $I_0$, allows the value of $l^*$ to be calculated at any time. The diffusion coefficients can be fit with the measured $l^*$ value of the sample as a function of time and a best fit for $\tau$ could be found. From the value of $\tau$, $D$ was calculated. The apparent particle radius is calculated using the Stokes-Einstein relationship. In summary, DWS allows the simultaneous investigation of the static and
dynamic behaviour of colloidal particles in fairly concentrated suspensions. Static properties of the sample are reflected by the temporal interparticle spatial organization, as determined by the value of \( l^* \). Dynamic properties of the sample are represented by the average particle self-diffusion coefficient \( (D) \) obtained by probing the colloidal mobility over a very short length scale.

It should be noted that all these calculations hold true only in freely diffusive systems, hence, a direct correlation between the decay time and the diffusion coefficient would only be applicable before strong interparticle interactions happens. As soon as the particle dynamic is changed to a subdiffusive motion (e.g., the point after which a liquid-like colloidal suspension is converted to a more gel-like state), the quantity \( t/\tau \) in eq. 2.5 must be replaced by \( k_0(\Delta r^2 (t))/6 \) (Weitz and Pine 1993; Gaygadzhiev et al. 2008).

Theoretically, \( l^* \) is determined by the scattering form factor \( F(q) \) and the structure factor \( S(q) \)

\[
l^* \propto (\int F(q)S(q)q^3 dq)^{-1} \quad \text{[Eq. 2.8]}
\]

For a highly dilute system, all particles are far away from each other and interparticle interactions are negligible, \( S(q) = 1 \). However, for more concentrated dispersions, the interparticle forces and the spatial correlation between particles cannot be ignored. A change in the value of \( l^* \) in such a system are due to the alteration of particle-particle interactions, if all other physical properties of the scatterers such as particle size, concentration, and refractive index contrast remain constant. The turbidity parameter, \( 1/l^* \),
then describes the positional organization of the particles within the system and the arising interparticle forces, and it is directly proportional to $S(q)$ and $F(q)$.

### 2.6.3 Chromatographic techniques

#### 2.6.3.1 Ion chromatography

The separation in ion exchange chromatography is based on difference in the ion exchange affinities of the individual analyte. In this research, total, soluble and diffusible calcium and phosphate were measured. Determination of the calcium was carried out using non-suppressed ion chromatography (Rahimi-Yazdi et al. 2010) and the determination of phosphate was carried out using a CO$_2$ suppressed ion chromatography. To detect the total, soluble and diffusible calcium in milk, all forms of the calcium need to be change into free ionized calcium as the measurements are based on Ca$^{2+}$ conductance. In this research, to decrease the influence of the di- and tri-carboxylic acids present in milk (mainly citrate), the samples were diluted and directly acidified with hydrochloric acid to precipitate the proteins and release all the micellar calcium phosphate. The soluble calcium and phosphate are defined as those in the serum phase after centrifugation at 100,000 x g. The centrifugal supernatants of milk are then filtered using ultrafiltration tubes (Corning, US) with molecular weight cut-off of 10 kDa to obtain the diffusible part, which will be used for the determination for the diffusible calcium and phosphate. The ultrafiltration tubes were centrifuged at 4000 x g for 1 h to separate the residual soluble proteins from the calcium and phosphate fraction unbound to protein.

The non-suppressed ion chromatography method is used to measure determine the
calcium. For the sample preparation of total calcium, aliquots (666 µL) of milk samples were mixed with 400 µL of 1 M HCl and 266 µL HPLC water in an Eppendorf microcentrifuge tube. The mixture was then centrifuged at room temperature for 15 min at 7600 x g (Eppendorf centrifuge, 5415 D, Brinkmann Instruments Ltd., Mississauga, Ontario, Canada). The clear parts were then diluted to 300 times directly using HNO₃ acceptor solution (2mmol.L⁻¹ HNO₃ solution). The soluble and diffusible calcium are prepared by diluting 100 times with HNO₃ acceptor solution.

The phosphate in the milk present in two forms, the inorganic phosphate, which can be released by acidification, and organic phosphate covalently bound to peptide chains of caseins (Gaucheron et al. 1996), which cannot be released by acidification. The samples need to be ashed to fully release the phosphate. To prepare samples for the total and soluble phosphate measurements, 1 mL milk sample or supernatant was transferred to the Pyrex test tubes and then heated at 100°C overnight to dry the samples, followed by a mineralization at 500 °C for 6 h in an Isotemp muffle furnace (Fisher Scientific). The obtained ashes are dissolved with 1 mL of 1 mol.L⁻¹ HNO₃ and then diluted with HPLC water. The total and soluble samples are diluted 300 times with HPLC water before analysis by ion chromatography. The permeable fraction is diluted 200 times.

The instrument we are using to determine the calcium and phosphate is an 861 Advanced Compact IC (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland), which contains an injection valve, high pressure pump and a conductivity detector. The diluted samples will be put in the plastic tube and loaded with a 838 sample processor into 833 IC liquid handling Dialysis unit (both Ω Metrohm). The instrument is operated with the system of IC Net (v. 2.3, Metrohm). The samples are pumped by an autosampler
to one side of the dialysis membrane. Meanwhile, an acceptor solution (2 mM nitric acid for calcium and HPLC water for phosphate) was continuously pumped on the other side of the membrane to collect the ions. The ion enriched acceptor solution (20 μL) was then injected into the column and was eluted isocratically with a mobile phase. For the phosphate measurement, a suppressor is inserted between the separating column and the detector. Both the eluent and the analytes are chemically modified in the suppressor to reduce the self-conductivity of the eluent and increase the detectability of the analytes. The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration curves prepared with individual calcium or phosphate standards (TraceCERT, Fluka, Sigma Steinheim, Germany). Both column and detector temperatures were kept at 30°C.

For the determination of the calcium, a cation column (Metrosep C4/150, Metrohm) packed with 5 μm silica gel with weakly acidic carboxyl acid functional groups (RCO₂⁻) group was employed. Pyridine-2, 6-dicarboxylic acid (99% Acros Organics, Geel, Belgium) and 70% nitric acid were used to prepare mobile phase (0.7 mM dipicolinic acid and 1.7 mM HNO₃). Samples were eluted at a flow rate of 0.9 mL min⁻¹ (Rahimi-Yazdi et al. 2010). For the determination of phosphate, an anion column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5 μm polyvinyl alcohol with quaternary ammonium groups was employed. Sodium hydroxide and sodium carbonate solutions were used to prepare mobile phase (1.0 mM sodium carbonate and 4mM sodium hydroxide). Samples were eluted at a flow rate of 0.5 mL min⁻¹. The levels of colloidal calcium and phosphate in the samples can be estimated from the difference between the total amount and that measured in the centrifugal supernatant fraction.
2.6.3.2 RP-HPLC

Reverse-Phase high-performance liquid chromatography (RP-HPLC) is a partition chromatography which separates the molecules based on the hydrophobicity. The separation relies on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. Silica is the most commonly used support material for the stationary phase functional groups. Reversed-phase HPLC utilizes polar mobile phases, usually water mixed with methanol or acetonitrile. Increasing the polarity of the mobile phase usually increases solute retention, while increasing non polarity of the mobile phase decreases retention and results in faster elution. In practice, a gradient elution is the best way to get a better resolution (Reuhs and Rounds 2010).

In this research, the caseinomacropeptide (CMP), released from κ-casein due to the action of chymosin on Phe_{105}-Met_{106} bond, was measured. CMP is highly glycosylated with oligosaccharides that are O-linked to threonine and serine. A C2/C18 column was used for the determination of CMP released during renneting of milk (López-Fandiño et al., 1993). This column contains a liquid stationary phase (mixed Ethyl; C2 and Octadecyl; C18) covalently attached to a support of silica. A mixture of acetonitrile-water with 1% trifluoroacetic acid (TFA) as ion suppression and/or ion pairing agent was used as the mobile phase. TFA prevents band broadening and helps to maintain the pH in the system (Reuhs and Rounds 2010). The gradient elution was designed to improve the separation of the different molecules in the sample depending on their hydrophobicity. CMP lacks
aromatic amino acids like tryptophan and tyrosine and does not show absorption at 280 nm. Therefore, the detection was carried out at 210 nm.

2.6.4 Rheology

Rheology is the study of flow and deformation of matter under applied forces, which is measured routinely using a rheometer. The measurement of rheology is mainly performed on a liquid state, but also on “soft solids” or solids under conditions in which they respond with plastic flow rather deforming elastically in response to an applied force. Depending on the external conditions applied, the viscosity or the viscoelasticity of samples can be measured (Goodwin and Hughes 2000). Unlike Newton’s law of viscosity, which describes the flow behaviour of normal fluids, the relationship between stress and deformation describes the elasticity for metals and other elastic materials.

Viscosity is defined as a fluid’s resistance to flow under an applied shear stress. The apparent viscosity is the result of the stress (τ) divided by the shear rate (γ). For Newtonian fluids, shear stress is proportional to the shear rate and the viscosity is constant. Nevertheless, for most liquids, the viscosity term is not constant, but depends on the shear rate or shear rate history, and the fluid is called non-Newtonian fluid. Viscoelasticity is the property of materials that exhibit both viscous and elastic characteristics while undergoing deformation. It is usually denoted by the shear modulus (G), which is defined as the result of shear stress (τ) divided by the shear strain (ϒ). The shear strain is defined as the resultant deformation after the shear stress is performed (Daubert and Foegeding 2010).
In this thesis, a controlled stress rheometer was used for both the rotational and the oscillatory measurements. For the rotational measurement, a cone and plate geometry was used to measure the viscosity values for different samples. The cone has an angle of 2.09° and its special design insures the shear rate is constant throughout the cone-plate gap. A steady flow test (shear rate ramp from 0.1-300 s⁻¹) was employed to measure the flow behaviour and viscosity values of the concentrated milk. For the oscillation measurements, a concentric cylinder (28 and 30 mm inner and outer cylinders diameter, respectively) was used and an external water bath (Isotemp 3016, Fisher Scientific) was used to control the temperature. The method of small deformation rheology was employed to measure the sol-gel transition, as the material characterization was carried out within the linear viscoelastic range of the sample.

Dynamic testing, which involves an oscillatory applied strain or stress, can provide very useful information on the gel formation process (Daubert and Foegeding 2010). In this case, stress or strain is applied in an oscillatory, sinusoidal mode, and the response, strain or stress and the difference in the wave phase can be measured over time. As a result of both stress and strain vary sinusoidally with respond shifted out of phase by a phase angel δ. The phase angle ranges from 0° (ideal elastic response) to 90° (ideal viscous response), and is given by the equation δ = ωΔt, where ω is the angular frequency of the oscillation. In addition, the complex modulus (G*) can be calculated from both the shear stress (τ) and the shear strain (ϒ) amplitudes with the equation G* = ϒ/τ (Kissa 1999).

Some of the main parameters can be obtained include the elastic modulus (G’), which is a measure of energy stored per oscillation cycle, the viscous modulus (G’’).
which is a measure of energy dissipated as heat per cycle, and the loss tangent (\( \tan \delta \), where \( \delta \) is the phase angle), which is the ratio of the viscous to elastic properties. These parameters are described as follows:

\[
G' = G^* \cos \delta \quad \text{[Eq. 2.9]}
\]

\[
G'' = G^* \sin \delta \quad \text{[Eq. 2.10]}
\]

\[
\tan \delta = \frac{G''}{G'} \quad \text{[Eq. 2.11]}
\]

At the beginning of the gelation process, \( G'' \) is higher than \( G' \) and the phase angle is high (\( \delta \sim 90^\circ \)), indicating the milk sample is more viscous. As structure begins to form, the phase angle begins to decrease, eventually the \( G' \) starts to overcome \( G'' \), indicating a more elastic character of the gel. In this work, the gelation point is defined as the point of crossover of \( G' \) over \( G'' \), at this point \( \tan \delta = 1 \) (Lucey et al. 2000). Based on the changes of these parameters, it is possible to determine the onset of the gelation and the properties of the final gels.
CHAPTER 3

CHANGES IN THE PHYSICO-CHEMICAL PROPERTIES OF CASEIN MICELLES IN THE PRESENCE OF SODIUM CHLORIDE IN UNTREATED AND CONCENTRATED MILK

3.1 Abstract

The addition of NaCl to milk is known to alter the mineral equilibrium and the structure of casein micelles. The objective of this study was to better understand the light scattering and rheological properties of milk as a function of NaCl addition (0-500 mmol. L⁻¹), for fresh skim milk and 2x concentrated milk, prepared using osmotic stressing. NaCl was added while dialyzing milk with milk serum (permeate) for 18 h. The presence of NaCl decreased the pH of milk and the zeta-potential of casein micelles. When measured under diluted conditions, using dynamic light scattering, the average radius of the casein micelles increased. Total calcium and phosphate decreased with NaCl concentration, with a corresponding increase of the permeable calcium and phosphate, and of non sedimentable caseins. In both untreated and concentrated milk, the viscosity increased with NaCl concentration. The characteristic decay time (τ) and turbidity measured under non-diluted conditions using diffusing wave spectroscopy showed a gradual decrease with NaCl addition. Addition of NaCl caused disruption of the internal structure of the casein micelles and changes in the composition of the serum phase, affecting the bulk viscosity and the light scattering properties of the continuous phase, due to a change in the refractive index contrast between the micelles and the serum.

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3.2 Introduction

Caseins constitute up to 80% of total proteins in bovine milk. In native milk, they are associated into colloidal particles known as casein micelles. The composition and structure of casein micelles have been widely reported (see for example, Dalgleish and Corredig 2012; Holt 2013). Casein micelles have a core composed primarily of \( \alpha_S \)- and \( \beta \)-caseins, associated to one another by non-covalent interactions and by calcium phosphate nanoclusters of a few nanometer size, linked to the serine residues of the casein proteins (De Kruif and Zhulina 1996; Dalgleish and Corredig 2012). \( \kappa \)-casein molecules are mostly located on the surface of the casein micelle, with their hydrophilic portions partly extended into the aqueous phase, yielding a polyelectrolyte brush of great importance to the colloidal stability of the protein particles (De Kruif and Zhulina 1996).

Casein micelles stability plays a crucial role in the overall processing properties of milk, and changes in mineral balance have important consequences to the stability of the micelles. A wide variety of studies have investigated the effect of NaCl on physicochemical characteristics of milk. The addition of NaCl to bovine milk causes a decrease in pH (Grufferty and Fox 1985; Gaucheron et al. 2000) and an increase in the voluminosity of casein micelles (Creamer 1985; Van Hooydonk et al. 1986). In addition, NaCl increases the level of non sedimentable calcium and magnesium (Grufferty and Fox 1985; Gaucheron et al. 2000). There are conflicting findings about the influence of NaCl on the non-sedimentable phosphate. Some researchers report that NaCl addition has little effect on the non-sedimentable phosphorus (Le Graët and Gaucheron 1999; Gaucheron et al. 2000; Huppertz and Fox 2006a), while others have shown that NaCl significantly increases the diffusible phosphorous concentration (Famelart et al. 1999; Karlsson et al.
Most of the studies have been carried out on reconstituted skim milk or casein concentrates (Famelart et al. 1999; Huppertz and Fox 2006). Few studies have been conducted on the effect of salt addition on concentrated milk. Huppertz and Fox (2006) studied the effect of NaCl, directly added to milk, on various processing properties of caseins. The addition of NaCl to concentrated milk increased the pH of maximum heat stability and the maximum heat coagulation time. There were no differences in the average casein micelle size, but there was a reduced net charge of the casein micelles and a reduction of pH, also in concentrated milk. As for unconcentrated milk, soluble and ionic calcium increase by addition of NaCl, but the level of soluble inorganic phosphorus does not vary (Huppertz and Fox 2006).

In this work, the effect of NaCl on casein micelles properties was studied on untreated skim milk and the same milk concentrated using osmotic stressing. Using this process, the concentration of casein micelles can be carried out non-invasively, unlike evaporation or ultrafiltration (Bouchoux et al. 2009). The aim of this research was to further investigate the NaCl-induced changes of physico-chemical properties of concentrated milk, compare them to those of fresh skim milk, and to study such properties in situ, without dilution, using diffusing wave spectroscopy and rheology.

3.3 Materials and Methods

3.3.1 Skim milk and permeate preparation

Fresh raw milk was obtained from the Elora Dairy Research Farm of University of Guelph (Elora, ON, Canada). Sodium azide was added immediately at a concentration of 0.02 % (w/v) (3mmol.L⁻¹) as a bacteriostatic agent. The milk was skimmed by
centrifugation at 4000 × g for 25 min at 4 °C (J2-21 centrifuge, Beckman Coulter Canada Inc, Mississauga, Canada) and then filtered four times through Whatman fibreglass filter (Fisher Scientific, Mississauga, Ontario, Canada). The skim milk was kept at refrigerated (4 °C) until use.

Skim milk permeate was prepared by ultrafiltration of skim milk (Crown Dairy, Guelph, Ontario, Canada) by passing it through an OPTISEP® Filter module (Smartflow Technologies, Apex, NC, USA) with 10 kDa molecular mass cutoff at ambient temperature. The acquired permeate was added with 0.02 % (w/v) sodium immediately and kept at refrigeration temperature until it was used.

3.3.2 Concentration of milk using osmotic stressing

Polyethylene glycol (PEG) (35,000 Da, Fluka, Oakville, Ontario, Canada) was added to milk permeate at a final concentration of 7%. Regenerated cellulose dialysis bags (Fisher Scientific, Whitby, Ontario, Canada) with a molecular mass cut off of 6–8 kDa used with Milli-Q water and conditioned with permeate. Milk samples (40 mL) were then placed in the dialysis bags and immersed in a 1 L of permeates containing additional concentrations of NaCl ranging from 0 to 500 mmol.L^{-1}. The dialysis allowed the exchange of small molecules such as water, ions, and lactose but not caseins or PEG (Bouchoux et al. 2009). Concentrated samples (2x) were obtained after dialysis for 18 h at 4°C. When necessary, the final volumes of the 2x retentates was adjusted with ultrafiltration (UF) permeate (see above). Non-concentrated (1x) skim milk was also dialyzed in 1 L UF permeate with different NaCl concentrations as controls.

3.3.3 Zeta potential and average casein micelle size

The particle size and the apparent zeta potential of the casein micelles were
measured by dynamic light scattering (DLS) method (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). After concentration, the milk samples were diluted 500 times with filtered (0.2 μm nylon filters, Fisher scientific) UF permeates containing the same concentration of added NaCl and analyzed. All the measurements were conducted at 25 °C.

3.3.4 Diffusing wave spectroscopy (DWS)

Transmission DWS was used in this research which allow to investigate colloidal behavior of casein dispersion in situ without dilution. The output results from DWS for a free diffusing system, is a time-dependent correlation function, with a characteristic decay time:

\[ \tau = \frac{1}{k_o^2 D} \]  

[Equation 3.1]

Where \( k_o \) is the wave vector \( k_o = \frac{2\pi n}{\lambda} \), where \( n \) is the refractive index of the continuous phase and \( \lambda \) is the wavelength), \( D \) is diffusion coefficient. The photon transport mean free path \( l^* \) was also measured (Alexander and Dalgleish 2005); this parameter is related to the length scale over which the direction of the scattered light has been totally randomized. Turbidity was calculated as the inverse of the \( l^* \) parameter. The apparent radius of the casein micelles was derived from diffusion coefficient through the Stokes-Einstein relation. Standard latex spheres of diameter 260 nm (Portland Duke Scientific, Palo Alto, CA, USA) were used to calibrate the laser intensity daily.

The sample was poured into an optical glass cuvette (Hellma Canada Ltd., Concord, Ontario, Canada) with a 5 mm path length and then placed in a water bath maintained at 25°C. The sample was illuminated by a solid-state laser light with a wavelength of 532 nm and a power of 350 mW (Coherent, Santa Clara, CA, USA).
Scattered light intensity was collected in transmission mode as previously described (Sandra et al. 2011) and analyzed using software developed specifically for the equipment (Mediavention Inc., ON, Canada). Correlation functions and intensity of transmitted scattered light were measured at intervals of 3 min. The viscosity and refractive index of the continuous phase used to calculate the radius were $1.021 \times 10^{-3}$ Pa.s and 1.34, respectively (Alexander and Dalgleish 2005; Alexander et al. 2006; Sandra et al. 2011).

### 3.3.5 Rheological measurements

Advanced Rheometer AR 1000 (TA Instruments Ltd., New Castle, DE, USA) equipped with a Peltier temperature controller was used to measure the viscosity. All samples were subjected to a steady flow test (shear rate ramp from 0-100 s$^{-1}$), using a cone and plate geometry, with a set gap of 0.51 mm. The values are reported for 100 s$^{-1}$. All measurements were conducted at 25°C.

### 3.3.6 Non sedimentable protein

The soluble protein present in the casein micelles dispersion was defined as the fraction that did not sediment after ultracentrifugation at 100,000 × g for 1 h at 20 °C (OptimaTM LE-80K ultracentrifuge with rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada). The supernatants were given two sequential filtrations using 0.45 μm and then 0.22 μm (low protein binding, Fisher Sci.).

The amount of protein present in the supernatant was measured using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) and the protein
concentration was determined using 6.38 as conversion factor. The protein composition of supernatants was subsequently analyzed by SDS-PAGE under reducing conditions; the resolving and stacking gel contained 15% and 4% acrylamide, respectively. Gels were run at 175 V for 1 h using Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Hercules, CA). The gels were stained with Coomassie blue in a 5 : 1 : 4 mixture of methanol, acetic acid and Milli-Q water for 30 min and destained in a 4.5 : 1 : 4.5 mixture of methanol, acetic acid and Milli-Q water for 1 h for two times. Destained gels were scanned in a Bio-Rad Gel Doc EZ Imager (Bio-Rad Power Pac HC, Hercules, CA) equipped with Image Lab 3.0 (Bio-Rad Power Pac HC, Hercules, CA) software.

3.3.7 Determination of calcium and phosphate

For the determination of total calcium, aliquots (666 µL) of milk samples were mixed with 400 µL of 1 M HCl and 266 µL in an Eppendorf microcentrifuge tube using HPLC water. The mixture was then centrifuged at room temperature for 15 min at 4500 × g (Eppendorf centrifuge, 5415 D, Brinkmann Instruments Ltd., Mississauga, Ontario, Canada). The clear supernatants were then diluted with HNO₃ acceptor solution (2 mM HNO₃ solution) to 300 times. For determination of diffusible calcium, the supernatants separated by centrifugation (see section 3.3.6) were transferred to ultrafiltration tubes with molecular weight cutoff of 10 kDa; it was then centrifuged at 4000 × g for 1 h to get rid of the proteins. The obtained permeates were diluted 200 times with HNO₃ acceptor solution.

The ion separation was carried out using 861 Advanced Compact IC (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland) composed of an injection valve, high
pressure pump and a conductivity detector. A cation column (Metrosep C4/150, Metrohm) packed with 5μm silica gel with weakly acidic carboxyl acid functional groups (RCO$_2^-$) group was employed. Pyridine-2, 6-dicarboxylic acid (99% Acros Organics, Geel, Belgium) and 70% nitric acid were used to prepare mobile phase (0.7 mM dipicolinic acid and 1.7 mM HNO$_3$). The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration curves prepared with calcium standards (TraceCERT, Fluka, Sigma Steinheim, Germany). Samples were eluted at a flow rate of 0.9 mL·min$^{-1}$ (Rahimi-Yazdi et al. 2010).

For the determination of total and diffusible phosphate, same sample preparation methods were adopted as calcium. However, in this case, HPLC grade water was used as acceptor solution in the phosphate measurement and the samples were eluted on an anion column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5 μm polyvinyl alcohol with quaternary ammonium groups was employed. Sodium hydrogen carbonate and sodium carbonate solutions were used to prepare mobile phase (1.0 mM sodium hydrogen carbonate and 3.2mM sodium carbonate). Samples were eluted at a flow rate of 0.5 mL.min$^{-1}$.

3.3.8 Statistical analysis

Three or four replicates were performed for each test. ANOVA and Tukey HSD were carried out on the experimental measurements with 95% confidence level using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).
3.4 Results

3.4.1 Influence of NaCl on pH and zeta potential of the casein micelles

The values of pH and of zeta potential of the casein micelles in milk after equilibration with NaCl are summarized in Table 3.1. The addition of NaCl caused a reduction in pH for both control (1×) and concentrated (2×) milk, in full agreement with previous reports (Gaucheron et al. 2000; Grufferty and Fox 1985; Huppertz and Fox 2006). The pH of control skim milk reduced gradually from 6.74 to 6.56 for milk dialyzed against permeates containing 500 mmol.L\(^{-1}\) NaCl; similarly, the pH of 2× concentrated milk decreased from 6.76 to 6.47.

The zeta-potential of casein micelles in the absence of NaCl was about -21 mV. Addition of NaCl to unconcentrated and 2× concentrated milk led to a decrease of zeta potential to -16 mV (Table 3.1). This change is expected as the higher ionic strength of the solution will cause a decrease in the electrostatic charge and thickness of the electrical double layer (Bouchoux, et al. 2009; Bouchoux et al. 2010), and will reduce the electrophoretic mobility of the colloidal particles (Walstra 2003).

3.4.2 Influence of NaCl on mineral balance

Milk samples were dialyzed against milk permeate (the serum portion of milk) containing varying concentration of NaCl. The presence of PEG in the permeate caused the milk to be concentrated 2× in 18 h.
Table 3.1 Effect of NaCl (0-500 mmol.L⁻¹) on the pH and zeta potential of casein micelles in untreated and 2× concentrated milk. Results are the average and standard deviation of three independent experiments. Within a concentration, different letters show significant differences for p<0.05.

<table>
<thead>
<tr>
<th>NaCl (mmol.L⁻¹)</th>
<th>pH</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.74 ± 0.01ᵃ</td>
<td>-21.2 ± 0.1ᵃ</td>
</tr>
<tr>
<td>1× 100</td>
<td>6.67 ± 0.01ᵇ</td>
<td>-20.2 ± 0.7ᵃᵇ</td>
</tr>
<tr>
<td>300</td>
<td>6.65 ± 0.01ᵇ</td>
<td>-19.2 ± 0.8ᵇ</td>
</tr>
<tr>
<td>500</td>
<td>6.56 ± 0.01ᶜ</td>
<td>-16.2 ± 0.3ᶜ</td>
</tr>
<tr>
<td>0</td>
<td>6.76 ± 0.01ᵃ</td>
<td>-21.9 ± 0.2ᵃ</td>
</tr>
<tr>
<td>2× 100</td>
<td>6.69 ± 0.01ᵇ</td>
<td>-20.3 ± 1.0ᵃ</td>
</tr>
<tr>
<td>300</td>
<td>6.56 ± 0.03ᶜ</td>
<td>-16.1 ± 0.2ᵇ</td>
</tr>
<tr>
<td>500</td>
<td>6.47 ± 0.01ᵈ</td>
<td>-15.4 ±0.9ᵇ</td>
</tr>
</tbody>
</table>
Figure 3.1 illustrates the changes in total and soluble (defined as permeable, see methods) calcium and phosphate in the samples after dialysis against different concentrations of NaCl. In the absence of NaCl, with or without PEG in the milk permeate, the milk samples maintained the chemical potential of all ions in the native state, as previously demonstrated (Bouchoux et al. 2009). With additional NaCl, a chemical potential difference occurred between milk samples and permeates, resulting in an exchange of ions during dialysis. The total calcium in milk decreased gradually with NaCl addition (Figure 3.1A). The total calcium contents were reduced from 30.9 ± 0.3 mM and 44.9 ± 0.6 mM in unconcentrated and 2× concentrated milk, respectively, to 20.6 ± 0.1 mM and 23.0 ± 0.2 mM when 500 mmol.L⁻¹ NaCl was added (Figure 3.1A). Similarly, total phosphate contents, in both unconcentrated and 2× concentrated milk showed a significant decrease after addition of 300 mmol.L⁻¹ NaCl, although to a lower extent than calcium (Figure 3.1B). The total calcium decreased 49% for 2× milk and 33% for 1× milk, while the phosphate decreased only 4.6% and 12.4% for 2× and 1× milk, respectively.

The decrease in the total calcium concentration in milk with the addition of NaCl corresponded to an increase in diffusible calcium, as clearly shown in Figure 3.1C. It was concluded that with the addition of NaCl, dissociation of calcium from the casein micelles occurred. Only about 32% and 23% of the original colloidal calcium was recovered in unconcentrated and 2× concentrated milk, respectively, in samples containing 500 mmol.L⁻¹ NaCl.
**Figure 3.1** Influence of added NaCl on total calcium (A), total phosphate (B), diffusible calcium (C) and diffusible phosphate (D) in untreated (filled triangle) and 2× (empty triangle) concentrated milk. Values are average of triplicate experiments, with the standard deviation indicated by vertical error bars.
On the other hand, at this NaCl concentration, there was less than a 10% increase in the amount of diffusible phosphate recovered in unconcentrated milk, and about 20% (from 13.7 to 16.5 mM) for 2× concentrated milk (Figure 3.1D). It was concluded that most phosphate remained associated to the proteins even after the addition of 500 mM NaCl, resulting only in a slight decrease of the total concentration of phosphate (Figure 3.1B).

Previous researchers have reported that the addition of NaCl to casein micelle suspensions leads to the solubilisation of calcium and phosphorus from micelles, and increased the casein voluminosity and hydration (Karlsson et al. 2005; Hussain et al. 2011). The increase in soluble calcium is due to the exchange between sodium and calcium during dialysis. As calcium phosphate clusters are crucial to the internal structure and contribute to the rigidity of the casein micelles, the replacement of calcium by sodium within the casein micelles could decrease protein-protein interactions and loosen the internal structure of the casein micelles.

### 3.4.3 Influence of NaCl on milk apparent viscosity

The addition of NaCl up to 500 mM progressively increased the apparent viscosity of both control, unconcentrated and 2× milk. Figure 3.2 summarizes the changes in apparent viscosity measured at 100 s⁻¹. In untreated milk, the apparent viscosity showed a change from 1.7 to 1.9 mPa.s with 500 mM NaCl added. The apparent viscosity of 2× concentrated milk changed from 2.8 to 3.5 mPa.s. These results were fully aligned with reports in the literature (Carr et al. 2002; Karlsson et al. 2005).
Figure 3.2 Viscosity of control (▲) and 2× (△) concentrated milk as a function of NaCl. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.
Previous work attributed the increased viscosity to an increased osmotic pressure and an effective increase in the volume fraction of the casein micelles (Carr et al. 2002). In addition, the increase of NaCl concentration will cause a decrease in the approach distance between casein micelles because of a decrease in electrostatic repulsion, affecting the bulk milk viscosity (De Kruif 1998). Similar results have also been reported for UF concentrates in the presence of NaCl (Karlsson et al. 2005).

3.4.4 Light scattering measurements

The light scattering properties of unconcentrated and concentrated milk were measured using dynamic light scattering, under diluted (DLS) or concentrated (DWS) conditions. Correlation functions obtained using DWS are shown in Figure 3.3. In all cases, the correlation functions showed a slowing down of the characteristic decay time $\tau$, evidenced by a shift of curves toward longer times, with NaCl concentration. There was a difference in the $\tau$ for unconcentrated milk compared to $2\times$ concentrated milk, and the smaller value for concentrated milk was due to the increase in the volume fraction of the casein micelles, their decreased average distance, or possible changes in the viscosity of the continuous phase. The relationship between volume fraction and diffusivity can be predicted for hard spheres using the Beenaker-Mazur formalism (Beenakker and Mazur 1984). The average radius of the casein micelles was then derived from the diffusion coefficient (corrected for crowding) using the Stokes-Einstein equation (Beenakker and Mazur 1983). These apparent sizes were then compared to those measured by DLS, after extensive dilution of the samples in milk permeate with same amounts of NaCl (Table 3.2).
Figure 3.3 Typical correlation functions (DWS) for unconcentrated milk (A) and 2× concentrated milk (B) as a function of NaCl concentration: 0 (solid line), 100 (dotted line), 300 (short dash line), 500 (dash-dot line) mmol.L$^{-1}$. The results shown are for representative runs.
Table 3.2 Apparent radius for untreated (1x) and concentrated (2x) milk measured by DLS and DWS. Values are means of data from triplicate experiments, ±standard deviation. Within concentration, values with a different superscript letter are significantly different (p<0.05). In 2x milk, diffusion coefficients were corrected according to Bennaker-Mazur formalism (Beenakker and Mazur 1983).

<table>
<thead>
<tr>
<th>NaCl (mmol.L⁻¹)</th>
<th>DLS (nm)</th>
<th>DWS (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>84 ± 2ᵃ</td>
<td>128 ± 1ᵃ</td>
</tr>
<tr>
<td>1x</td>
<td>100</td>
<td>85 ± 1ᵃᵇ</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>87 ± 1ᵇ</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>88 ± 1ᵇ</td>
</tr>
<tr>
<td>2x</td>
<td>100</td>
<td>85 ± 1ᵃ</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>86 ± 1ᵃ</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>90 ± 1ᵇ</td>
</tr>
</tbody>
</table>
The difference in the size values derived from DLS and DWS for control milk (with no NaCl) is due to the high dilution necessary in DLS measurement as well as the instrumental set up. When measured with DLS, there was an increase in size of the casein micelles from 84 nm to 88 nm at the high concentration of NaCl and all samples showed a monomodal distribution (not shown here). When casein micelles were measured in situ, undiluted, using DWS, the apparent radius values were much larger than those measured by DLS (Table 3.1). While in the absence of NaCl there was no difference in the apparent radius of the casein micelles between unconcentrated and 2× concentrated milk, in both cases there was an increase in the radius of the casein micelles with NaCl concentration. When measured in situ, the decrease in size is a consequence of the restricted diffusivity of the casein micelles. This may have been caused by an increase in the voluminosity of the casein micelles in the presence of NaCl, or/and by an increase the viscosity of the continuous phase, due to changes in the composition of the serum phase, i.e. dissociation of protein from the casein micelle (see below). These results supported earlier reports from transmission electron microscopic observations showing the presence of large rough micellar structures when NaCl is added to UF concentrate (Karlsson et al. 2005). However, it is important to note that the present work demonstrates for the first time an increase of casein micelles size (a decrease in their diffusivity) in the presence of NaCl both under diluted (DLS) and concentrated (DWS) conditions. Other researchers have reported no significant effect of NaCl on the size of casein micelles for unconcentrated milk and 2× concentrated milk (Home and Davidson 1986).

Using DWS is also possible measure the static properties (turbidity) of milk as a function of NaCl concentration. The value of \( I^* \) is a function of the refractive index
contrast, the physical properties of the scattering particles, their volume fraction, and it can also give some indication of spatial interparticle correlation. Hence, assuming no changes in the size, shape or refractive index contrast of the scattering particles, by comparing $l^*$ values from one sample to the next it may be possible to identify structural differences in the dispersion (Alexander et al. 2006). The addition of NaCl clearly decreased the milk turbidity, to a much larger extent for 2× milk than for unconcentrated milk. In the case of unconcentrated milk, there were no significant differences in the $1/l^*$ between samples with 300 mmol.L$^{-1}$ and 500 mmol.L$^{-1}$. The change in turbidity was larger for 2× concentrated milk, the addition of NaCl led to progressive decrease of $1/l^*$ value (Figure 3.4), from 2.26 to 0.98 mm$^{-1}$. As casein micelles are the main components that affect the turbidity of milk, all these changes also suggest a modification of the physical properties of the casein micelles, most probably caused by the increase in size and a change of their internal structure.

3.4.5 Soluble protein content and composition

To better understand the extent of disruption of the casein micelles with addition of NaCl, the amount of non-sedimentable protein was measured in control and 2× concentrated milk as a function of NaCl, as shown in Figure 3.5. As expected, 2× concentrated milk had more soluble protein than untreated milk. In both cases there was an increase in the non sedimentable protein with increasing NaCl concentration. NaCl caused disruption of the casein micelles and loosening of their internal structure.
Figure 3.4 Influence of added NaCl on $1/l^*$ of unconcentrated (-▲-) and 2× (-Δ-) concentrated milk. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.
Figure 3.5 Influence of added NaCl on the serum protein content of unconcentrated (▲) and 2× (△) concentrated milk. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.

The release of protein will increase the viscosity of serum phase and decrease the diffusivity of casein micelles, resulting in a significant change of the diffusion coefficient measured by DWS. Nonetheless, the significant, albeit small difference in the casein micelles radius measured by DLS under diluted conditions suggested that in addition to the increase in medium viscosity, the casein micelles also changed their size with the addition of NaCl.
To better understand the composition of the soluble fraction, supernatant fractions were analyzed by SDS-PAGE electrophoresis (Figure 3.6). In both 1× and 2× milk, the band intensity of $\alpha_S$-, $\beta$- and $\kappa$-caseins increased with NaCl addition. No significant changes were observed in the whey proteins bands. These results are in contrast with previous publications (Huppertz and Fox 2006b), where the intensity of casein bands in the non sedimentable fraction was the largest in control, and decreased with NaCl addition. The discrepancy in the results may be attributed to the use of fresh milk (present study) and reconstituted milk (earlier study).

3.5 Conclusions

It is known that $\alpha_S$- and $\beta$-caseins are highly phosphorylated and linked to calcium phosphate nanoclusters via their phosphoserine residues (Dalgleish 2011). $\kappa$-caseins do not participate in the formation of nanoclusters as they do not possess phosphate centers. Hence, they are present on the surface of the casein micelles, providing steric and electrostatic repulsion against aggregation. After addition of NaCl, sodium can replace calcium and may alter the structure of nanoclusters.
Figure 3.6 SDS-PAGE electrophoresis of centrifugal supernatant from untreated and concentrated milk. Casein solution (lane 1) and whey protein solution (lane 10) were used as standard. Supernatants from unconcentrated milk containing 0 (lane 2), 100 (lane 3), 300 (lane 4), 500 (lane 5) mmol.L\(^{-1}\) NaCl and 2×concentrated milk containing 0 (lane 6), 100 (lane 7), 300 (lane 8), 500 (lane 9) mmol.L\(^{-1}\) NaCl. Supernatants from unconcentrated milk and 2×concentrated milk were diluted 1 time and 2 times with deionized water, respectively. Results are representative of three replicate experiments.

The data shown in Figure 3.1 clearly demonstrated that the addition of NaCl caused partial solubilisation of the colloidal calcium phosphate and weakened protein-protein interactions within the casein micelle. The size of the casein micelles increased at high concentration of NaCl added. With the addition of NaCl there was an increase in the amount of non sedimentable \(\alpha_s\)- and \(\beta\)-caseins (see Figure 3.6). This partial dissociation affected the refractive index contrast of the protein particles, and caused a decrease in
their diffusivity, as shown by DWS measurements. Although the increased ionic strength caused partial shielding of the protein charges, with a decrease in the electrostatic repulsion between micelles, aggregation was still at least partly prevented through steric stabilization. There was a small, albeit significant change in the average apparent diameter of casein micelles.

Casein micelle have been viewed as hard-spheres from the experiment results of diffusion coefficient (Alexander et al. 2002), viscosity (Dahbi et al. 2010) and voluminosity (Liu et al. 2012). After the addition of NaCl, replacement of calcium by sodium caused a change in the internal structure, but most importantly, caused drastic changes to serum composition, as reflected by the increase in non sedimentable protein and free calcium and phosphate. Hence, it was possible to conclude that the addition of NaCl does not only cause a change in the hydration of the casein micelles, but, and more importantly, a loosening of the internal structure, an increase in the soluble protein in the continuous phase, and a slight change in the apparent size of the casein micelles.
CHAPTER 4

INFLUENCE OF SODIUM CHLORIDE ON THE COLLOIDAL AND RENNET COAGULATION PROPERTIES OF CONCENTRATED CASEIN MICELLES SUSPENSIONS

4.1 Abstract

This paper aims to investigate the influence of NaCl on the colloidal and rennet coagulation properties of concentrated milk. Milk was concentrated to 1x, 3x and 5x, using ultrafiltration. The results showed that the addition of NaCl into milk caused calcium phosphate solubilization, decreases in pH and negative charge on the surface of casein micelles. Increasing the volume fraction caused the formation of stiffer gels for both samples with or without NaCl. The addition of NaCl caused a significant increase in the bulk viscosity of the milk concentrated 5x, and a decrease in turbidity. The concentration has no effect on the gelation time of control samples, nor on the kinetics of CMP release. Rennet gelation was retarded by the addition of NaCl, and the gels showed lower elastic moduli compared to those obtained with control milk.

4.2 Introduction

Skim milk is a dispersion of proteins, minerals and lactose. The major proteins in milk are caseins, which constitute 80% of total milk protein. In milk, they are present in the form of casein micelles, which play an important role in the functionality of milk (Dalgleish 2011; Dalgleish and Corredig 2012). The structure of casein micelle has attracted the attention of scientists and many models have been proposed to elucidate the structure of casein micelles over the past 50 years (Holt 1992, 1998; Horne 2006;
Dalgleish and Corredig 2012). Colloidal calcium phosphate (CCP) nanoclusters, with an average radius of 2.3 nm, are present in the inner core of the casein micelles, and are surrounded by the phosphorylated caseins (αS- and β-caseins) (Holt 1998; Holt 2004). κ-casein molecules are located on the surface of the casein micelle, providing a polyelectrolyte brush of great importance to the colloidal stability of these protein particles (De Kruif and Zhulina 1996).

The structure of casein micelles is affected by changes of environment, such as pH, temperature and the presence of other minerals (Grufferty and Fox 1985; Le Graët and Gaucher 1999; Carr et al. 2002; Huppertz and Fox 2006). It has been reported that the addition of sodium chloride (NaCl) causes the solubilization of colloidal calcium phosphate, increases dissociation of caseins, and improves the solubility of milk concentrates (Mao et al. 2012); however, it may influence the processing functionality of casein micelles (Famelart et al. 1999; Gaucheron et al. 2000). Furthermore, the addition of NaCl decreases milk pH (Huppertz and Fox 2006) and increases the hydration of casein micelle (Van Hooydonk et al. 1986b).

Recent research demonstrated that when NaCl was added to 1x and 2x concentrated milk, there was dissolution of the colloidal calcium phosphate and release of caseins, with consequent changes of viscosity and turbidity (Zhao and Corredig 2015). However, few studies conducted on the influence of NaCl on the concentrated milk can be found and further research about the NaCl on the processing properties of casein micelles is important to optimize the use of NaCl in dairy products.

Rennet coagulation is a critical processing step in cheese making. The rennet gelation involves two overlapping stages. The primary stage is the cleavage of κ-casein;
once enough κ-casein (about 85%-90%) is cleaved, the second stage, aggregation of the casein micelles occurs with the formation of a gel network (Lucey 2002; Liu et al. 2014). Concentration of skim milk using ultrafiltration increases the proteins and colloidal minerals in the retentate and decreases the average distance between casein micelles (Sandra et al. 2011). Previous researchers found that the concentrated milk has a longer coagulation time and higher gel firming rate than non-concentrated milk (Dalgleish 1980; Waungana et al. 1999). However, recent research (Sandra et al. 2011) showed that concentration has no significant effect on the release of caseinmacropeptide and coagulation time when same amount of rennet is added to milk. Addition of NaCl was reported to increase the coagulation time of milk (Sbodio et al. 2006). It is still unknown how NaCl will influence the rennet coagulation of concentrated milk.

This research aims to better understand the influence of addition of NaCl prior to concentration on the structure of casein micelles and on the rennet gelation behaviour of the concentrated milk. The concentration (1x, 3x and 5x) was achieved by ultrafiltration based on the volume fraction. In our study, the rennet gelation process was followed by rheology and diffusing wave spectroscopy (DWS). Prior to rennet gelation, the changes in the physico-chemical properties of concentrated milk were characterized by measuring the total and soluble protein, total and diffusible calcium and phosphate, hydrodynamic size, zeta potential and light scattering properties, using diffusing wave spectroscopy (DWS).
4.3 Materials and methods

4.3.1 Preparation of concentrated milk

Skim milk was obtained from a local dairy company (Crown Dairy, Guelph, Ontario, Canada). 0.01% sodium azide was added immediately to prevent the bacterial growth. 300 mmol L\(^{-1}\) sodium chloride added to milk and stirred for 15 min at room temperature. All the samples were then equilibrated overnight in the refrigerator. After equilibration, aliquots of samples (1L) were concentrated to different concentrations (1x, 3x and 5x) using an ultrafiltration cartridge (10 kDa Millipore CDUF001LG, Fishier scientific, Missauga, ON, Canada) based on the volume reduction. All permeates were collected.

4.3.2 Determination of total and soluble protein content

The total protein was determined directly using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) and the protein concentration was calculated using 6.38 as conversion factor.

The soluble protein was defined as the fraction that did not sediment after ultracentrifugation at 100,000 x g for 1 h at 20 °C (OptimaTM LE-80K ultracentrifuge with rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada). The supernatants were then filtered through the 0.45 μm membrane (low protein binding, Fisher Sci.) and then measured by the Dumas nitrogen analyzer.
4.3.3 Determination of total and diffusible calcium and phosphate

In this research, the 861 Advanced Compact IC (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland) was used to measure the total and diffusible calcium and phosphate in milk. The diffusible calcium and phosphate were defined as those present in the supernatant but not combined with the serum proteins. The sample preparation for both total and diffusible calcium and phosphate has been described in our previous research (Zhao and Corredig 2015).

The calcium was determined using nonsuppressed ion chromatographic method, as described before (Rahimi-Yazdi et al. 2010). On the other hand, a CO$_2$ suppressed ion chromatographic method was used to determine the total and diffusible phosphate. An anion exchange column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5 µm polyvinyl alcohol with quaternary ammonium groups was employed. Sodium hydrogen carbonate and sodium carbonate solutions were used to prepare mobile phase (1.0 mM sodium carbonate and 4mM sodium hydroxide). Samples were eluted at a flow rate of 0.5 mL.min$^{-1}$.

4.3.4 Rennet gelation

Prior to the addition of rennet, milk samples were equilibrated at 30 °C for at least 20 min. The rennet used was Chymax Ultra (CHR Hansen., Milwaukee, USA) with average strength of 790 (±5%) IMCU/mL. The rennet was diluted 100-fold in MilliQ water before addition to milk. The diluted rennet was added to milk with a proportion of 4µL per mL milk. The final concentration of rennet in milk was 0.031 IMCU/mL. Milk
samples were stirred for 30 s after rennet addition prior to further analysis. The gelation process was carried out at 30 °C.

The release of caseinomacropeptide (CMP) by rennet was monitored using an established method (Lopez-Fandino et al. 1993). Rennet at the concentration defined above was added to the milk sample which was then immediately divided into 2 mL aliquots in different test tubes. Samples were incubated at 30 °C and the reaction was stopped at designated times (every 10 min) by addition of 4 mL of 3% trichloroacetic acid (TCA). After overnight storage in refrigerator, the supernatant was collected and then centrifuged at 4500 x g for 15 min (Eppendorf centrifuge, 5415D, Mississauga, Canada). The obtained supernatants were then filtered through using 0.45 μm filters (syringe driven filters, Fisher Scientific). The content of caseinmacropeptide was then determined using RP-HPLC. The HPLC was an Ultimate3000 LC (Thermo Scientific) with degasser, pump, auto-sampler, and UV detector (set to 210 nm). The sample (100 μL) was injected on a column (Pharmacia Biotech μRPC C2/C18 ST 4.6/100 Piscataway, NJ, USA with a Vydac C-4 guard column, Mandel, USA) and elution was carried out with 0.1% TFA in water and 0.1% TFA in 90% acetonitrile in a non-linear gradient (Lopez-Fandino et al. 1993) at 40 °C. The total peak area of the chromatograms was integrated using Chromeleon™ 7.2 Data System Software (Thermo Scientific, Burlington, ON, Canada). The maximum peak area at plateau was considered as 100% of the CMP released.

4.3.5 Light scattering

Zeta potential and the average apparent radius of the casein micelles were
determined by the Dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). All samples were diluted 1,000 times with corresponding filtered (0.2 μm nylon filters, Fisher scientific) UF permeates and analyzed.

Transmission diffusion wave spectroscopy (DWS) was used to investigate the colloidal properties and to monitor the rennet gelation process of casein micelles, as previously reported (Alexander et al. 2006; Corredig and Alexander 2008). The output result from DWS is a time-dependent correlation function, as expressed by the following equation:

\[ g(t) \approx \frac{\left(\frac{L}{\tau^*}+\frac{q}{\tau}\right)^{1/2}}{\left(1+\frac{8\pi^2}{3}\sinh\left[\frac{L}{\tau^*}\left(\tau\right)^{1/2}\right]^{1/2}\frac{L}{\tau^*}\cosh\left[\frac{L}{\tau^*}\left(\tau\right)^{1/2}\right]^{1/2}\right)^{1/2}} \quad \text{(Eq. 4.1)} \]

Where L is the sample thickness and τ characteristic decay time, τ = 1/q^2D, q is the wave vector (q = 2πn/λ, where n is the refractive index of the continuous phase and λ is the wavelength (Weitz et al. 1993). \( l^* \) is the transport mean free path, which represents the length scale over which the direction of the light passing through a sample has been fully randomized (Alexander et al. 2006). The value of \( l^* \) can be calculated from the total intensity of the scattered light from the sample and previous calibrated the laser intensity using a standard latex spheres of diameter 260nm (Portland Duke Scientific, Palo Alto, CA, USA). However, this relation only holds true in freely diffusive systems. Once the particle dynamics is changed to a sub-diffusive motion the quantity \( t/\tau \) in eq(1) must be substituted by \( k_0 = \langle \Delta r^2(t) \rangle / 6 \). In this case, the mean square displacement (MSD) can be used to determine changes in the particles’ degrees of freedom, which is defined as the mobility of a scattering particle around its average position (Sandra et al. 2007; Cucheval et al. 2009).
Prior to the measurement, all samples were transferred into an optical glass cuvette (Hellma Canada Ltd., Concord, Ontario, Canada) with a 5 mm path length and then placed in a water bath maintained at 25°C. A solid-state laser light with a wavelength of 532 nm and a power of 350 mW (Coherent, Santa Clara, CA, USA) was used to illuminate the samples. Scattered light intensity was collected in transmission mode and analyzed using software designed specifically for the equipment (Mediavention Inc., ON, Canada). Correlation functions and intensity of transmitted scattered light were measured at intervals of 2 min. The viscosity and refractive index of the continuous phase used to calculate the radius were $1.021 \times 10^{-3}$ Pa.s and 1.34, respectively (Gaygadzhiev et al. 2008). For renneting measurements, correlation functions and intensity of transmitted scattered light were measured for 2 min (118s collection, 2s break) until gelation.

4.3.6 Rheology

The rheometer was used to measure the apparent viscosity of samples and follow the gel formation process. The apparent viscosity of different samples before renneting was measured using a shear rate ramp from 10-100 s$^{-1}$ applied with a controlled stress rheometer (AR 1000, TA Instruments Ltd., New Castle, DE, USA) at 25 °C. The sample was loaded on a cone and plate geometry with a set gap of 0.51 mm. The values are reported for viscosity measured at 100 s$^{-1}$.

The gel formation process of skim and concentrated milk was followed by oscillatory measurements, using a constant strain of 0.01 and a frequency of 1Hz. In this case, a concentric cylinder (28 and 30 mm inner and outer cylinders diameter, respectively) and an external water bath (Isotemp 3016, Fisher Scientific) was used to
control the temperature. Aliquots (20 mL) of sample were transferred to cylinder within 5 min after the addition of chymosin. The milk gelation point is defined as the point when the elastic modulus (G’) and viscous modulus (G’”) cross over (\(\tan \delta = 1\)) (Lucey et al. 1998; Lucey and Horne 2009). This point corresponds to a steep increase in the storage modulus (G’) over time.

4.3.7 Statistical analysis

Experiments were carried out at least in triplicate. ANOVA and Tukey HSD were carried out on the experimental measurements with 95% confidence level using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).

4.4 Results

4.4.1 Influence of NaCl and concentration on soluble protein, soluble calcium and phosphate and \(\zeta\)-potential of casein micelles

Three different levels of concentration were tested, with and without addition of 300 mM NaCl, corresponding to 1x, 3x, 5x of the original skim milk, and a total protein of 3.2, 9 and 13% (w/v) (Table 4.1). The amount of soluble protein was statistically different in samples of 1x milk containing 300 mM NaCl (p<0.05) compared to control. On the other hand, no differences were detected for the 3x and 5x concentrated samples. In all cases, about 30% of protein was recovered in the centrifugal supernatants. As previously reported in the literature (Grufferty and Fox 1985; Gaucheron et al. 2000; Huppertz and Fox 2006a), the addition of NaCl in milk significantly (p<0.05%) reduced the pH of both unconcentrated and concentrated milk, from 6.66 to 6.54 for milk.
containing 300 mM (Table 4.1). It is also important to note that the pH values of milk were not significantly different from 1x milk after concentration, in contrast with what was reported in the literature (Waungana et al. 1999; Karlsson et al. 2007). The difference is due to the fact that ultrafiltration was combined with diafiltration, causing further changes in mineral equilibrium.

Table 4.1 Effect of NaCl and concentration on pH, total and soluble proteins of milk, and zeta potential of casein micelles. Values are means of data from triplicate experiments, ±standard deviation. Values with a different superscript letter are significantly different (p<0.05).

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>pH</th>
<th>Total protein (%)</th>
<th>Soluble protein (%)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x 0 mM 3x 5x</td>
<td>6.66±0.01a</td>
<td>3.29±0.01a</td>
<td>0.84±0.04a</td>
<td>-21.7±1.2a</td>
</tr>
<tr>
<td>3x 3x 1x 5x 300 mM</td>
<td>6.64±0.01a</td>
<td>9.31±0.45b</td>
<td>2.47±0.14c</td>
<td>-20.3±0.6a</td>
</tr>
<tr>
<td>5x 3x 1x 300 mM</td>
<td>6.65±0.01a</td>
<td>13.13±0.29c</td>
<td>4.21±0.19d</td>
<td>-20.1±1.0a</td>
</tr>
<tr>
<td>1x 3x 5x 300 mM</td>
<td>6.53±0.01b</td>
<td>3.28±0.04a</td>
<td>1.02±0.04b</td>
<td>-19.1±0.9a</td>
</tr>
<tr>
<td>3x 3x 1x 300 mM</td>
<td>6.54±0.01b</td>
<td>8.79±0.36b</td>
<td>2.47±0.08c</td>
<td>-15.9±0.5b</td>
</tr>
<tr>
<td>5x 3x 1x 300 mM</td>
<td>6.54±0.01b</td>
<td>13.5±0.58c</td>
<td>4.41±0.15d</td>
<td>-15.6±0.5b</td>
</tr>
</tbody>
</table>
Table 4.1 also summarizes the values of ζ-potential of casein micelles, which in control milk was around -21 mV, and showed no significant influence with concentration (Table 4.1). However, it was clearly noted that the addition of 300 mM NaCl significantly decreased the zeta potential of casein micelles, especially for the concentrated samples, which reached values of about -16 mV. This decrease was induced by the screening effect of NaCl, which will cause a decrease in the electrostatic charge and thickness of the electrical double layer (Bouchoux et al. 2009; Bouchoux et al. 2010). The presence of NaCl reduced the electrophoretic mobility of the colloidal particles. Both NaCl addition and concentration had no influence on the hydrodynamic radius of casein micelles (data not shown). Similar results were also reported before (Huppertz and Fox 2006).

Figure 4.1 describes the changes in total and diffusible calcium and phosphate as the three concentrations for milk with and without NaCl. The total calcium and phosphate increased with concentration, and the addition of NaCl caused a decrease in the amount of calcium and phosphate recovered in the suspensions for 3x and 5x milk. After the addition of NaCl, the diffusible calcium and phosphate increased significantly compared to milk without NaCl (Figure 4.1C and D). These results clearly indicated that the addition of NaCl caused the solubilization of calcium and phosphate from the casein micelles. The results are in full agreement with previous reports (Le Graët and Gaucheron 1999; Huppertz and Fox 2006a; Sbodio et al. 2006). Addition of NaCl clearly decreased the colloidal calcium and phosphate present in the micellar suspensions; the value of colloidal calcium decreased from 309±26 mg/kg/g for 1x milk to 270±30 mg/kg/g after the addition of 300 mM NaCl.
**Figure 4.1** Influence of NaCl addition on total (A, B) and diffusible (C, D) calcium (A, C) and phosphate (B, D). Samples concentrated 1x, 3x, and 5x with (filled circles) and without (empty circles) 300 mM NaCl. Values are average values from triplicate experiments, with the standard deviation indicated by error bars. Lines are drawn to guide the eye.
4.4.2 Apparent viscosity

Figure 4.2 shows the changes in the viscosity, measured at 100 s\(^{-1}\) for milk samples concentrated with or without NaCl. The viscosity of milk increased with concentration. With the addition of NaCl the bulk viscosity increased significantly; in 5x concentrated milk the values changed from 8.7±0.1 to 12.1±0.3 mPa.s. It has been previously reported that the addition of NaCl causes a reduction of the mean free distance between casein micelles (Karlsson et al. 2007), and the closer proximity of the micelles is the reason for the increase in the milk viscosity. It has been recently shown that the addition of NaCl also increases the concentration of soluble casein, and this can also contribute substantially to an increase in the bulk viscosity of the casein suspensions (Famelart et al. 1999; Zhao and Corredig 2015). Other authors reported a similar effect of NaCl in sodium caseinate solutions, and the increase in viscosity was attributed to an increase in osmotic pressure as well as an increase in the protein voluminosity (Abd El-Salam et al. 1987). The presence of NaCl decreases the overall charge of the proteins, causing an increase in protein-protein interactions. All these effects ultimately result in the higher bulk viscosity noted for NaCl containing concentrated milk.

4.4.3 Diffusing wave spectroscopy measurements

The turbidity parameter \(1/l^*\) was measured by transmission DWS (Figure 4.3). As expected there was an increase in the turbidity from 1x to 3x concentrated milk, due to the increased scattering events in a given space (Sandra et al. 2011). The closer spacing between casein micelles will cause the light scattering to be randomized faster, resulting in the decrease of the \(l^*\).
Figure 4.2 Viscosities of milk at different concentration, with (filled symbols) or without (empty symbols) 300 mM NaCl. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.
The additional increase in concentration from 3x to 5x did not further increase the turbidity and no significant difference could be detected between these two concentrations. According to the previous research, the turbidity of milk fit well to the theoretically predicted values for a hard-sphere system up to a concentration factor 3.2 (Dahbi et al. 2010). A maximum turbidity value was obtained at a concentration factor 3.9 and further increase of concentration factor led to decrease of turbidity, due to the occurrence of inter-particle interactions at high concentration (Nair et al. 2014). The addition of 300 mM NaCl to milk caused a significant decrease of the turbidity parameter at all concentrations. The turbidity parameter is a function of the optical properties of the scattering particles as well as a structure factor. At the low concentration (<4x for milk), the system can be regarded as free diffusing (Nair et al. 2014). The negative charge on the surface of casein micelles decreased only slightly with NaCl addition, suggesting that there was still sufficient charge repulsion between these protein particles. The interparticle interactions therefore could be considered negligible, \( S(q) = 1 \). Therefore, the turbidity parameter is only influenced by the scattering form factor \( F(q) \), which is related to the size of casein micelles and refractive index contrast between the scatterers and serum. From the results of DLS, all samples have a hydrodynamic radius value around 85 nm and no significant change in the radius of casein micelles was detected with addition of NaCl. The turbidity parameter change can be attributed to the differences in the refractive index between the scatterers and the serum and it is probably due to the changes of serum refractive index as a result of dissolution of colloidal calcium phosphate and casein release (Zhao and Corredig 2015).
Figure 4.3 Influence of NaCl addition and concentration factor on the turbidity parameter for milk with (filled circles) or without (empty circles) 300 mM NaCl. Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.
4.4.4 Rennet induced gelation

The effect of NaCl on the kinetics of CMP release is shown in Figure 4.4. As previously shown for milk concentrated by ultrafiltration (Sandra et al. 2011), there were no differences in CMP release with concentration. When the CMP release was compared to control milk with no NaCl, it was shown that the presence of 300 mM NaCl caused a decrease in the rate of the CMP release, which is in agreement with previous literatures (Gatti and Pires 1994; Famelart et al. 1999). However, the curves for concentrated milk with NaCl were superimposed and were not significantly different (p<0.05), regardless of concentration factor. All three samples reached about 85% cleavage of caseinmacropeptide at about 80 min, compared to those of control milk, which required only 60 min to reach the critical point for aggregation.

Figure 4.5 shows the changes of the DWS parameters as a function of time, during rennet induced coagulation. The apparent radii were corrected by the Beenakker-Mazur formalism (Beenakker and Mazur 1984) and the 1/\(l^*\) was normalized for better comparison of the gelation behaviour between different treatments. The initial 1/\(l^*\) parameter increased with the concentration (Table 4.2) and increased with gelation time prior to the gelation point, fully consistent with previous literature observations (Sandra et al. 2011), and similar behaviour was shown for samples with or without NaCl. It is important to note that even if the gelation time differed between milk with and without NaCl, the onset of change for 1/\(l^*\) did not show significant differences with concentration or with NaCl.
**Figure 4.4** Amount of caseinomacropeptide (CMP) released as a function of time from the addition of the rennet into the milk samples. 1x untreated milk (○), 1x (●), 3x (▲), and 5x milk (■) containing 300 mM NaCl. Data are the average of three separate experiments, with bars representing standard deviation.
Figure 4.5 Development of normalized $1/l^*_t$ (● ○) and apparent radius (▲ △) during rennet gelation of milk with (filled symbols) and without (empty symbols) NaCl addition and concentration. Data are representative of three separate experiments for 1x (A), 3x (B) and 5x (C).
The initial changes in $1/l^*$ are due to the cleavage of CMP. At this stage, the remaining steric repulsion on the surface of casein micelles can still prevent aggregation. However, once enough CMP has been cleaved, interparticle interactions start to occur and changes of turbidity are noted. The $1/l^*$ increased quickly and significant differences were noted in the evolution of the turbidity parameter during renneting, between control milk and milk with 300 mM NaCl, at all concentrations. The corresponding times for the sharp increase in $1/l^*$ were around 20, 30 and 64 minutes for 1x, 3x and 5x samples respectively (Table 4.2).

In control samples, aggregation occurred around 60 min, consistent with the data reported for CMP release (Table 4.2). The aggregation point did not change with concentration. The addition of NaCl caused a delayed increase of radius (Figure 4.5). Unlike the case of control milk with no NaCl, there was a difference in the onset of aggregation for these samples, whereby the rapid increase of radius happened after 100 min for 1x milk, and the time shifted to 80 and 65 min for 3x and 5x, respectively. The differences in the aggregation behaviour as a function of time with concentration were not related to the primary stage of renneting, as the CMP release was not significantly different between samples containing NaCl at different concentration.

The development of elastic ($G'$) modulus and the loss tangent (tan $\delta$), during the rennet gelation process are summarized in Figure 4.6. Both samples with or without NaCl showed the formation of a stiff network, but with different gelation times.
Table 4.2 Rennet gelation parameters of concentrated milk with or without the addition of NaCl. Values are means of three separate experiments. Values with a different superscript letter are significantly different (p<0.05).

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>Concentration factor</th>
<th>Gelation time from rheology (min)</th>
<th>initial l*/1°</th>
<th>Gelation time from DWS</th>
<th>Elastic modulus after 45 min of gelation point (Pa)</th>
<th>Tan δ plateau value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>1x</td>
<td>59.4±1.4a</td>
<td>1.57±0.05a</td>
<td>58.8±2.0a</td>
<td>14±1a</td>
<td>0.2662±0.0009a</td>
</tr>
<tr>
<td></td>
<td>3x</td>
<td>60.0±1.1a</td>
<td>3.31±0.01b</td>
<td>60.6±1.7a</td>
<td>353±12b</td>
<td>0.2666±0.0008a</td>
</tr>
<tr>
<td></td>
<td>5x</td>
<td>57.4±1.9a</td>
<td>3.46±0.08c</td>
<td>59.8±1.2a</td>
<td>1403±159c</td>
<td>0.2708±0.0006b</td>
</tr>
<tr>
<td>300mM</td>
<td>1x</td>
<td>104.0±4.4b</td>
<td>1.35±0.01a</td>
<td>102.6±0.7b</td>
<td>13±0.7a</td>
<td>0.2596±0.0010c</td>
</tr>
<tr>
<td></td>
<td>3x</td>
<td>80.6±2.8c</td>
<td>2.83±0.05e</td>
<td>83.1±0.9c</td>
<td>286±22d</td>
<td>0.2606±0.0040bc</td>
</tr>
<tr>
<td></td>
<td>5x</td>
<td>67.1±4.8d</td>
<td>2.84±0.07e</td>
<td>70.0±2.1d</td>
<td>933±123e</td>
<td>0.2615±0.0033c</td>
</tr>
</tbody>
</table>

a defined as when delta<45°

b defined as the first value after the addition of rennet

c defined as the crossover of two tangent lines of the radius over time
Table 4.2 also summarizes the stiffness of the gels after 45 min from the gelation point. The curd-firming rate increased with concentration, in full agreement with previous results (Sharma and Hill 1993; Guinee et al. 1996). It is well understood that gels formed from UF-concentrated milk are much stronger compared to gels made from control unconcentrated milk, simply due to the higher numbers of bonds forming between casein micelles. In addition, the increased volume fraction of casein micelles due to UF-concentration reduced the average distance between micelles, which increased the frequency of collisions and the contact area in the gel network, contributing to the increased curd-firming rate (Waungana et al. 1997; Mishra et al. 2005).

In the presence of NaCl, consistent with the DWS observations, there was an increase in the time of gelation, and there was a significant difference with concentration. All samples showed lower stiffness than the same samples without NaCl. The loss tangent values for samples without NaCl addition reached a plateau around 0.27 regardless of concentration, as previously reported (Sandra et al. 2011). Similar loss tangent plateau values (0.26) were reached for samples containing 300 mM NaCl. Higher tan δ value indicates higher rearrangement and flexibility of the structure and more susceptibility to syneresis (Mishra et al. 2005). All the gels formed in this research had similar tan δ values, indicating both the NaCl addition and concentration have no effect on the final milk gel types. It has been reported that addition of NaCl not only can affect the cleavage rate of κ-caseins, but can also influence the aggregation of renneted casein micelles. At NaCl concentrations higher than 200 mM, the rate of aggregation significantly decreases (Bansal et al. 2008).
Figure 4.6 Development of elastic modulus $G'$ (●○), and tan $\delta$ (▲△) during rennet gelation of milk with (filled symbols) and without (empty symbols) NaCl addition and concentration. Data are representative runs for 1x (A), 3x (B) and 5x (C).
4.5 Conclusions

The replacement of Ca$^{2+}$ by Na$^+$ has been reported to cause a change in the voluminosity of the casein micelles (Karlsson et al. 2007). In the present study, no change in the hydrodynamic radius was shown after addition of NaCl. Partial solubilization of the colloidal calcium phosphate weakened the internal bonds between micelles. The total protein and total calcium phosphate increased with concentration (Table 4.1). Addition of NaCl led to the increased soluble calcium phosphate, thus more diffusible calcium phosphate was removed during the ultrafiltration process, resulting in the lower total calcium and phosphate amounts compare to the samples without NaCl (Figure 4.1). NaCl also reduced the negative charge on the surface of casein micelles Table 4.1.

Addition of NaCl led to a significant increase in the rennet coagulation time. This effect could only partly attributed to a decrease in the rate of κ-casein hydrolysis. In the presence of NaCl, there were differences in the gelation time depending on concentration, and at the higher concentrations, gelation occurred before 85% of the CMP was released. This change can be attributed to the decreased average distance between casein micelles as a result of the increased solvation after NaCl addition (Famelart et al. 1999). In addition, the negative charge of casein micelles decreased significantly at high concentrations in the presence of NaCl, as shown in Table 4.1. The presence of high concentration NaCl can also inhibits the aggregation of renneted casein micelles. The slower formation of intra- and intermolecular bonds and decreased colloidal calcium phosphate content (Figure 4.1) in the presence of NaCl lead to the decreased gel stiffness at the defined measure time. It is important to note that during the UF process, the sodium chloride present in the serum phase was also removed, which will change the final
concentration of NaCl in the samples. The final concentration of NaCl added for 3x and 5x samples were 280 mmol.L\(^{-1}\) and 260 mmol.L\(^{-1}\) respectively.

The mechanism for the negative effect of NaCl on the coagulation of milk is still a source of debate. It has been ascribed to the screening of the positively charged cluster of κ-casein and of the chymosin, which inhibit the interaction between chymosin and κ-casein and decreased the enzymatic rate (Visser et al. 1987). On the other hand, concentration had no influence on the gelation time of raw milk and all the samples gelled at about 60 min regardless of concentration (See table 4.2). Similar result was also found in previous research and it was concluded that the changes in rennet coagulation with concentration are merely a cause of crowding effect (Sandra et al. 2011). By contrast, the gelation time of milk with 300 mmol.L\(^{-1}\) addition decreased significantly with concentration, from 104 min to 66 min for 5x concentrated milk. The addition of NaCl influenced both the primary and secondary stage of rennet coagulation. However, all the samples with different concentrations showed similar kinetics of CMP release, indicating that the concentration has no influence on the primary stage of rennet coagulation. The decreased gelation time with concentration is mainly due to changes of aggregation behaviour of renneted casein micelles. At higher concentrations, the decreased average distance negative charge on the surface of casein micelles allows them to diffuse closer to each other and more easily to aggregate with each other. This research can help us better understand the changes of the structure of casein micelles as a function of environmental conditions and processing. The investigation of the structure-function properties of casein micelles can be beneficial for the application of modified casein micelles as ingredient in food products.
CHAPTER 5

EFFECT OF PH-MODIFICATION AND RE-EQUILIBRATION ON MILK SERUM COMPOSITION

5.1 Abstract

The objective of this work was to investigate the properties of casein micelles after pH-adjustment and subsequent re-equilibration, as a function of the temperature of re-equilibration (4 or 22°C). The changes in turbidity, the average radius of the casein micelles and the composition of the soluble phase were measured. Acidification led to the solubilisation of colloidal calcium phosphate and decrease of the average radius of the micelles. With re-equilibration, casein dissociation occurred. In milk with pH values greater than 6.0, the average radius was recovered after re-equilibration. At pH values greater than neutral there was an increase of the radius of casein micelles and increased dissociation of the casein. After re-equilibration, the radius of micelles and soluble protein in the serum decreased. The results were not affected by the temperature of re-equilibration. This research clearly demonstrated that partial changes to the calcium phosphate equilibrium can lead to important changes of the functionality of casein micelles.
5.2 Introduction

Caseins constitute about 80% of the total protein in milk. They are present in the form of casein micelles (Dalgleish and Corredig 2012). During acidification, there is a release of colloidal calcium phosphate from the micellar phase to the soluble phase, with a change in the supramolecular structure of the casein micelles. A number of methods have been adopted to demineralise casein micelles, including acidification (Dalgleish and Law 1988; Dalgleish and Law 1989; Lucey et al. 1996) and the use of chelating salts (Griffin et al. 1988; Ward et al. 1997; Augustin and Clarke 2008; Ozcan et al. 2011). In all these studies, it was reported that demineralization causes the disruption of the structure of the casein micelles and changes in their processing functionality.

In 1960, McGann and Pyne used dialysis to re-equilibrate the composition of the soluble phase to study modifications to the colloidal calcium phosphate fraction in milk. Dialysis has since been adopted as a tool in the study of mineral equilibrium in milk, to investigate, for example, the role of calcium phosphate on the heat stability (On-Nom et al. 2010; On-Nom et al. 2012) and acid gelation (Anema 2009; Ozcan et al. 2011), or to re-equilibrate the soluble phase in the samples. However, it is not known if the temperature during re-equilibration by dialysis has an effect on the composition of the serum phase. This has important consequences not only in better standardize experimental practices, but to better understand the changes that may occur when modified milk ingredients are incorporated in milk during processing.

Recently, it was shown that partial acidification, followed by re-equilibration, causes demineralization and disruption of casein micelles. However, in this research, all the samples were freeze dried and then reconstituted, and all the re-equilibration
experiments were conducted at 4°C (Silva et al. 2013). The dissociation of caseins is temperature-dependent and the amount of soluble casein is higher at 4°C than at room temperature (Dalgleish and Law 1988; Law and Leaver 1998). Hence, it is not known whether re-equilibration after acidification will be affected by temperature. The objective of this research was to study the impact of re-equilibration temperature on casein dissociation during re-equilibration. The milk was adjusted to various pH values at 4°C and then re-equilibrated by dialysis against skim milk at 4 and 22°C.

5.3 Materials and Methods

5.3.1 Sample preparation

Fresh untreated milk (Elora Dairy Research Station, Elora, ON, Canada) was skimmed by centrifugation at 4000 x g for 25 min at 4 °C (J2-21 centrifuge, Beckman Coulter Canada Inc, Mississauga, Canada) and then filtered four times through a Whatman fibreglass filter (Fisher Scientific, Mississauga, Ontario, Canada). Pasteurized skim milk (Crown Dairy, ON, Canada) was also employed to prepare permeate, by filtration with an OPTISEP® filter module (Smartflow Technologies, Apex, NC, USA) with a molecular weight cut-off of 10kDa, at room temperature (22°C). Sodium azide was added at a concentration of 0.01 % (w/v) to prevent bacterial growth.

Fresh skimmed untreated milk was acidified to different pH values at 4°C by adding different concentrations of glucono-delta-lactone (GDL). Two samples were also prepared by adjusting the pH to 7.1 and 8.0 using 2 mol.L⁻¹ NaOH. All samples were left at 4°C overnight. Before re-equilibration by dialysis, samples were left for 2 h at room temperature. Cellulose dialysis bags (Fisher Scientific, Whitby, Ontario, Canada) with a molecular mass cutoff of 6–8 kDa were used, to allow the exchange of small molecules
such as water, ions, and lactose but not proteins (Bouchoux et al. 2009). Milk samples (40 mL) were placed in the dialysis bags and immersed in 1 L pasteurized skim milk. To determine if the temperature of the re-equilibration has an effect on the composition of the serum phase, the dialysis process was conducted at both 4 and 22°C for 18 h. The pH values after re-equilibration were measured and all samples showed similar values to untreated milk.

5.3.2 Physico-chemical characterization of the suspensions

In this research, Transmission diffusing wave spectroscopy (DWS) was used to measure the turbidity parameter (1/l*) as previously explained (Weitz et al. 1993; Gaygadzhiev et al. 2008).

The particle size of the casein micelles was measured by dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). The milk samples were diluted 1000 times with filtered (0.2 μm nylon syringe-driven filter, Fisher scientific) permeates that prepared by ultrafiltration and adjusted to the same pH values as milk samples with 1 mol.L⁻¹ HCl and NaOH, if needed.

The serum phase was separated by ultracentrifugation at 100,000 x g for 1 h at 20°C (Optima TM, LE-80K Beckman Coulter Canada Inc., Mississauga, Canada), after equilibration of the samples at room temperature for 2 h. The supernatants were filtered using 0.45 μm filters (syringe driven filters, Fisher Scientific) and then further analyzed for soluble calcium phosphate and protein.

The amount of total and soluble calcium present in the samples was determined using non-suppressed ion chromatography as previously described in detail (Rahimi-
Yazdi, Ferrer, & Corredig, 2010). The soluble calcium was defined as the calcium in the serum phase after centrifugation at 100,000 x g. In addition to soluble calcium, diffusible calcium, which was defined as the free calcium in the serum and was prepared by filtering the soluble serum phase using ultrafiltration tubes (Corning, New York, US) with a nominal molecular weight cut-off of 10KDa. The colloidal calcium was calculated by subtracting the amount of soluble calcium from the total calcium.

Protein concentration in the serum phase was determined using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) using 6.38 as conversion factor. The protein composition of supernatants was subsequently analysed by SDS-PAGE under reducing conditions; the resolving and stacking gel containing 15% and 4% acrylamide, respectively. All the soluble samples were diluted 1:1:1 with Milli-Q water and sample buffer (0.5M Tris-HCl, pH 6.8, 20g/kg SDS, 190g/ kg glycerol, 0.5g/ kg 2-mercaptoethanol, 0.1g.kg$^{-1}$ bromophenol blue) and then heated for 5 min at 95 °C. Samples (6 μl) were loaded and gels were run at 175V for 45 min using Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Hercules, CA), stained with Coomassie blue and destained with a mixture of methanol, acetic acid and Milli-Q water according to manufacturer’s instructions.

5.3.3 Statistical analysis

Three or four replicates were performed for each test. ANOVA and Tukey HSD were carried out on the experimental measurements with 95% confidence level (Minitab, V. 15, Minitab Inc., State College, PA, USA).
5.4 Results and Discussion

5.4.1 Distribution of Calcium between serum and colloidal phases

Calcium equilibrium between serum and colloidal phases plays an important role in the casein micelle structure and affects the processing functionality of the casein micelles (Holt et al. 1986; Lewis 2011). It has been previously shown that the composition of the soluble fraction of milk is affected by temperature. Hence, the objective of this work was to better understand how the temperature of re-equilibration may affect the composition of the soluble phase and the physico-chemical characteristics of the casein micelles.

In this research, both the soluble and diffusible calcium and phosphate before and after re-equilibration, as a function of the pH were measured, as shown in Figure 5.1. After pH-adjustment, soluble and diffusible calcium and phosphate increased with decreasing pH, in agreement with previous research (Anema 2009; Silva et al. 2013). There were no significant differences between samples re-equilibrated at 4 °C and 22 °C (p<0.05). A similar behaviour was also noted for diffusible calcium (Figure 5.1B) and phosphate (Figure 5.1D). The concentration of diffusible calcium was also constant and fully recovered compared to the control, with no differences with temperature. The colloidal calcium and phosphate were also calculated as the difference between the total calcium and soluble calcium. As expected, acidification caused the dissolution of colloidal calcium phosphate, after re-equilibration, the total and colloidal calcium and phosphate declined gradually with decreasing pH (data not shown).
Figure 5.1 Amount of soluble (A, C) and diffusible (B, D) calcium and phosphate before (○-) and after re-equilibration at 4 (▲-) or 22 °C (△-). Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.

It has been previously reported that increasing the pH of skim milk may lead to the decrease of Ca$^{2+}$ concentration in the serum phase (Lucey and Horne 2009; Ozcan et al. 2011). In this work, the amount of total calcium and phosphate, and more importantly, colloidal calcium and phosphate, did not show significant changes after re-equilibration against milk regardless of the temperature. Previous research has shown that the solubilisation of colloidal calcium was not influenced by the temperature of acidification – albeit, the same author showed a difference in casein dissociation from the micelles (Dalgleish and Law 1989).
5.4.2 Soluble protein in the serum

The amount and composition of soluble protein recovered in the samples as a function of pH before and after re-equilibration is illustrated in Figure 5.2. The amount of soluble protein was not significantly different for all samples between pH 5.5 and 7.0, and showed a significant increase at pH 8.0. The results shown in Figure 5.2A clearly demonstrate that the re-equilibration greatly affected the integrity of the casein micelles, in milk acidified at pH <6.2. Samples acidified at pH 6.0 and 5.5, after re-equilibration, regardless of temperature, showed a substantial increase in the amount of soluble protein, from 1% to 1.3% and 2.4%, respectively. It is known that the casein dissociation during acidification is temperature–dependent, with more casein dissociated at 4 °C than 20 °C (Dalgleish and Law 1988). The results shown in Figure 5.2A show no effect of re-equilibration on dissociation.
**Figure 5.2 A.** Concentration of soluble protein before (-●-) and after re-equilibration at 4 (-▲-) or 22 °C (-∆-). Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars. SDS-PAGE under reducing conditions of centrifugal supernatants: before re-equilibration (B) after re-equilibration at 4 °C (C) and after re-equilibration at 22 °C (D). Each lane represent sample with different pH: Lane 1 (pH 5.5), lane 2 (pH 6.0), lane 3 (pH 6.3), lane 4 (pH 6.7), lane 5 (pH 7.1), lane 6 (pH 8.0). The main proteins are identified: αs-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin.

There was a significant difference between the amount of soluble protein before or after re-equilibration for the milk with pH 8.0. It has been previously reported (Vaia et al. 2006) that dissociation of the micelles occurs at this pH. After re-equilibration, the soluble protein concentration in the serum phase decreased, again, regardless of temperature. It was concluded that during re-equilibration, protein-protein interactions occurred causing precipitation of the caseins.

To further understand the changes of soluble protein composition during pH-adjustment and re-equilibration process, the supernatants were analyzed by SDS-PAGE under reducing conditions, as shown in Figure 5.2B-D. Before re-equilibration,
increasing pH to 8 significantly increased the amount of soluble caseins present in the supernatant (Figure 5.2B). Figures 5.2C and D show the composition of the serum fraction after re-equilibration at 4 °C and 22 °C, respectively. The intensity of the casein bands for samples acidified at pH 6.0 and 5.5 increased greatly. On the other hand, re-equilibration of alkalinized samples decreased the casein bands, when compared to Figure 5.2B. In summary, there was no difference in the whey proteins bands. There was no effect of re-equilibration temperature on the protein composition of the samples after re-equilibration by dialysis.

5.4.3 Influence of pH-adjustment and re-equilibration on light scattering parameters

Both acidification and alkalinization caused a decrease in turbidity (Figure 5.3). Changes in turbidity of milk have been previously reported (Lieske et al. 1999; Orlien et al. 2010). Acidification increased soluble calcium, reduced the negative charge on the surface of the caseins, causing partial collapse of the κ-casein polyelectrolyte layer. There were no significant differences in the amount of soluble casein in the serum phase, as shown in Figure 5.2A. Hence, the decrease in turbidity shown in Figure 5.3A was caused by a change in the refractive index contrast between the casein micelles and the serum phase, and a change in the hydrodynamic size of the casein micelles. At pH 8, the turbidity was also lower than at natural pH, but in this case, there was an increase in the amount of soluble caseins (Figure 5.2A). After re-equilibration to the original pH, regardless of temperature, the turbidity of all samples previously acidified at pH <6.5 declined significantly. This was a consequence of a significant disruption of the casein micelles, as shown in Figure 5.2. However, no difference was found between samples re-
equilibrated at 4 °C and 22 °C. In contrast with the acidified samples, in the milk adjusted to pH >7, the turbidity parameter increased after re-equilibration. This may be a result of increased protein-protein interactions (Nair et al. 2014).

The radius of pH-modified casein micelles before and after re-equilibration was determined by DLS (Figure 5.3B). Before re-equilibration, the radius of casein micelles decreased gradually with acidification, with a large drop in the diffusivity of the casein micelles at pH 5.5. Alkalisation led to the increase of the average radius of the casein micelles. Similar results have been reported before, and have been attributed to the partial collapse of the hairy layer and the change in hydration of the casein micelles (Le Graët and Gaucheron 1999; Anema et al. 2007). No data is available on the size of the casein micelles after re-equilibration at different temperatures.
Figure 5.3 $1/l^*$ (A) and hydrodynamic radius (B) of milk as a function of initial pH before re-equilibration (●-●), after re-equilibration at 4 (-▲-) or 22 °C (-Δ-). Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars.
After re-equilibration of milk adjusted between pH 6.0 and 6.8 there were no significant differences in the radius of casein micelles. For the sample acidified at pH 5.5, a smaller size was measured after re-equilibration, consistent with extensive disruption of the casein micelles. No difference could be found between samples re-equilibrated at 4°C and 22°C. Samples adjusted to pH >6.8, after re-equilibration, showed a smaller, albeit significant change in the size of the casein micelles. In this case, there was a clear effect of temperature: the average radius of casein micelles after re-equilibration at 4°C was significantly higher than that of micelles re-equilibrated at 22°C (p<0.05).

5.5 Conclusions

Acidification decreased the colloidal calcium phosphate content, while alkalinisation increased the solubilisation of the caseins from the micelles. Re-equilibration of acidified samples caused dissociation of the casein micelles, while in samples originally at pH above 7, there was a decrease in the soluble caseins. There were no differences in the calcium and soluble protein composition after dialysis at 4 or 22°C, indicating that re-equilibration temperature did not affect the properties of the micelles.
CHAPTER 6

DETERMINATION OF CHANGES IN VOLUMINOSITY OF CASEIN MICELLES WITH PH BY MEASURING THEIR COLLOIDAL BEHAVIOUR AS A FUNCTION OF CONCENTRATION

6.1 Abstract

Milk acidification causes changes to the structure and stability of the casein micelles. In this research the physico-chemical properties of casein micelles were studied in milk suspensions adjusted to pH 6 and 5.6, as a function of concentration, and compared to suspensions at the native pH (6.7). Skim milk was acidified by addition of glucono-δ-lactone and concentrated using osmotic stressing. The apparent radius of the casein micelles, measured after redilution in their corresponding permeate, decreased with decreasing pH, but showed no differences with concentration. Furthermore, the casein micelles showed free diffusive behaviour at all three pH values. Only suspensions at pH 5.6 showed hindered diffusion at 4x concentration. At protein concentrations > 6% there was a larger amount of dissociated casein with decreasing pH. The relative viscosity of the concentrated suspensions was modeled according to the behaviour of colloidal hard spheres, taking into account hydrodynamic interactions. The voluminosity of the casein micelles was estimated to be 3.5 and 3.1 for pH 6 and 5.6, respectively. The maximum packing volume was estimated by fitting the relative viscosity as a function of volume fraction. At pH 6.0 the casein micelles suspensions still showed a high maximum packing
volume, due to their decrease in voluminosity, the release of calcium phosphate and the presence of sufficient charge repulsion. On the other hand, at pH 5.6, the stability of the micelles was reduced, and they reached maximum packing at 0.38 volume fraction. Furthermore it was suggested that at 3x and higher concentration, the voluminosity values should further decrease, as the fit was no longer valid at these concentrations. This research contributed to a better understanding of the colloidal properties of casein suspensions as a function of pH and concentration, allowing optimization of their utilization as ingredients in foods.

6.2 Introduction

Caseins constitute 80% of the total protein in milk, with a concentration of about 25 g.L⁻¹. At the native pH of milk, caseins are self-assembled in a supramolecular structure referred to as the casein micelle. Casein micelles are composed of four different caseins, \( \alpha_{s1} \), \( \alpha_{s2} \), \( \beta \) and \( \kappa \)-casein in approximate ratios of 4:1:3.5:1.5, together with calcium phosphate (Dalgleish 2011). The highly phosphorylated caseins (\( \alpha \) and \( \beta \)-caseins) interact with nanoclusters of calcium phosphate (CCP), constituting approximately 8% (w/w) of the total micelles weight (Holt 1992; De Kruif et al. 2012; Dalgleish and Corredig 2012). The \( \kappa \)-casein is mainly present on the surface of casein micelles, providing electrostatic and steric repulsion forces that impart colloidal stabilization (De Kruif and Zhulina 1996). The distribution of calcium phosphate between the colloidal phase and the serum phase is in a dynamic equilibrium, which is affected by changes of environment, such as pH, temperature and other minerals (Holt 1998; Zhao and Corredig 2014).
Although casein micelles have been widely studied, understanding their structural changes during processing steps such as membrane filtration, concentration and acidification, is still a challenge (De Kruif 1998; Dalgleish and Corredig 2012; Liu et al. 2012; Nair et al. 2013; Li and Corredig, 2014). This is of particular relevance with the widespread application of pre-treatments to retentates before or during membrane concentration, to modify the functionality of the casein micelle (Eshpari et al. 2014; Marella et al. 2015). For example, the injection of carbon dioxide before membrane concentration has been shown to increase the solubility of the powders, due to the increased dissociation of the casein from the micelle (Marella et al. 2015).

It is known that the viscosity of casein micellar suspensions increases with increasing concentration in a non-linear fashion, and a transition occurs from Newtonian to non-Newtonian behaviour at a volume fraction around 0.4 (Bouchoux et al. 2009; Nair et al. 2014). Furthermore, it has been reported that the diffusivity of casein suspensions closely follows the behaviour of hard-sphere suspensions up to volume fraction 0.4 (De Kruif 1992; De Kruif 1998; Nair et al. 2014), and their rheological behaviour fits well with the Mendoza model for solid spheres suspensions (Mendoza and Santamaría-Holek 2009) reaching maximum packing volume fractions of about 0.8. This model takes into account the hydrodynamic interactions between colloidal particles. The critical packing volume fraction for casein micelles in untreated skim milk has been shown to be about 0.8 (Karlsson et al. 2005; Nair et al. 2014).

The effect of processing on casein micelles voluminosity is not fully understood. Voluminosity is defined as the ratio between the volume occupied by the particles and their protein concentration. It has been recently shown (Liu et al. 2012) that during the
evaporative concentration process, water is removed preferentially from the serum, and it was concluded that the voluminosity of the casein micelles remains constant until high volume fraction (4x). However, this may not be the case for acidified milk. During the acidification process, the CCP dissociates gradually (Jacob et al. 2011), the net charge of casein micelles decreases and caseins are released in the serum phase, depending on the temperature (Gastaldi et al. 1994). Changes in pH result in changes in the physico-chemical properties that affect their processing functionality (Augustin and Clarke 2008; Nair et al. 2014).

Little research is available on the influence of concentration on the colloidal behaviour of pH-modified casein micelles. In particular, the changes in size and voluminosity of the micelles are often estimated using methods that apply extensive changes to the environment of the casein micelles. The solubilisation of colloidal calcium phosphate is affected by the volume fraction of the casein micelles and with concentration, there is an increased amount of casein dissociated in the soluble fraction (Li and Corredig 2014). These changes will affect the voluminosity of the casein micelles. The objective of this study was to investigate the influence of pH-modification on the colloidal properties of the casein micelles in concentrated milk. Milk was acidified to pH 6.0 and 5.6 and then concentrated using osmotic stressing, a gentle technique that minimizes shear effects and maintains a constant composition of the serum phase. Hence, the rheological properties of casein micelles at different pH values could be studied as a function of volume fraction, while maintaining a constant serum composition. It was hypothesized that at a lower pH, the release of casein from the micellar fraction and the release of calcium and phosphate would affect the apparent diameter and voluminosity of
the casein micelles, as well as their estimated maximum packing volume and their
diffusivity. A better understanding of the changes occurring at pH values above the
isoelectric point of the caseins is necessary to optimize processing conditions.

6.3 Materials and Methods

6.3.1 Sample preparation

Fresh raw milk was obtained from the Elora Dairy Research Farm of University
of Guelph (Elora, ON, Canada). Sodium azide (0.1 g.L⁻¹) was added to the milk
immediately as a bacteriostatic agent. The milk was skimmed by centrifugation at 4,000 x
g for 25 min at 4 °C (J2-21 centrifuge, Beckman Coulter Canada Inc, Mississauga,
Canada) and then filtered four times through Whatman fibreglass filter (Fisher Scientific,
Mississauga, Ontario, Canada).

To reach pH 6.0 and 5.6, glucono-delta-lactone was added to skim milk at
concentrations of 0.35%, and 0.57%, respectively. The milk was stirred for 5 min and
then refrigerated.

Osmotic stressing was used to concentrate the milk suspensions (Nair et al. 2014).
A standard regenerated cellulose dialysis tube (Spectra/Por 1, Fisher Scientific, Whitby,
Ontario, Canada) with a molecular mass cut-off of 6-8 kDa was used. This pore size
ensured the exchange of water, ions, and lactose but not proteins or PEG. The dialysis
membrane was washed three times with Milli-Q water and then conditioned in Milli-Q
water before experiments. Samples (40 mL) of the pH-adjusted milks were placed in
dialysis bags and immersed in 1 L milk adjusted to the pH of the micellar suspensions,
and containing different concentrations of stressing polymer, polyethylene glycol (PEG).
All milk contained 0.1 g.L⁻¹ sodium azide as a bacteriostatic agent. The use of milk at the
The corresponding pH values ensured the same ionic composition across the dialysis membrane. The dialysis process was conducted for 18 h at 4°C with stirring, to minimize sample degradation.

**6.3.2 Analysis of the soluble fraction**

The soluble fraction was defined as the fraction that did not sediment after ultracentrifugation at 100,000 x g for 1 h at 20 °C (Optima™ LE-80K ultracentrifuge with rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada) (Nair et al. 2013). All of the concentrated milk samples were equilibrated at room temperature for 1 h before centrifugation. The separated supernatants were carefully removed and then filtered using 0.45 μm filters (low protein binding, Fisher Scientific). Protein analysis was carried out using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) and protein content was calculated using 6.38 as conversion factor.

Total, soluble and diffusible calcium and phosphate were measured. The soluble calcium and phosphate were analyzed from the centrifugal supernatant (see above). Diffusible calcium and phosphate were also measured, filtering the supernatants with 10 kDa ultrafiltration tubes (Corning, US). The tubes were centrifuged at 4,000 x g for 1 h to separate the residual soluble proteins from the calcium and phosphate fraction unbound to protein.

To determine the total calcium, aliquots (666 μL) of milk samples were mixed with 400 μL of 1 M HCl and 266 μL HPLC water in an Eppendorf microcentrifuge tube. The mixture was then centrifuged at room temperature for 15 min at 7,600 x g (Eppendorf centrifuge, 5415 D, Brinkmann Instruments Ltd., Mississauga, Ontario,
Canada). The clear supernatants and corresponding diffusible parts after ultrafiltration of the supernatants were then diluted directly with HNO$_3$ acceptor solution (2 mmol.L$^{-1}$ HNO$_3$ solution). For determination of total calcium, the supernatants were diluted 300 times. For the determination of soluble and diffusible calcium, the supernatants were diluted 100 times.

For total and soluble phosphate measurements, 1 mL milk sample or supernatant was transferred to the Pyrex test tubes and then heated at 100°C overnight to dry the samples, followed by a mineralization at 500 °C for 6 h in an Isotemp muffle furnace (Fisher Scientific). The residual ashes were dissolved in 1 mL of 1 mol.L$^{-1}$ HNO$_3$. Samples were diluted 300 times with HPLC water before analysis by ion chromatography. The diffusible fraction was diluted 200 times.

Calcium and phosphate measurements were carried out using 861 Advanced Compact IC (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland) (Rahimi-Yazdi et al., 2010). To determine the calcium, a cation exchange column (Metrosep C4/150, Metrohm) packed with 5 μm silica gel with weakly acidic carboxyl acid functional groups (RCO$_2^-$) group was employed. Pyridine-2, 6-dicarboxylic acid (99% Acros Organics, Geel, Belgium) and 70% nitric acid were used to prepare the mobile phase (0.7 mM dipicolinic acid and 1.7 mM HNO$_3$). For the determination of phosphate, an anion exchange column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5 μm polyvinyl alcohol with quaternary ammonium groups was employed. Sodium hydrogen carbonate and sodium carbonate solutions were used to prepare mobile phase (1.0 mol.L$^{-1}$ sodium carbonate and 4 mol.L$^{-1}$ sodium hydroxide). The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration.
curves prepared with calcium or phosphate standards (TraceCERT, Fluka, Sigma Steinheim, Germany). Samples were eluted at a flow rate of 0.9 and 0.5 mL.min\(^{-1}\) for calcium and phosphate measurement, respectively.

### 6.3.3 Light scattering measurements

The hydrodynamic radius of casein micelles was determined by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Worcestershire, UK), after extensive dilution with milk permeates. Milk permeates with different pH values were prepared using an ultrafiltration cartridge (10 KDa Millipore CDUF001LG, Fisher Scientific), and were then filtered through 0.2 μm nylon filters (Fisher Scientific) before use. All of the concentrated micellar samples were diluted 1,000 times into the filtered permeates immediately before the measurements.

To further investigate the colloidal properties of casein micelles as a function of pH, without dilution, transmission diffusion wave spectroscopy (DWS) was employed as previously described (Alexander et al. 2002). The turbidity parameter (1/\(l^*\)) was used to determine the optical properties of different samples. The photon transport mean free path \(l^*\) represents the length scale over which the direction of the scattered light has been totally randomized. In addition, for a freely diffusing system, the apparent diffusion coefficient (D) can be derived from the characteristic decay time of the intensity autocorrelation functions and used to calculate the apparent particle radius using Stokes-Einstein relation (Alexander et al. 2006; Sandra et al. 2011). When the particle dynamics are changed to a sub-diffusive motion, then the mean squared displacement (MSD) values can be calculated to probe the system dynamics instead of apparent diffusion coefficient.
(D). When the measurement time is much smaller than the characteristic time of the system, the MSD can be written as:

\[ \langle \Delta r^2 (t) \rangle \propto t^p \] \hspace{1cm} \text{Eq. 6.1}

The value of the exponent p is 1 for a freely diffusing particle while in an arrested system the value of p is always less than 1 (Krall and Weitz 1998; Romer et al. 2000).

All samples were poured into an optical glass cuvette (Hellma Canada Ltd., Concord, Ontario, Canada) with a 5 mm path length and then placed in a water bath maintained at 25°C. A solid-state laser with a wavelength of 532 nm and a power of 350 mW (Coherent, Santa Clara, CA, USA) was used to illuminate the samples. Scattered light intensity was collected in transmission mode and analyzed using software developed specifically for the equipment (Mediavention Inc., ON, Canada). Correlation functions and intensity of transmitted scattered light were measured at intervals of 2 min.

### 6.3.4 Rheology measurements

The viscosity of the concentrated suspensions, as well as the centrifugal supernatants was determined using a controlled stress Rheometer AR 1000 (TA Instruments Ltd., New Castle, DE, USA) equipped with a Peltier temperature controller. The viscosity of the supernatant was used to derive the relative viscosity of the concentrated casein micelles suspensions. All samples were subjected to a steady flow test (shear rate ramp from 0.1-300 s\(^{-1}\)), using a cone and plate geometry, with a set gap of 0.51 μm. The values of viscosity measured at 300 s\(^{-1}\) were used for the fit of the Mendoza model for solid sphere suspensions. in the calculation of the relative viscosity (Mendoza and Santamaría-Holek 2009). This model takes into account the hydrodynamic
interactions between the colloidal particles.

\[ \eta = \eta_0 \left[ 1 - \left( \frac{\varphi}{1-c\varphi} \right)^{5/2} \right] \quad \text{Eq. 6.2} \]

Where \( \eta \) is the sample viscosity, \( \eta_0 \) the soluble phase viscosity, \( \varphi \) the volume fraction. The value of \( \varphi = C \cdot v \), where \( C \) is the protein mass concentration (g.mL\(^{-1}\)) and \( v \) is the protein voluminosity. The value of voluminosity for casein micelles in native milk is 4.4 cm\(^3\)g\(^{-1}\) (Holt, 1998; Nair et al. 2014). In the equation, \( c \) represents the ratio between \( [(1- \varphi_{\text{max}})/\varphi_{\text{max}}] \), where \( \varphi_{\text{max}} \) is the critical packing volume fraction of the dispersed particles. This is the concentration where the suspension loses its fluidity. It is important to note that this model could be applied to particles of different shape by changes to the value of the exponent (Mendoza and Santamaria-Holek, 2009); however, in this study, it was assumed that casein micelles could be approximated to spherical particles.

The Mendoza model has been shown to fit well both unheated and heated milk as a function of volume fraction (Nair et al. 2013; 2014). By assuming a hard sphere behaviour for the casein micelles, and by deriving their size from the average apparent diameter measured by light scattering, the maximum volume fraction as a function of pH for the concentrated protein suspensions could be derived, by best fit calculations using sigma plot (version 12.1, Systat Software Inc., San Jose, CA, USA).

### 6.3.5 Statistical analysis

Three or four replicates were performed for each test. ANOVA and Tukey HSD were carried out on the experimental measurements with 95% confidence level using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).
6.4 Results and Discussion

6.4.1 Composition of the soluble phase

Casein micelles are very sensitive to pH changes; decreasing the pH in milk leads to a decrease in the charge of the proteins and the dissociation of colloidal calcium phosphate and caseins from the micellar phase (Dalgleish and Law 1988; Dalgleish and Law 1989). These changes are also function of casein micelles volume fraction (Li and Corredig, 2014). In this work, the concentration of soluble protein was measured in centrifugal supernatants after separation at 100,000 x g. The results are shown in Figure 6.1 for suspensions at three pH values: pH 6.7, 6.0 and 5.6, as a function of concentration. In all samples, the amount of protein in the serum fraction increased with increasing total protein concentration. This was expected, as both caseins and whey proteins are retained by the membrane during the osmotic concentration process.

The results clearly indicated that at low protein concentrations (up to 5.5%), there were no significant differences in the amount of soluble protein recovered as a function of pH. The extent of dissociation was similar, regardless of the pH of the suspension. At protein concentrations >6%, the amount of soluble protein increased in the suspensions with lower pH, and the extent of dissociation increased with decreasing pH. The lowest level of soluble protein was found at the native pH of milk (pH 6.7). At this pH, the increase with concentration seemed to follow a linear behaviour, with a slope of about 0.31. It is known that as the pH decreases, colloidal calcium phosphate dissociates and caseins are released into the serum (Dalgleish and Law 1988; Moitzi et al. 2011; Li et al. 2015).
Figure 6.1 Concentration of protein in the supernatant after centrifugation at 100000 x g as a function of total protein concentration, for samples at pH 6.8 (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average values of triplicate experiments, with the standard deviation indicated by error bars.

At pH 6 and 5.6, the protein concentration as a function of total concentration was no longer linear; suspensions at pH 5.6 showed the highest concentration of protein in the supernatant, followed by casein suspensions at pH 6.0. The dissociation of protein from the micelles will affect their colloidal behaviour.
The concentration of soluble and diffusible calcium and phosphate as a function of protein concentration in the various suspensions was also measured, as shown in Figure 6.2. It is known that the equilibrium of minerals between the serum phase and the colloidal phase is affected by environmental changes as well as protein concentration (Holt et al., 1986; Li and Corredig, 2014) and it plays an important role in the processing functionality of the casein micelles (Lucey et al. 1996; Dalgleish and Corredig, 2012). When milk is concentrated using osmotic stressing, the small ions and water diffuse across the dialysis membrane, keeping the serum phase relatively constant, similarly to what would occur during membrane concentration (such as UF and MF).

Previous work already demonstrated that at the native pH of milk, the amount of soluble calcium increases with protein concentration in the dispersions in a linear fashion (Anema 2009; Liu et al. 2012; Nair et al. 2014). This is clearly shown in Figure 6.2A (circles). This behaviour is different than what reported for milk concentrated by evaporation, where the total solids in the serum phase increase, and there is a decrease of soluble calcium phosphate (Liu et al. 2012).

As shown in Figure 6.2A, the amount of soluble calcium increased as a function of concentration in all suspensions, but if increased to a larger extent in suspensions at pH 6.0 and 5.6, and at a higher rate compared to suspensions at the native pH of milk. These results were in full agreement with the data reported on protein solubility (Figure 6.1).
Figure 6.2 Changes of soluble (A and B) and diffusible (C and D) calcium and phosphate as a function of concentration for samples with different pH: pH 6.7 (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average of three independent experiments, and bars represent standard deviation. Lines are drawn to guide the eye. Note that symbols may be larger than error bars.
The highest amount of soluble calcium was shown in suspensions at pH 5.6. Similarly to calcium, the amount of soluble phosphate also increased as a function of protein concentration (Figure 6.2B) with a higher extent for suspensions at pH 6.0 and 5.6, however, in this case the amount of phosphate released in the soluble phase seemed to reach a plateau at pH 6.0.

To distinguish the ions associated to the protein from the soluble ions, the concentration of ions present in ultrafiltered serum (diffusible ions) was also measured, as shown in Figure 6.2 C and D for calcium and phosphate, respectively. Although acidification of milk is reported to progressively solubilise the colloidal calcium phosphate with a marked increase in the solubilisation below pH 5.6 (Lucey and Horne 2009), no information is available as a function of concentration for pH-modified milk suspensions.

Figure 6.2 compares for the first time the data of soluble with diffusible calcium and phosphate, leading to an increased understanding of the difference between the inorganic calcium and phosphate and that associated with the protein. Diffusible calcium and phosphate showed similar trends than what shown for soluble calcium and phosphate. There was a higher recovery of ions in the soluble fraction compared to the diffusible fraction, because of the difference in calcium and phosphate associated to the proteins in the soluble phase. As expected, there was a significantly higher soluble and diffusible calcium and phosphate at pH 5.6 than at 6.8. There was a larger difference with pH in the amount of calcium present, while in the case of phosphate, there was little difference between concentrations in the soluble phase at pH 6 and 5.6. It is important to note that in addition to the soluble phosphate and the phosphate released from the colloidal calcium
clusters, the amount of phosphate present in the supernatant also includes the phosphate present on the phosphorylated sites of the protein. The little difference in soluble phosphate in dispersions adjusted to pH 6 and pH 5.6, would suggest that at pH 5.6, albeit a higher concentration of protein was present in the supernatant (Figure 6.1) compared to pH 6.0, the amount of phosphorylated caseins was similar.

The amount of colloidal calcium phosphate residual in the casein micelles suspension was calculated as the difference between the total calcium and phosphate and that present in the soluble fraction. As expected, the amount of colloidal calcium associated with the micellar fraction decreased with decreasing pH. At 1x concentration, the amount of colloidal calcium in control skim milk was \(689 \pm 21\) mg kg\(^{-1}\) and decreased to \(342 \pm 86\) and \(229 \pm 50\) mg kg\(^{-1}\) at pH 6.0 and pH 5.6, respectively. This corresponded to 26 mg/g of caseins in dispersions at pH 6.8, and 8.8 mg/g of caseins for dispersions at pH 5.6. At 4x concentration, the amount of colloidal calcium decreased from \(2643 \pm 209\) mg.Kg\(^{-1}\) in control milk at pH 6.8, to \(1677 \pm 22\) and \(1236 \pm 107\) mg kg\(^{-1}\) for pH 6.0 and pH 5.6, respectively. As expected the amount of colloidal calcium phosphate was affected by pH, but to a much less extent by the protein concentration.

Native casein micelles can be regarded as hard-spheres whose κ-casein surface layer can resist compression at very high concentrations (Bouchoux et al. 2009; 2010). It was hypothesized that the release of colloidal calcium phosphate, along with the increase in solubilisation of caseins with concentration and pH did not affect the supramolecular structure of the native casein micelles. This may be the case for caseins concentrated at the native pH of milk; but in acidified samples, the polyelectrolyte layer surrounding the micelles is weakened, affecting the ability of the caseins to maintain steric stability. At
the pH tested in this work (6.0 and 5.6) the proteins were still sufficiently charged, as still far from their isoelectric point, and an increased protein-protein repulsion may occur due to the dissolution of colloidal calcium phosphate (Lucey et al. 1997). Further decrease in pH to 5.6 the attractive forces may overcome the electrostatic repulsion. At these pH values, internal rearrangements may take place within the casein micelle, as previously proposed also by other researchers (Moitzi et al. 2011; Ouanezar et al. 2012).

6.4.2 Light scattering parameters measured as a function of pH and concentration

As shown in Figures 6.1 and 6.2, the combination of acidification and concentration led to changes in the composition of the casein micelles. Figure 6.3 illustrates the hydrodynamic radius of the casein micelles as measured by dynamic light scattering. To maintain the physico-chemical environment, the concentrated milk suspensions were rediluted extensively in their corresponding serum. In all cases there was no evidence of aggregation, as the apparent radius was constant as a function of protein concentration. By decreasing the pH there was a significant decrease of the radius of casein micelles, from about 80±1 nm to 71±0.4 nm at pH 5.6. The decrease in size of the casein micelles has been attributed to the collapse of the κ-casein layer as its charge is decreased (Alexander et al. 2006; Donato et al. 2007). However, recent research has suggested that acidification of skim milk not only leads to the decrease of micellar mass and radius, but also to a redistribution of mass within the casein micelles (Moitzi et al. 2011; Ouanezar et al. 2012). Indeed, the acidification process also results in increased turbidity (Alexander and Dalgleish 2005). It is not at this stage possible to determine whether the reduced radius arise because of a general shrinkage of the casein micelles or
from the collapse of the layer of κ-casein. However, when these concentrated, pH modified casein micelles suspensions were rediluted in their serum, no changes in the radius with concentration could be detected. The remaining charge on the surface of the casein micelles was sufficient to maintain a stable suspension even at pH 5.6, as no aggregation was measured by light scattering as a function of concentration.

To further understand the influence of pH-modification and concentration on the colloidal properties of casein micelles, diffusing wave spectroscopy was also used to determine the light scattering properties of the casein micelles in situ. Figure 6.4 illustrates the changes in the turbidity parameter (1/l*) of milk samples with different pH values as a function of concentration. The turbidity of a colloidal suspension is influenced by the refractive index contrast, particle size, concentration, as well as by the interparticle interactions (Corredig and Alexander 2008; Gaygadzhiev et al. 2008).

During the acidification process, the dissolution of colloidal calcium phosphate and release of caseins changed the refractive index contrast between the casein micelles and the serum phase, resulting in the decrease of turbidity. At the native pH of milk, the turbidity parameter increased with the concentration of the suspensions. For the dispersions at pH 6, the turbidity was lower than for control skim milk suspensions at the same concentration. At pH 5.6, the turbidity was even lower and the values reached a plateau at a concentration of protein of about 8%, corresponding to about 3x concentrated milk.
Figure 6.3 Apparent hydrodynamic radius as a function of concentration for samples with different pH: pH 6.7 (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average values from triplicate experiments, with the standard deviation indicated by vertical bars. Lines are drawn to guide the eye.

It is expected that with the increase of protein content, more scattering events happen in a given space and the light becomes randomized faster, resulting in the increase of the turbidity parameter $1/l^{*}$. The decrease in turbidity of the acidified suspensions can be attributed to a decrease in the refractive index contrast, due to the release of calcium and phosphate in the serum phase and an increase in the dissociation of the caseins.
To better understand the diffusivity behaviour of the pH-modified casein micelles as a function of concentration, the mean squared displacement (MSD) was also measured using DWS (Figure 6.5). The casein suspensions at the native pH of milk showed a free diffusing behaviour at the concentrations tested, with a linear relation between time and MSD, confirming previous reports of a free diffusing, hard-sphere behaviour up to volume fractions of 0.54 (Dahbi et al. 2010; Nair et al. 2014). All p values (see methods) for all suspensions at different pH and concentration were not statistically different from 1 up to 3x concentration. However, a drop of slope value to 0.92 was observed for 4x samples at pH 5.6, suggesting restricted diffusivity of the casein micelles under these conditions.

### 6.4.3 Rheological properties of concentrated milk

It has been previously reported that the viscosity of milk increases with concentration and at $\phi > 0.4$ (corresponding to about 12% protein) a shear thinning behaviour is observed (Dahbi et al. 2010; Nair et al. 2013). At low protein concentrations ($\phi < 0.3$), the casein micelles are non-interacting and their colloidal properties can be well modelled to those of a colloidal hard-sphere system, both using turbidity, diffusivity or rheological data (Alexander et al. 2002).
Figure 6.4 Changes in turbidity as a function of concentration for concentrated control pH 6.7 (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average values from triplicate experiments, with the standard deviation indicated by vertical bars.
Figure 6.5 Mean squared displacement as a function of concentration for samples with different pH: pH 6.7 (A), suspensions acidified at pH 6.0 (B) and pH 5.6 (C). From left to right: 1x (●), 2x (○), 3x (▲), 4x (Δ).
On the other hand, at high protein concentration, interparticle interactions occur and casein micelles will behave as deformable colloids like microgel particles (Bouchoux et al. 2009; Bouchoux et al. 2010; Nöbel et al. 2012; Olivares et al. 2013). However, this behaviour may be observed at high volume fractions (>0.78) where a transition from hard-spheres to a viscoelastic gel will occur (Bouchoux et al. 2009). It is still unclear how the interpenetration of the polyelectrolyte layer of κ-casein present on the surface may influence the deformability of the casein micelles at these high concentrations, and how the polydispersity may affect the overall diffusivity of the micelles. Although this behaviour has been studied for milk at its native pH, very little is known about milk suspensions at lower pH values.

Figure 6.6 illustrates the changes in viscosity measured at 300 s⁻¹ for the suspensions and their corresponding serum phase as a function of protein concentration. The viscosity for milk suspensions increased non-linearly as a function of concentration for all the pH values, fitting to an exponential equation (p<0.01%) (Figure 6.6A). Suspensions at pH 6.0 and 6.7 showed a similar viscosity behaviour, however, at concentrations >8% protein, the values of viscosity were lower for pH 6 than 6.8 suspensions. This lower viscosity can be attributed to the decrease in the size of the casein micelles, as shown in Figure 6.3. A further decrease in pH (to pH 5.6) instead led to the increase of bulk viscosity, especially at protein concentrations >8%.
Figure 6.6 Viscosity of the suspension (A) and serum phase (B) as a function of concentration for samples with different pH: 6.7 (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average values from triplicate experiments, with the standard deviation indicated by error bars. Note that symbols may be larger than error bars.
In this case, it may be possible to consider a higher solubilisation of the calcium phosphate as well as caseins from the micellar phase, and an increase in protein-protein interactions (Ouanezar et al. 2012). It is also important to note that at this pH the apparent radius of the casein micelles was significantly lower than for pH 6 and pH 6.8.

The viscosity of the serum phase was also measured and results are summarized in Figure 6.6B. At the native pH of milk (circles), the serum viscosity increased linearly with concentration reaching values of about 1.8 mPa.s at 13% protein. On the other hand, at pH 6.0, the serum viscosity remained similar to that of suspensions at pH 6.8 up to <8% protein, and increased significantly at higher protein concentrations. The changes were in line to the results for protein dissociation shown in Figure 6.1. Suspensions at pH 5.6 showed a linear increase in the serum viscosity, with much higher values of serum viscosity than those of suspensions at pH 6.8. Once again, this higher serum viscosity reflected the increase in soluble protein present in the serum phase after centrifugation at 100,000 x g.

It has been previously reported that during concentration by evaporation, water is removed preferentially from the serum phase instead of the colloidal phase (Liu et al. 2012). In the present work it was assumed that the hydration of the casein micelles at the concentrations studied remained unchanged. It was then hypothesized that with a decrease in pH, there would be a change in the voluminosity and maximum packing volume fraction ($\phi_{\text{max}}$) for casein micelles. To test this hypothesis, the rheological properties of the pH-modified suspensions were plotted as a function of volume fraction.
(Figure 6.7). To estimate volume fraction, a value of $4.4 \text{ cm}^3 \cdot \text{ml}^{-1}$ was used in all cases. The fit of the curve would prove the validity of such assumption.

**Figure 6.7** Changes in relative viscosity as a function of concentration for protein suspensions at native pH (circles), pH 6.0 (triangles) and pH 5.6 (squares). Values are average values from triplicate experiments, with the standard deviation indicated by vertical error bars. The dotted lines correspond to theoretical predictions of relative viscosity using Mendoza Model for interacting colloidal hard spheres.
It has been previously reported that for untreated skim milk, the relative viscosity \( (\eta/\eta_0) \) can be modelled after a model for colloidal hard spheres (Mendoza and Santamaria-Holek 2009; Nair et al. 2014). By keeping the value of voluminosity at 4.4 cm\(^3\).g\(^{-1}\), it was not possible to obtain good statistical fit for equation 6.2, for colloidal suspensions at pH 6.0 and 5.6. It was then assumed a change in the voluminosity of the casein micelles simply taking into account their decrease in apparent radius (Figure 6.3). A value of 4.4 cm\(^3\).g\(^{-1}\) was used as voluminosity of the casein micelles at pH 6.8. The volume fraction of casein micelles at pH 6.0 and 5.6 was calculated using a volume \( v=4\pi r^3/3 \) (r is the radius calculated by dynamic light scattering). The estimated values of volume fraction were 80 and 70% of the volume fraction of control micelles suspensions, for pH 6.0 and 5.6, respectively (see Figure 6.8). The colloidal behaviour of milk at pH 6.8 fit well, with an estimate value of \( \varphi_{\max} \) of 0.80, in full agreement with a previous report (Nair et al. 2014). On the other hand, best fits were obtained using Equation 6.2 for \( \varphi_{\max} \) values 0.90 and 0.36 for pH 6.0 and 5.6, respectively (Figure 6.8). It is important to note that in the case of pH 5.6, it was possible to obtain a high \( R^2 \) value of the fit only when not including points at concentrations higher than 3x, as shown in Figure 6.7, the relative viscosity at high volume fraction showed much lower values than expected. There are two possible reasons for such discrepancy. On one hand, it is possible that at those high volume fractions the composition of the serum phase was not accurately estimated, because of the low amounts of serum recovered and the size of the protein aggregates present in the supernatant. On the other hand, it may be possible that at high volume fraction, the properties of the casein micelles changed, showing a further
decrease in voluminosity and hydration, and causing an incorrect estimation of the volume fractions, for dispersions at

![Graph showing voluminosity vs pH](image)

**Figure 6.8** Voluminosity (circles) of casein micelles and corresponding critical packing volume fraction (triangles) estimated as a function of pH. Lines are drawn to guide the eye.
pH 5.6 and at concentration of protein >8%. This would be fully in line with the diffusivity data shown in Figure 6.5.

Previous research has reported that the dissociation of caseins is low at pH 6.0 and increases at lower pH values (Dalgleish and Law 1988), although the dissolution of colloidal calcium phosphate is substantial at this pH. Hence it is possible to assume that at pH 6.0 the surface charge of casein micelles decreases but the steric repulsion provided by the κ-casein layer is still high enough to keep the casein micelles away from each other. In addition, the internal charge of the protein is high enough to cause an increase in intermolecular charge repulsion. It may be possible that under those conditions, the concentration will lead to internal rearrangements of the micelles. Microstructural analysis suggested that casein micelles are more homogeneous and show a higher average density at this pH (Moitzi et al. 2011).

The rheological behaviour of suspensions at pH 6.0 fitted to equation 6.2 showed a value for \( \varphi_{\text{max}} \) value of about 0.9. Such high maximum packing volume fraction will result from smaller size of the casein micelles (Figure 6.3) and the lower viscosity of the suspensions (Figure 6.6). Further decrease of pH to 5.6, the hydrodynamic radius of casein micelles continue to decrease. The release of caseins increased greatly and only little colloidal calcium phosphate remained within the casein micelles. However, at this pH, the negative charge provided by the surface κ-casein layer is also decreased greatly and this decreased the steric stabilization of the caseins, resulting in a significantly lower \( \varphi_{\text{max}} \) for these suspensions. Furthermore, at this pH, the viscosity values of the suspensions at a volume fraction >0.3 could not be fitted to the hard sphere behaviour (Menzdoza and Santamaria-Holek, 2009). These results imply that at pH 5.6, the
colloidal stability of casein micelles at pH 5.6 is largely impaired at the volume fractions >0.3.

6.5. Conclusions

This research demonstrated that concentration of casein suspensions at pH 6 or 5.6 causes extensive dissociation of caseins from casein micelles and colloidal calcium solubilisation. Dissociation increased with a decrease in pH, but most importantly, with concentration, and a comparison between soluble and diffusible calcium and phosphate suggested that at about pH 6 the amount of phosphate released reaches a plateau. There was very little difference in the soluble phosphate present in samples at pH 6 and 5.6, in spite of an increase in calcium release and soluble protein concentration.

At pH 6.0 and 5.6, the casein micelles showed a decrease in the apparent radius. The turbidity of the samples also decreased with pH, because of a decrease in the refractive index contrast between the micelles and the serum phase and the dissociation of caseins from the micellar phase. Acidification did not affect the diffusivity of the casein micelles, and all the suspensions up to 3x, showed to be free diffusing. The size of the casein micelles, after redilution in their corresponding serum also demonstrated that there was no aggregation with concentration. The sizes were stable for all samples, which illustrates that no irreversible aggregation occurs up to 4x even for the milk with the lowest pH 5.6.

This work allowed the estimation of voluminosity values for casein micelles at pH 6 and 5.6, as summarized in Figure 6.8. Values of voluminosity decreased from 4.4 at pH 6.8 to 3.1 at pH 5.6. The maximum packing volume fraction significantly at pH 5.6, to
values around 0.36, suggesting that at this pH, concentration >3x will cause the shift from free diffusing to physically arrested colloidal systems. This work contributes to a better understanding of the colloidal properties of casein suspensions at acidic pH, and it is of great importance in understanding dairy processes such as cheese making or other means of concentration.
CHAPTER 7

EFFECT OF PH-MODIFICATION ON THE RENNET COAGULATION OF
CONCENTRATED CASEIN MICELLES SUSPENSIONS

7.1 Abstract

Although the effect of concentration on the texture and microstructure of rennet induced milk gels has been studied, much less is known on the aggregation behaviour of pH-modified casein micelles after concentration. In this research, milk was adjusted to different pH values and then concentrated to 3x using osmotic stressing. All the concentrated samples were added to untreated skim milk with or without re-equilibration to the original milk pH. Rennet induced aggregation was then followed using rheology and light scattering. Rennet induced gelation was then studied compared to skim milk. Concentrated samples at pH 6.8 (control skim milk) showed no significant effect. On the other hand, when the concentrated acidified casein micelles were added back to untreated milk, the gels had higher elastic modulus compared to control treatments. Results were profoundly different when the dispersions were added to milk and followed by re-equilibration. These mixtures did not show gelation. The lack of gelation could not be attributed to changes in diffusible calcium and phosphate, as these concentrations were similar to those of the original milk, and there was also no significant change in the colloidal calcium phosphate; the difference was in the extent of casein solubilization. The results clearly demonstrate that processing history of concentrated casein micelles strongly affect their renneting functionality, and that both the soluble caseins and
diffusible calcium phosphate play important roles in the rennet coagulation of casein micelles. These results clearly outline how it is possible to modulate processing functionality by modifying concentrated milk retentates.

7.2 Introduction

Rennet coagulation of milk is the main step of the cheese making process. Rennet gelation has been well studied, and it is described as including two overlapping stages (Dalgleish 1980). The primary stage is the enzymatic cleavage of κ-casein; in this stage, chymosin (rennet) specifically acts on the Phe 105-Met 106 bond of κ-casein and releases its C-terminal region. During this stage, the diffusion coefficient of the micelles increases and the viscosity decreases due to the gradual removal of their polyelectrolyte hairy layer (De Kruif 1992; Tuinier and De Kruif 2002). Once a sufficient amount of κ-casein has been hydrolyzed, the steric and electrostatic repulsion forces generated by the few remaining κ-casein hairs are insufficient to stabilize the casein micelles, so, they begin to aggregate with each other and eventually form a network of protein particles. In untreated skim milk at native pH, this second stage of the reaction occurs when at least 85%-90% of the casein-macropetide is released into solution (Sandra et al. 2007).

It is known that the structure of casein micelles is affected by environmental changes and by the ionic equilibrium between the colloidal and soluble fractions. Rennet induced aggregation of casein micelles is very sensitive to any changes on the surface of the casein micelles (Waungana et al. 1997; Gaygadzhiev et al. 2012). With pH, both the surface and internal structure of casein micelles change (Le Graët and Gaucheron 1999; De Kruif 1999). Colloidal calcium phosphate gradually dissociates from casein micelles.
(Jacob et al. 2011), the net charge on the surface decreases and some caseins are released in the serum phase (Renault et al. 2000). The extent of mineral solubilisation continues with acidification, and is almost complete at about pH 5.0. On the micellar surface, the decrease of the negative charge due to diminishing pH leads to the collapse of the κ-casein layer. Thus, both the electrostatic and steric repulsions decrease and the micelles can diffuse closer to each other. This facilitates the rennet gelation process (Dalgleish and Corredig 2012).

It is reported that both the rate of the primary phase of proteolysis as well as the extent of the aggregation increase with lower pH (Renault et al. 2000). A mild pH reduction (to pH 6.3) can improve the rennet coagulation properties of milk (Law and Leaver 1998). Acidification of milk, followed by neutralization, has been shown to result in improved rennet properties, probably due to an elevated Ca$^{2+}$ activity in the serum (Lucey et al. 1996). However, the ionic equilibrium and the role played by the changes in composition of the soluble fraction (especially with the presence of soluble caseins and diffusible calcium) are not fully understood. Mixed acid and rennet coagulation processes are quite common, and also cause variations in the aggregation behaviour of the casein micelles and in the properties of the final gels (Salvatore et al. 2011).

Very little is understood on the effects occurring during renneting when only a portion of the casein micelles have been treated before renneting. It is understood that for gelation to occur, most of the CMP has to be released into solution, therefore it can be hypothesized that the presence of a portion of pre-acidified casein micelles should not cause any effect to the rennet behaviour of milk. However, some preliminary evidence of the contrary has been previously reported (Li and Dalgleish 2006).
Concentration is an important process step for manufacturing cheese and dairy ingredients or for milk standardization. In spite of the practices being widespread, very little is understood on the mechanisms involved in the gelation of milk when modified milk ingredients are added. The effect of concentration on rennet induced aggregation kinetics is still under debate. Concentrated milk forms stiffer gels due to an increased number of bonds in the network (Waungana et al. 1999). Some researchers showed a shorter coagulation time and higher gel firming rate for concentrated milk compared to non-concentrated milk (Dalgleish 1980; Waungana et al. 1999) while others (Sandra et al. 2011) reported that concentration has no significant effect on the release of caseinmacropeptide and coagulation time when same amount of rennet is added. It is unclear how the concentration will influence the rennet coagulation behavior of pH-modified casein micelles after their concentration.

The objective of this research was to investigate the rennet gelation behaviour of casein micelles as a function of pH-modification and concentration. All acidified milk was concentrated 3x using osmotic stressing. This methodology minimized shear and maintained the composition of the serum phase constant between samples at the same pH. All concentrated samples were added to untreated milk, and differences in coagulation behaviour were then observed.

7.3 Materials and Methods

7.3.1 Sample preparation

Fresh raw milk was provided by the Elora Dairy Research Farm of University of Guelph (Elora, ON, Canada). Sodium azide (0.1 g.L$^{-1}$) was immediately added to prevent bacterial growth. Skim milk was prepared by centrifuging at 4000 x g for 25 min at 4 °C.
(J2-21 centrifuge, Beckman Coulter Canada Inc, Mississauga, Canada) and filtering four times through Whatman fibreglass filter (Fisher Scientific, Mississauga, Ontario, Canada). To reach a final pH of 6.7, 6.0 and 5.5, Glucono-δ-lactone (GDL) (0%, 0.35%, and 0.57%, respectively) was added to skim milk, stirred for 5 min at room temperature, and then kept in the refrigerator overnight.

Milk was concentrated to 3x using osmotic stressing, as previously described (Nair et al. 2014). Milk was placed in a dialysis bag (standard regenerated cellulose spectra/Por 1, Fisher Scientific, Whitby, Ontario, Canada) with a molecular mass cut-off of 6-8 kDa, then immersed in skim milk containing polyethylene glycol (PEG) and 0.1 g.L⁻¹ sodium azide, also adjusted to the corresponding pH with GDL. The pore size of the dialysis membrane ensured the exchange of water, ions, and lactose but not proteins or PEG. The dialysis membrane was washed three times with Milli-Q water and then conditioned in Milli-Q water for 30 min before experiments. Skim milk samples (40 mL) were placed in the dialysis bags and immersed in 1 L milk containing about 10% PEG. 3x concentrated samples were obtained by dialyzing 18 h at 4°C with stirring, to minimize sample degradation.

To investigate the effect of pH-modified casein micelles on the coagulation behavior of untreated milk, the concentrated milk suspensions with different pH were then mixed with untreated skim milk at a ratio 1:1. The 1:1 mixtures were also dialyzed against the original milk overnight, to eliminate the influence of pH. All samples were re-equilibrated for 2h at room temperature and then analyzed.
7.3.2 Chemical characterization

The soluble serum was obtained by the ultracentrifugation at 100,000 x g for 1 h at 20 °C (OptimaTM LE-80K ultracentrifuge with rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada) and subsequent filtration through 0.45 µm filter (low protein binding, Fisher Sci.). The diffusible phase was defined as the serum after diffusing through a filter with a molecular cut off of 10 kDa as previously described (Zhao and Corredig 2015).

Total and soluble protein concentration in the serum phase was determined using a Dumas nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI) using 6.38 as conversion factor.

Total, soluble and diffusible calcium were measured as previously published (Rahimi-Yazdi et al. 2010; Zhao and Corredig 2015). On the other hand, an ashing procedure was used to prepare the total and soluble phosphate. The milk sample or the serums (1mL) were placed in the Pyrex test tubes and then heated at 100°C overnight to dry the samples, followed by a mineralization at 500 °C for 6 h in an Isotemp muffle furnace (Fisher Scientific). The HPLC water was used to dilute the phosphate samples. The phosphate was determined using 861 Advanced Compact IC (Ω Metrohm ion analysis, Metrohm Ltd., Herisau, Switzerland). An anion column (Metrosep A Supp5-150/4.0, Metrohm) packed with 5 µm polyvinyl alcohol with quaternary ammonium groups was employed. Sodium hydrogen carbonate and sodium carbonate solutions were used as mobile phase (1.0 mM sodium carbonate and 4mM sodium hydroxide). Samples were eluted at a flow rate of 0.5 mL.min⁻¹.
7.3.3 Gelation

Prior to the addition of rennet, milk samples were equilibrated at 30 °C for at least 20 min. Chymax Ultra (CHR Hansen., Milwaukee, USA) with average strength of 790 (±5%) IMCU/mL was diluted 100-fold in MilliQ water immediately before addition to milk mixtures. The diluted rennet (4μL per mL milk) had a final concentration of 0.031 IMCU/mL. Milk samples were stirred for 30 s after rennet addition, and then incubated at 30 °C.

The gel formation process of all milk samples was followed by oscillatory measurements, using a constant strain of 0.01 and a frequency of 1 Hz. The rheometer (AR 1000, TA Instruments Ltd., NewCastle, DW, USA) was equipped with concentric cylinder (28 and 30 mm inner and outer cylinders diameter, respectively) and an external water bath (Isotemp 3016, Fisher Scientific). Milk (20 mL) was transferred to the cylinder within 5 min of rennet addition. The milk gelation point was defined as the point when the elastic modulus (G’) and viscous modulus (G’’) cross over (tan δ, where δ is the phase angle = 1) (Lucey et al. 1998; Lucey and Horne 2009).

Transmission DWS was also used in this research to follow the changes in light scattering of the milk suspensions during rennet gelation. This technique allows investigation of the static and dynamic behaviour of the casein micelles, in situ, without dilution. The changes of the turbidity parameter, 1/l*, during rennet coagulation was determined. The l* value is the photon transport mean free path, which represents the length scale over which the direction of the scattered light has been completely randomized. The turbidity parameter is a function of the concentration of the scattering particles, their radius and refractive index as well as the particle spatial organization.
The dynamic properties of the casein micelles can be determined by the measurement of their diffusion coefficient (Weitz et al. 1993).

The light source for the DWS was a solid state diode pumped Nd:YAG laser type of a wavelength 532 nm and a power of 350 mW (Coherent, Santa Clara, CA, USA). All samples were poured into an optical glass cuvette (Hellma Canada Ltd., Concord, Ontario, Canada) with a 5 mm path length and then placed in a water bath maintained at 25°C. Data was analyzed using software developed specifically for the equipment (Mediavention Inc., ON, Canada). Correlation functions and intensity of transmitted scattered light were measured at intervals of 2 min (118s collecting followed by a 2s break). Measurement of DWS and rheology were made simultaneously on two fractions of the same renneted milk.

### 7.3.4 Statistical analysis

ANOVA and Tukey HSD were carried out on the experimental measurements with 95% confidence level using Minitab statistical package release 15 (Minitab Inc., State College, PA, USA).

### 7.4. Results and Discussion

#### 7.4.1 Characterization of milk samples

Three values of pH were tested in this work, pH 6.7 (initial pH), pH 5.9 and 5.5. After concentration, there were no differences in the milk pH compared to the initial 1x milk (Table 7.1). On the other hand, when the acidified concentrate was added to milk, the final pH was 6.7, 6.1 and 5.9, for the three different treatments. As pH and ionic
equilibrium strongly affect the renneting properties of milk, for better comparison, the mixtures were dialyzed against untreated milk overnight, to re-establish similar pH values.

**Table 7.1** pH, total and soluble protein content of different samples after different treatments. Values are means of from triplicate experiments, ±standard deviation. Values with a different superscript letter are significantly different (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Total protein (%)</th>
<th>Soluble protein (%)</th>
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<tbody>
<tr>
<td>Control 1x</td>
<td>6.72±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.29±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control 3x</td>
<td>6.69±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.13±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.53±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed with milk (1:1)</td>
<td>6.69±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.27±0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.54±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed and dialyzed</td>
<td>6.66±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.96±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.37±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.9 1x</td>
<td>5.97±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.26±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.97±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.9 3x</td>
<td>5.83±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.27±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.20±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed with milk (1:1)</td>
<td>6.15±0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.87±0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.06±0.21&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mixed and dialyzed</td>
<td>6.52±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.67±0.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.08±0.22&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.5 1x</td>
<td>5.53±0.12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.29±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.02±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.5 3x</td>
<td>5.54±0.08&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.59±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.94±0.31&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Mixed with milk (1:1)  & 5.87±0.05<sup>b</sup> & 6.00±0.67<sup>c</sup> & 2.60±0.15<sup>b</sup> \\
Mixed and dialyzed & 6.48±0.13<sup>ad</sup> & 5.60±0.44<sup>c</sup> & 3.73±0.05<sup>f</sup> \\

After dialysis against untreated milk of the mixtures, the pH of the two acidified samples was about 6.5 (for both 5.9 and 5.5 treatment); these values were slightly lower than those for control milk (pH 6.6).

In addition to the pH values for all treatments, Table 7.1 also reports the values of total and soluble protein for all the samples tested. The 3x concentrated samples reached 9% total protein. When mixed to untreated milk, the final concentration was about 5.9% with no significant changes after dialysis. The amount of soluble protein, defined as the protein recovered in the supernatant after centrifugation at 100,000 x g for 1 h, was different between treatments. For the unconcentrated samples (1x), there was no significant difference in the soluble protein content among different pH values. In all cases, 1% soluble protein was recovered. However, after concentration, the amount of soluble protein increased with decreasing pH, from 2.5% at pH 6.7, to 3.2 and 3.9% for pH 5.9 and 5.5 samples. After mixing the 3x samples with skim milk, the amount of soluble protein was 1.5, 2 and 2.6% for the three pH treatments, again reflecting the increase in soluble protein for samples at the lower pH values. After dialysis of the mixtures, there were no statistically significant differences for samples acidified at pH 5.9 compared to control, however for the treatment of pH 5.5, after dialysis, the soluble protein increased to 3.7% compared to 2.6% before dialysis. These results clearly indicate that the re-equilibration of the samples with a serum of similar composition to control skim milk caused further dissociation of the caseins when the milk was acidified at pH
Previous work has demonstrated that the calcium phosphate equilibrium between the serum phase and micellar phase plays a key role in the structural integrity of the casein micelles and their renneting functionality (Choi et al. 2007; Sandra and Corredig 2013). Hence, both the soluble and diffusible calcium and phosphate for all the samples was measured for all treatments, to evaluate possible differences in ionic composition. The colloidal calcium and phosphate were also calculated, which were defined as the difference between total calcium and phosphate and diffusible calcium and phosphate. The results are shown in Figure 7.1.

Both soluble and diffusible calcium increased with decreasing pH, indicating the dissociation of colloidal calcium phosphate from casein micelles (Dalgleish and Law 1989). The colloidal calcium and phosphate were significantly lower in acidified samples compared to control milk in both 1x and 3x concentrated samples (Figure 7.1E and F). The 3x concentrated milk samples had significantly more soluble and diffusible calcium and phosphate than their corresponding 1x samples. It is noteworthy to observe that in the 3x suspensions, the difference in the diffusible and soluble calcium between pH treatments was much higher than in the 1x suspensions. As a consequence, the colloidal portion of calcium phosphate (shown in Figure 7.1E and F) was much lower in the 3x suspensions acidified at pH 5.5 compared to control, with the samples at pH 5.9 being at intermediate values.

After mixing 3x concentrated milk samples with untreated skim milk, there was still a difference in the soluble and diffusible calcium and phosphate. However, after re-equilibration (dialysis) against skim milk, this difference was less between the samples at
different pH, especially in the diffusible (free) calcium and phosphate, indicating that the dialysis recovered a similar amount of free calcium and phosphate in all three treatments, making the rennet induced aggregation studies more comparable.

**Figure 7.1** Effect of concentration, mixing and re-equilibration processes on the soluble calcium (A) and phosphate (B), diffusible calcium (C) and phosphate (D), and colloidal calcium (E) and phosphate (F).
calcium (E) and phosphate (F) of milk with different pH. From left to right: 6.7 (black bar), pH 6.0 (white), pH 5.6 (grey).

It is important to note that the discrepancy between soluble and diffusible calcium in the mixtures dialyzed against milk indicate that the difference was due to the amount of mineral associated to the soluble protein, as for milk at low pH, more soluble protein was present, as shown in Table 7.1. After re-equilibration, as expected, there were no differences in the amount of diffusible calcium and phosphate between treatments (Figure 7.1 C and D), although there was a significant difference in the soluble fraction, due to the presence of different amounts of soluble phosphoproteins in solution. In addition, the amount of colloidal calcium was 250 mg/kg/g of casein for 1x milk, and 263 for 3x milk at pH 6.7. For samples acidified at pH 5.5 the values were 95 and 64 mg/kg/g casein for 1x and 3x, respectively. In the mixtures before equilibration, the colloidal calcium concentrations were 267, 171, 121 mg/kg/g of caseins for the mixtures obtained with suspensions adjusted at pH 6.7, 5.9 and 5.5. The amount of colloidal calcium phosphate after equilibration was similar to that of the mixtures before equilibration.

The results indicated that the mineral equilibrium between the colloidal phase and the serum phase can be influenced by the pH-adjustment and concentration, and further influenced by mixing the pre-treated casein suspensions with milk.

7.4.2 Rennet induced gelation

Figure 7.2 summarizes the development of $G'$, $G''$ and $\tan \delta$ for milk samples treated at the three different pH.
Figure 7.2 Development of elastic modulus and ten δ during rennet gelation of milk samples with different pH values: 6.7 (A, D), pH 6.0 (B, E) and pH 5.6 (C, F). ● (1x), ○ (1:1 before re-equilibration), ▲ (1:1 after re-equilibration), Δ (3x). Only representative runs are shown.

Table 7.2 summarizes the average values for the rheology parameters measured.
For the untreated control milk, all samples showed a gelation point of about 55 min after rennet addition. The same gelation point was also noted for 3x concentrated milk, as well as for the concentrated milk mixed with skim milk (Table 7.2), in agreement with what observed in previous research (Sandra et al. 2011). While the tan δ at plateau did not show a significant difference for control untreated milk, regardless of treatment, the value of G’ and the gel network formation were significantly affected by the concentration. The firmness of the final gels increased with increasing protein concentration. The elastic modulus measured at 45 min after gelation point increased significantly with concentration. The increase in G’ with protein concentration is simply due to the higher protein molecules in a given space and the higher number of bonds between proteins (Waungana et al. 1999).

For milk samples acidified at pH 6.0 and 5.6, a much shorter gelation time (Table 7.2) was found, as pre-acidification facilitates the rennet gelation of casein micelles (Renault et al. 2000). A lower pH affects the chymosin activity, and decreases the electrostatic and steric repulsion of the casein micelles (Karlsson et al. 2007). Furthermore, the elastic modulus increased at a faster rate for the samples with higher protein concentration, as a result of reduced distance between micelles, increased collision frequencies, and the increased bonds and contact area in the gel network (Mishra et al. 2005). In the acidified suspensions, the gelation time was significantly delayed with concentration (Table 7.2), in spite of the higher levels of soluble and diffusible calcium and phosphate (Figure 7.1). The corresponding gelation time increased from 9.0±2.0 and 6.0±0.5 to 20±1.0 and 13±0.0 min, for suspensions adjusted at pH 5.9 and 5.5, respectively.
Table 7.2 Rennet gelation parameters of different samples measured by rheometer.

Values are means of from triplicate experiments, ±standard deviation. Values with a different superscript letter are significantly different (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Gelation time from rheology (min)</th>
<th>Gelation time from DWS (min)</th>
<th>Elastic modulus after 45 min from gelation point (Pa)</th>
<th>Tan δ plateau value</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 1x</td>
<td>55±2.5a</td>
<td>53±1.3a</td>
<td>16±2.0a</td>
<td>0.2618±0.006a</td>
</tr>
<tr>
<td>Control 3x</td>
<td>58±3.0a</td>
<td>59±5.0a</td>
<td>392±31b</td>
<td>0.2697±0.020a</td>
</tr>
<tr>
<td>1:1 before dialysis</td>
<td>54±2.0a</td>
<td>55±2.0a</td>
<td>146±10c</td>
<td>0.2600±0.003a</td>
</tr>
<tr>
<td>1:1 after dialysis</td>
<td>52±2.0a</td>
<td>56±3.0a</td>
<td>188±28c</td>
<td>0.2627±0.000a</td>
</tr>
<tr>
<td>pH 5.9 1x</td>
<td>9.0±2.0b</td>
<td>10.6±0.5b</td>
<td>106±8.0d</td>
<td>0.2925±0.006ab</td>
</tr>
<tr>
<td>pH 5.9 3x</td>
<td>20±1.0c</td>
<td>20±1.2c</td>
<td>977±81e</td>
<td>0.3338±0.007c</td>
</tr>
<tr>
<td>1:1 before dialysis</td>
<td>22±0.5c</td>
<td>24±3.0c</td>
<td>357±34b</td>
<td>0.2898±0.002ab</td>
</tr>
<tr>
<td>1:1 after dialysis</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>pH 5.5 1x</td>
<td>6.0±0.5b</td>
<td>8.0±1.0b</td>
<td>55±5.0f</td>
<td>0.4420±0.028d</td>
</tr>
<tr>
<td>pH 5.5 3x</td>
<td>13±0.0d</td>
<td>15±1.5d</td>
<td>640±73e</td>
<td>0.5093±0.042d</td>
</tr>
<tr>
<td>1:1 before dialysis</td>
<td>16±2.0cd</td>
<td>18.2±0.5cd</td>
<td>372±30b</td>
<td>0.3651±0.027c</td>
</tr>
<tr>
<td>1:1 after dialysis</td>
<td>-------</td>
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</table>

The value of tan δ at plateau was also higher, indicating the final gels have different properties than that of control milk. A pH reduction (to pH 5.9) improved the rennet coagulation properties of milk, the gelation happened at an earlier time and the final gel had a much higher elastic modulus (Table 7.2). A decrease to pH 5.5 in the suspensions before concentration led to further samples with a much lower gelation time; however, the elastic modulus was also lower compared to the milk with pH 6.0, for both
unconcentrated and concentrated samples. All these results were consistent with previous research (Lucey et al. 1996; Law and Leaver 1998; Renault et al. 2000). During the pH-reduction process, the decrease of the negative charge on the surface of casein micelles reduces the repulsion between casein micelles and colloidal calcium phosphate dissociated into the serum phase, the combined effects of reduced repulsion and elevated Ca\(^{2+}\) activity increased the aggregation rate during the rennet coagulation of milk and leads to the increased elastic modulus (Dalgleish 1983). However, once the pH has been further reduced, the larger amount of soluble caseins present in the serum phase affects the aggregation behaviour of the casein micelles, by causing the formation of a weaker gel network. It has been shown in previous research that the presence of soluble protein can affect the gelation behaviour of milk (Gaygadzhiev et al. 2012). The increase in the gelation time for the concentrated, acidified samples may be due to the increase in the soluble protein present (as shown in Table 7.1).

After mixing 3x pH-modified milk with skim milk, while the gelation point did not show a significant difference from that of the concentrated sample, the value of G' after 45 min was intermediate, indicating the effect of volume fraction. After re-equilibration of the samples by dialysis against milk, no gelation was detected in the mixes.

To better characterize the influence of pH-modification and concentration on the aggregation behaviour of casein micelles, especially at the earlier stages of rennet-induced gelation, diffusing wave spectroscopy was used to follow the changes of radius and turbidity of the casein micelles as a function of renneting time (Figure 7.3). The starting value of 1/\(l^*\) increased with protein concentration, simply due to the increased
number of scattering events within the corresponding scattering volume. For the raw milk samples, all systems showed similar kinetic aggregation behaviour, the turbidity increased gradually with time. The error for the turbidity increased after the gelation point. This typical rennet-induced aggregation behaviour of casein micelles for unconcentrated and concentrated milk has been described in detail in previous papers (Sandra et al. 2007; Sandra et al. 2011). The development of the radius was also in full agreement with the rheology data and increased greatly after the gelation point (Table 7.2).

For the acidified milk samples without concentration, the gelation happened much faster than the raw milk, as already indicated by the rheology experiments (Figure 7.2) both the turbidity and radius increased very quickly and fluctuated greatly after the gelation point. After concentrated to 3x, the average distance between casein micelles is much smaller and they can interact strongly over short distance (Karlsson et al. 2005), thus, the stability of casein micelles were expected to be highly sensitive to changes in the steric and electrostatic repulsions caused by the hydrolysis of κ-casein prior to coagulation and it was confirmed by the initially decreased of G' value after the addition of rennet (Figure 7.2). The turbidity and radius for 3x samples increased greatly after the gelation point.
Figure 7.3 Development of apparent radius and turbidity parameter (1/l*) for milk samples with different pH values: 6.7 (A, D), pH 6.0 (B, E) and pH 5.6 (C, F), after different treatments. ● (1x), ○ (1:1 before re-equilibration), ▲ (1:1 after re-equilibration), △ (3x). Only representative runs are shown.

but gelation happened at longer times compared to unconcentrated samples, in agreement with previous research (Karlsson et al. 2007). It was also proposed by Karlsson (2007)
that complete hydrolysis of κ-casein took more time due to lower rennet-casein ratio in concentrated than unconcentrated skim milk and this led to the longer coagulation time for concentrated sample. However, it cannot explain why the concentration has no effect on the coagulation time of original milk. In addition, it was reported that concentration of raw milk had no influence on the final degree of CMP release needed for clotting to occur and all samples gelled at about 90% CMP release (Sandra et al. 2007). On the other hand, for the acidified unconcentrated milk with pH 5.8, the gelation happened at only 50% cleavage of the κ-casein while the gelation point for the concentrated samples happened at a much lower degree of κ-casein hydrolysis (Karlsson et al. 2007). All these results indicated that the increase of the gelation time for the concentrated acidified sample is due to the inhibition of the combination of rennet with the casein micelles, probably due to the increased soluble protein, as the increase in Ca$^{2+}$ activity would favourably affect aggregation, and concentration would cause a decreased mean free distance between renneted micelles.

After mixing 3x concentrated sample with raw milk with a ratio 1:1, all samples showed good rennet gelation behaviour, the turbidity and radius increased after rennet addition. However, after re-equilibration by dialysis against the raw milk, only slight increase of the turbidity could be detected and there was no significant increase in the casein micelles radius (i.e. no changes in their diffusivity). This result fully supported the hypothesis that the second stage of aggregation is inhibited in these samples. It is important to note that the pH values of all samples after re-equilibration were similar, and more importantly, the mixes had similar diffusible calcium and phosphate contents (Figure 7.1). In addition, the colloidal calcium phosphate concentration was also not
different between mixtures before and after dialysis. As discussed in previous papers, the increased \( \text{Ca}^{2+} \) activity was able to facilitate the rennet coagulation of milk (Lucey et al. 1996) while the soluble caseins inhibited the rennet gelation process (Sandra and Corredig 2013). In this case, the changes in the diffusible calcium phosphate and dissociation of caseins during the dialysis process might be the factors that inhibit the rennet-induced coagulation of casein micelles.

7.5. Conclusions

Acidification and concentration changed the mineral equilibrium of the suspensions, and their rennet induced gelation properties. The elevated \( \text{Ca}^{2+} \) facilitated the rennet coagulation of casein micelles and resulted in the formation of gels with higher elastic modulus. The rennet gelation happened at earlier times for acidified samples due to the decreased steric and electrostatic repulsions. For untreated control milk, concentrated sample had similar gelation time as the unconcentrated sample and the turbidity and radius increased greatly after the gelation point. On the other hand, for the acidified samples, the concentration increased the gelation time. The firmness of rennet gels increased with concentration due to increased protein molecules and bond numbers in a given space. The samples with pH 6.0 had the highest elastic modulus. After mixing the 3x concentrated samples with raw milk with a ratio 1:1, the soluble and diffusible calcium phosphate increased with decreasing pH. The final gels for the milk mixed with acidified milk sample had higher elastic modulus compared to that of raw milk. No difference was found between samples with pH 6.0 and 5.6. After dialysing all the mixed samples against raw milk for 18 h, the pH and diffusible calcium phosphate were
recovered to values close to the raw milk. On the other hand, the soluble proteins and soluble calcium phosphate increased after re-equilibration, indicating the disruption of casein micelles. When added with rennet, no gelation was detected for the acidified samples, only a slight increase of turbidity and no change in the apparent radius could be detected. The results indicated that the integrity of casein micelles plays an important role in the rennet gelation process, as well as the Ca\textsuperscript{2+} activity and the soluble caseins. This research clearly demonstrated that the structure and organization of casein micelles can be modified by changing the mineral equilibrium between the serum phase and colloidal phase, which can help to optimize the application of casein micelles as food ingredients. The results show the potential of designing milk concentrates ingredients that can modulate the cheese making properties of milk.
CHAPTER 8

OVERALL CONCLUSIONS

The investigation about the structure-function of casein micelles has drawn great attention in recent years, not only because it can help us better understand the changes of the structure and organization of casein micelles during processing, but also can help us produce products with improved properties. For example, highly concentrated milk suspensions can be obtained, with elevated solubility and stability. In this research, we attempted to characterize the changes or the organization and functionality of casein micelles as functions of modifications of minerals and volume fraction. The changes of mineral were performed by adding NaCl or changing the pH.

The addition of NaCl was able to solubilise the colloidal calcium phosphate and decrease the negative charge on the surface of casein micelles. The dissociation of caseins was influenced by the method of adding NaCl and concentration. The soluble proteins increased greatly with the concentration of NaCl while the NaCl was added and concentrated by dialysis. On the contrary, no significant change in the soluble proteins could be detected when 300 mM NaCl was added directly to the milk and concentrated with UF. In addition, no significant changes in the hydrodynamic radius of casein micelles were found after the addition of NaCl in both methods, which indicates the integrity of casein micelles was not changed. The changes in the light scattering properties ($1/l^*$ and correlation function) were due to the changes of internal structure of casein micelles and the composition of the serum phase. The rennet coagulation of milk was retarded by the addition of the NaCl, resulting in the longer RCT and formation of
Weaker gels. The kinetic of CMP release was inhibited by the presence of NaCl but was not influenced by the concentration factor. However, the RCT of milk after the addition of NaCl decreased with concentration, which indicates that the concentration factor only influenced the coagulation process of renneted casein micelles but without influencing the cleavage of κ-casein layer. This research first compared the influence of NaCl on the colloidal properties of casein micelles by adding and concentrated at different ways. We first found that the concentration can facilitate the rennet coagulation of casein micelles in the presence of NaCl by influencing the secondary stage of rennet coagulation.

The second method used in this research to modify the structure of casein micelles is acidification. Acidification led to the solubilisation of colloidal calcium phosphate, after re-equilibration by dialysing against the raw milk, significant decrease of radius and disruption of casein micelles was found at pH< 6.0. There were no differences in the serum composition after dialysis at 4 or 22°C, indicating that re-equilibration temperature have no influence on the properties of the micelles. The acidified casein micelles were then concentrated using the method of osmotic stressing, which is considered as a non-invasive method. The changes of viscosity of pH-modified milk with volume fraction was well predicted with a hard sphere behaviour model, which considering the interparticle interactions. From the model fitting, it was found that the voluminosity of casein micelles decreased with decreasing pH and milk with pH 6.0 had a highest maximum packing volume, due to the decrease in voluminosity, the reorganization of the internal structure and the sufficient steric repulsion on the surface. At the lowest pH 5.6, the stability was reduced and the surface change was not enough to keep the casein micelles away from each other at high concentrations. This leads to a much lower maximum packing volume.
fraction and further decrease of voluminosity at high concentrations.

The rennet gelation happened at earlier times for acidified samples due to the decreased steric and electrostatic repulsions. Concentrated milk increased the RCT slightly probably due to the increased soluble protein in the serum phase, which influences the diffusivity and combination of the rennet with κ-caseins. Samples with pH 6.0 had the highest gel strength for both unconcentrated and concentrated samples. After mixing the concentrated milk with raw milk, the rennet coagulation was facilitated and the gels happened at a much shorter time. However, after recovering the pH by dialysing against raw milk, no gel was formed and no changes in the average radius could be detected. This is probably due to the synergistic effect of decreased diffusible calcium and increased soluble proteins from the disruption of casein micelles.

In summary, this research extended our understanding about the changes of the organization and the functionality of casein micelles as a function of modification of the mineral equilibrium between the serum phase and colloidal phase by adding NaCl or acidification. It brought new understanding about the influence of the concentration on the functionality of the modified casein micelles, the changes of structure-function during the processing. These results allow us to improve the processing efficiencies of dairy products and to optimize the utilization of these different casein micelles as ingredients in foods.
CHAPTER 9

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