Colloidal Behaviour of Casein Micelles with Concentration

by

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Structure function changes of casein micelles were studied as a function of concentration using a non invasive concentration method, osmotic stressing. A combination of serum analysis, light scattering and rheological measurements were used to characterize the physico-chemical properties of casein micelles. In heated and unheated milk, rheological studies indicated that casein micelles behave as hard spheres of similar volume fractions, if the viscosity changes in the serum phase and the particle particle interactions are taken into account. The differences in the distribution of the heat induced complexes between colloidal and soluble phase affected the colloidal properties of casein micelles. Above 70 g L$^{-1}$ protein, the protein particles were no longer free diffusing. Re-dilution of the suspensions showed no irreversible aggregation. The data suggested that in the range of concentration studied casein micelles behave as hard spheres. Age gelation was also investigated on heated and unheated concentrated milk. In unheated concentrated milk proteolysis played an important role in imparting an increase in viscosity by causing aggregation of the casein micelles. On the other hand, in heated milk, there was a significant effect of the whey protein aggregates, which increased their interaction with the casein micelles over time. This effect, together with proteolysis caused age gelation in heated concentrated milk. The method of concentration used in this research, osmotic stressing, was then compared to ultrafiltration. It was demonstrated that these two methods are not equivalent, as shear and mixing during ultrafiltration cause rearrangements to the casein
micells. The differences were clearly demonstrated by adding soluble caseins to the milk before or after concentration. This project brings a better understanding on the effects of concentration on the structure-function of casein micelles and the interactions occurring in milk proteins during concentration.
Acknowledgements

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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCP</td>
<td>Colloidal calcium phosphate</td>
</tr>
<tr>
<td>CMP</td>
<td>Caseinomacropeptide</td>
</tr>
<tr>
<td>Cn</td>
<td>Casein</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Spectroscopy</td>
</tr>
<tr>
<td>DWS</td>
<td>Diffusing Wave Spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>FESEM</td>
<td>Field Emission Scanning Electron Microscope</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>Ion Chromatography</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma Spectroscopy</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix Assisted Laser Desorption Ionisation- Time of Flight Mass Spectroscopy</td>
</tr>
<tr>
<td>MSD</td>
<td>Mean squared displacement</td>
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<tr>
<td>NaCas</td>
<td>Sodium Caseinate</td>
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<tr>
<td>OS</td>
<td>Osmotic Stressing</td>
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<tr>
<td>PA</td>
<td>Plasmin Activators</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase-High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Poly Acrylamide Electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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</table>
TEM : Transmission Electron Microscopy
TFA : Trifluoroacetic acid
TMP : Trans Membrane Pressure
UF : Ultrafiltration
UHT : Ultra high heat treatment
CHAPTER 1

GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Caseins are a family of calcium binding secretory phosphoproteins contributing to about 80% of the protein content of cow’s milk (Horne, 2002). About 95% of the caseins in milk exist as particles of colloidal dimensions, generally referred to as casein micelles. These protein particles are polydisperse in size, ranging from 60 to 500 nm in diameter. Approximately 94% of the dry matter of micelles is composed of proteins, namely $\alpha_{\text{S}1}$, $\alpha_{\text{S}2}$, $\beta$ and $\kappa$-caseins, and 6% of salt and low molecular weight components, with the majority being referred to as colloidal calcium phosphate (Fox, 2003). The ratio between $\beta$-casein and $\kappa$-casein varies with the size of the casein micelles: the amount of $\kappa$-casein decreases as the micellar size increases, and the content of $\beta$-casein increases (Dalgleish et al., 1989; Marchin et al., 2007). The micellar calcium phosphate is distributed uniformly inside the micellar particles, in clusters of about 2.5 nm in diameter (Marchin et al., 2007); eight hundred of such clusters are present in an average micelle of 100 nm radius (Holt et al., 2003).

The understanding of the formation of the supra-molecular structure of native casein micelles and their changes during processing still holds many challenges. Models for the casein micelles have been described over the years; among various models, a modified nanoclusters model (Dalgleish, 2011; de Kruif et al., 2012) appears to successfully accommodate the behaviour of casein micelles in response to renneting, changes in pH, temperature, urea addition, or removal of calcium phosphate by EDTA. In the earlier
models, such as the submicellar or coat and core models (Morr, 1967; Slattery and Evard, 1973; Schmidt, 1980), as well as newer models like the dual binding model (Horne, 1998; Horne, 2002; Horne, 2009) the stability of the inner structure of the micelles was attributed to a balance of hydrophobic and electrostatic forces. While hydrophobic interactions may be important in the aggregation of the calcium phosphate/protein nanoclusters after they have been formed, once the proteins are at closer distance other short range interactions, such as calcium-bridging, hydrogen bond formation and Van der Waals interactions will occur. These forces are critical in maintaining the aggregate of micelles during cooling or acidification (de Kruijf, 1999; Marchin et al., 2007). The recent description of the casein micelles also take into account the role of water in maintaining micelle integrity and the structure adaptability, by hypothesising the presence of channels or domains of water in the inner core of the micelles (Bouchoux et al., 2010, Dalgleish, 2011, Trejo et al., 2011).

Indeed, the structure of casein micelles is very dynamic, and these particles are little perturbed to changes in serum environment, temperature and pressure (Horne, 2009). Further, the equilibrium of the soluble/bound calcium and phosphate ions in milk is markedly dependent on the temperature, total solids and pH (Holt, 1982).

When water is removed and the concentration of total solids in skim milk is increased, the distance between the casein micelles decreases; this forces the micelles to interact more frequently with each other. The viscosity of milk changes with concentration in a non-linear fashion, with a behaviour analogous to that of colloidal hard sphere suspensions (Dahbi et al., 2010). In addition, as the concentration increases there is a change from Newtonian to non-Newtonian behaviour (Walstra, 1984). A number of
workers have investigated the viscosity of concentrated milk prepared by heat evaporation (Ruiz and Barbosa-Cánovas, 1997; 1998), ultrafiltration (Karlsson et al., 2005; Karlsson et al., 2007; Waungana et al., 1999) or powder reconstitution (Alexander et al., 2002; Anema, 2008; 2009; Dahbi et al., 2010). The Eilers equation is a generally accepted rheological model for concentrated milk systems. However its credibility is questioned at higher volume fractions since it does not take into account the inter-particle interactions.

This project investigated the colloidal behaviour of casein micelles as a function of concentration (Chapters 3 and 4), as well as compositional differences in the serum phase. This information could help us better predict processes in which milk is subjected to concentration. In general, previous studies showed that the suspensions of casein micelles closely follow the behaviour of colloidal hard sphere systems with respect to their viscosity (Griffin et al., 1989, Dahbi et al., 2010), diffusivity (de Kruif, 1992), voluminosity (de Kruif et al., 2012) and light and neutron scattering behaviour (Holt et al., 2003; Horne and Davidson, 1993, Alexander et al., 2011). However, in most of these reported studies, either serum composition or processing history may have dramatically changed the structure-function of the casein micelles. In the present work, research was carried out on the fundamental interactions of the native casein micelles in concentrated milk while maintaining as close as possible the serum composition; this may not be the case in evaporation, drying or reconstitution. During ultrafiltration, the volume fraction of the protein is increased, while the soluble phase is not concentrated.

A better understanding of the physico-chemical properties of concentrated milk and the changes occurring with progressively increasing concentration levels is needed, to further
understand the dynamics of structure changes during concentration and ultimately determine better principles ruling the processing of concentrated milk. Chapter 3 aims at challenging some of the current views on the behaviour of casein micelles.

Results reported in Chapter 4 continue the study of concentrated milk, but after heat treatment. Indeed, our current understanding of how surface modifications with heating to the casein micelles and how these modifications affect their colloidal behaviour is still limited, at least under concentrated conditions. Heating does not seem to alter the structure of the casein micelles, but in milk, at temperatures higher than 70°C, whey proteins undergo extensive denaturation. During heating, the disulphide containing proteins, BSA, α-lactalbumin, αS2-casein, β-lactoglobulin and κ-casein form complexes affecting the processing functionality of milk (Corredig and Dalgleish, 1996; Anema et al., 2004). The role played by heat induced aggregates in regulating the structure function relationship of concentrated heated milk was investigated in Chapter 4.

For preparing concentrated casein micelles while maintaining the serum composition as close as possible to that of natural milk, this research employed osmotic stressing. This is an alternative process for concentrating milk at a laboratory scale and can be considered a non-invasive method, unlike evaporation or ultrafiltration. This technique had been previously employed to concentrate milk protein particles (Farrer and Lips, 1999, Bouchoux et al., 2009a). It was then possible to fine tune the final milk concentration by manipulating the operating conditions, and investigate the fundamental aspects of a colloidal system as a function of concentration, without applying shear or adding water to the sample to achieve high protein concentrations and remove lactose, as usually the case in membrane filtration.
In industrial operations such as evaporation and drying, the properties of micelles may change, because these operations affect the ionic balance of the system and the composition of the serum phase, in turn causing changes in the processing functionality of the proteins. In membrane filtration, on the other hand, the serum phase is transmitted through the filters, and the proteins, depending on the properties of the membrane, are selectively retained in the retentate. Generally ultrafiltration (UF) is considered to be non-invasive, and in Chapter 5 this process was compared to that of osmotic stressing. To evaluate any differences, we compared the renneting functionality of the casein micelles in milk concentrated at equivalent volume fractions, as well as the same milk with and without additional serum caseins added either before or after concentration. An in depth study on the effect of soluble caseins and calcium in retarding the renneting functionality of casein micelles in concentrated milk system were conducted in Chapter 6.

Finally, in Chapter 7 we report the effect of storage of concentrated milk. Indeed, although a plethora of work has been reported on the age gelation of ultrafiltered, concentrated or evaporated milk, the mechanisms leading to an increase in viscosity of the milk have not been fully elucidated. In Chapter 7, the causative mechanisms of age gelation in unheated and heated milk were studied.

The overall objective of this project was to extend the knowledge of the colloidal stability and behaviour of casein micelles as a function of their volume fraction. Very little work has been carried out in dairy systems using osmotic stress as a method of concentration. The understanding of the supra-molecular structure of native casein micelles and their changes during concentration can lead to better product formulation and novel processing techniques.
The working hypothesis is that the colloidal stability will be hampered at a critical micellar volume fraction, and that the properties of the micelles will change. Casein micelles are soft, flexible and adaptable structures, and studies currently employ techniques that do not allow for in situ determination of colloidal behaviour. In this thesis, in addition to conventional rheological methods, light scattering was employed to follow the colloidal behaviour and the dynamics of the casein micelles. In addition, for the first time, the serum phase was fully analyzed, to follow calcium exchanges between the colloidal and the soluble phase in milk as well as the soluble protein composition.

The main objective of the research was to study the concentration induced changes on the colloidal behaviour and functionality of casein micelles. The work was divided in five separate objectives:

1. Characterising the physicochemical behaviour and particle dynamics of casein micelles in untreated skim milk as a function of volume fraction.
2. Study the effect of changes in the serum composition because of heating on the physicochemical behaviour and particle dynamics of casein micelles in concentrated milk as a function of concentration.
3. Evaluating differences in the behaviour of casein micelles concentrated by ultrafiltration and osmotic stressing, by testing their rennet-induced gelation behaviour after processing.
4. Understanding the role of soluble caseins on the impaired rennetability of casein micelles in milk concentrated by osmotic stressing.
5. Identifying the leading causes of age gelation in concentrated milk.
CHAPTER 2

LITERATURE REVIEW

Caseins are calcium binding secretory phosphoproteins, mostly present in milk as a supramolecular aggregate (Fox, 2003). Their structural features have not been fully elucidated, because these proteins cannot be crystallized, hence cannot be analyzed by X-ray. The structure of casein micelles is very dynamic and adapts to the changes in environment like temperature, pH, ionic strength etc. These changes make the studies of the properties of the casein micelles a very interesting field of food colloid chemistry.

Several models have been proposed for the casein micelles and several reviews have been published (Farrell, 1973; Garnier, 1973; Slattery, 1976; Horne, 1998; Smyth et al., 2004; Fox and Brodkorb, 2008). These models tried to interpret the various existing physicochemical results on casein micellar suspensions, and often fell short at explaining the stability and integrity of the micelle under particular processing conditions. In general, the views on the casein micelle structure have slowly evolved over the years. There is general agreement on the main features of the structure, for example, the presence of a protective surface layer of κ-casein which provides steric as well as electrostatic stabilisation to the micelles (de Kruif and Zhulina, 1996; Dalgleish et al., 1989).

2.1. Casein micelle structure models

More than 40 years ago, the casein micelle was viewed as a uniform structure, containing subunits (Morr, 1967). Each subunit was thought to have a similar composition of $\alpha_s$- and κ-caseins both on the surface and the core, and be held together
by calcium phosphate bridging between the submicelles. Later it was suggested that the subunits are different in composition (Slattery and Evard, 1973), and primarily consist of either \( \alpha_s \)- and \( \beta \)-caseins or \( \alpha_v \)- and \( \kappa \)-caseins. In this case, the subunits are held together by hydrophobic interactions and the concept of \( \kappa \)-casein as a size limiting protein was introduced. Only later, more emphasis was given to the role of calcium phosphate as one of the principal holding forces between the submicelles (Schmidt and Payens, 1972).

Later, Walstra (1990) proposed an extension of submicelle model incorporating the steric stabilizing effect of \( \kappa \)-casein. This model also referred to electron microscopy images published in the early 70’s describing the casein micelle having a raspberry-like structure (Buchheim and Welsch, 1973). This appearance strengthened the view of the casein micelle as composed of subunits. Electron microscopy studies of McMahon and McManus (1998) ruled out this model, and suggested that the structure of the micelle is non-homogeneous and contains areas rich in water. Further Field Emission SEM experiments also described the micelles as a more heterogeneous structure, with no subunits and tubular structures of 10-20 nm protruding in the serum phase (Dalgleish et al., 2004). Recent calculations derived from cryo-TEM tomography (Trejo et al., 2011) also failed to identify any spherical protein substructure within the micelle. The major limitation of the submicelle model for casein micelles is that there is no explanation of the reasons behind the presence of two different submicelles during the synthesis in the cell, one rich in \( \kappa \)-casein and one without \( \kappa \)-casein.

A new model, with no submicelles, emerged from results obtained by neutron and X-ray scattering experiments (Holt et al., 2003). In the nanocluster model the micelle is depicted as a homogeneous protein matrix containing an ordered distribution of micellar
calcium phosphate nanoclusters. The model is based on the prediction data for a micelle of radius 108 nm, with a mass of $7.2 \times 10^8$ Da with 830 such clusters, with a mean spacing of 18 nm, acting as scattering points (Holt et al., 2003). The phosphoserine clusters control the surface area of the nanoclusters by forming an outer layer of phosphate groups. The interactions between these amorphous nanoclusters and the proteins cement the inner structure of the casein micelles. This model, however, did not include reasoning behind $\kappa$-casein as a size limiting protein on the micelle.

Another model of the inner structure of the casein micelles focused instead on the manner casein proteins interact with one another. The dual binding model (Horne, 1998; Horne, 2002; Horne, 2009) envisions the interactions between the casein proteins using polymer chemistry principles, describing two different driving forces to the assembly of the caseins in the micelle: 1) interactions of individual caseins through hydrophobic regions of the casein monomers and 2) bridging of caseins through calcium phosphate nanoclusters. The formation (and integrity) of the micelle is viewed as being controlled by a balance between attractive and repulsive forces in casein micelles, i.e., a localized excess of hydrophobic attraction over electrostatic repulsions shielded by calcium phosphate bridges. It is important to note that this model also explains well the presence of $\kappa$-casein on the surface of the particles, as it acts as a chain terminator, controlling the growth during the micelle assembly (Horne, 2002; Horne, 2009).

In spite of the advances in our understanding of the structure of the casein micelles, there are still a few details not fully explained. First of all, none of the models focused on the peculiar role of $\beta$-casein in the structure. This protein is released upon cooling, and its
release is reversible (Creamer at al., 1977). Hence, it is thought not to play an important structural role. However, the release of this protein into solution is limited.

The models outlined above did not fully discuss the reasons behind the high voluminosity of the casein micelle. It has been recently hypothesized that water is distributed unevenly in the interior of the micelles due to the presence of denser protein regions with hydrophobic domains (Dalgleish, 2011). These hydrophobic domains are also highlighted in new calculations deriving from X-ray and neutron scattering data (de Kruif et al., 2012).

The dynamic nature of casein micelles is related to the balance of short range interactions like hydrogen bonding, electrostatic interactions, Van der Waals forces, in addition to protein-protein interactions and calcium phosphate bridging (Dalgleish, 2011). As there is not sufficient \( \kappa \)-casein to stabilize the entire surface of the micelles (Dalgleish, 2011), \( \beta \)-casein also acts as a stabilizing protein, stabilizing the protein rich regions in the inner core of the micelle. It appears that this model could successfully accommodate the behaviour of casein micelles in response to renneting, changes in pH, temperature, urea addition, or removal of calcium phosphate by chelating molecules.

The study of the behaviour of casein micelles under concentrated conditions can further elucidate the structural features of these protein particles. Bouchoux and collaborators (2010) described the deformation behaviour of casein micelles under concentration, once again suggesting a heterogeneous structure of the casein micelle. The protein particles have hard and soft regions, depending on their ability to withstand compression. At the smallest length scale, the calcium phosphate nanoclusters, act as anchor points for casein
molecules. This fraction of the micelle is uncompressible. The intermediate level composed of hard regions of 10-40 nm in size, contains protein and calcium phosphate nanoclusters, on an average 7 nanoclusters, and these regions are connected with each other to form a continuous porous material. The final level of structure is the whole micelle which contains about 30 such hard regions, with considerable polydispersity, with soft regions filled with solvent.

2.2. Destabilization of caseins using rennet

Rennet is an important enzyme to study the functionality of casein micelles. This enzyme, chymosin, an aspartyl protease enzyme that specifically attacks the κ-casein at the Phe$^{105}$-Met$^{106}$ peptide bonds, causes the loss of steric stabilisation and formation of hydrophobic and calcium sensitive patches on the surface of casein micelles (Dalgleish, 1992; de Kruif, 1992). This reaction and the subsequent aggregation of the casein micelles are very sensitive to surface modification to the protein particles. The C terminal portion of κ-casein starting with Met$^{106}$ (called caseinomacropeptide or CMP) is glycosylated, making this part of the structure highly hydrated, providing the protective colloid properties to the micelles. When more than 90% of the CMP has been removed (Dalgleish, 1992), the secondary phase of rennet aggregation occurs, and the micelles aggregate in the presence of free Ca$^{2+}$ (Lucey, 2009). Modifications to the surface of the micelle are particularly critical to the secondary phase of rennet aggregation. For example, it is understood that the presence of sodium caseinate or whey protein heat-induced complexes in milk hinder aggregation (Vasbinder et al., 2003; Gaygadzhiev et al., 2012; Kethireddipalli et al., 2010; 2011).
The rennet reaction follows first order kinetics (Dalgleish, 1988). This may be expected since the Brownian motion of the casein micelles is negligible compared to that of the enzyme molecules, however, studies have not been conducted at high casein micelles volume fractions. Like conventional enzyme reactions, it also depends on both temperature and pH, with optimal temperature being 30°C and the optimum pH 6.0 (van Hooydonk et al., 1984; 1986a; 1986b; Fox et al., 2000).

Calcium ions play a very complex role in renneting and the effect of concentration in milk during gelation has been widely studied (Shalabi and Fox, 1982; Udabage et al., 2001; Sandra et al., 2012). It is generally recognized that the secondary stage of the reaction is mediated by soluble calcium. Ca$^{2+}$ ions form bridges between casein micelles and neutralize the negative charges, decreasing electrostatic and steric repulsion. In short, ionic calcium improves the rate of gel formation and gel strength; however, at high concentrations it seems to have the opposite effect (Lucey and Fox, 1993). Addition of CaCl$_2$ also affects milk pH, and mineral equilibrium. Moreover, the presence of 2-10 mM CaCl$_2$ in milk enhances the rate of gelation (Fox et al., 2000; Udabage et al., 2001; Choi et al., 2007).

In addition to soluble calcium, the equilibrium between colloidal and soluble calcium in milk is also very critical during cheese making (Choi et al., 2004). It has been shown that removal of colloidal calcium phosphate from casein micelles at constant ionic calcium activity results in fewer cross linking between casein micelles, which in turn produces weaker and more flexible gels (Lucey and Fox, 1993) with long term impact during ripening (Choi et al., 2007).
2.3. Heat treatment of milk

Unlike caseins, the structure of whey proteins is modified with heating. Heat-treatment of milk causes unfolding of whey proteins, which can then interact with themselves or with caseins in milk, to form a complex mixture of whey protein aggregates and whey protein coated casein micelles (Donato and Guyomarc’h, 2009).

With heating, the reactive thiol group of β-lactoglobulin (β-lg) is exposed and it is then free to form disulfide links with other thiol groups of β-lg or other proteins like α-lactalbumin (α-la), κ-casein, BSA, α_{s2}-casein, or other minor milk proteins (Hill, 1989, Mulvihill and Donovan, 1987; Corredig and Dalgleish, 1996). These heat-induced complexes are located partly on the surface of casein micelles or milk fat globules, and partly in the serum as small dispersed particles. Small changes of the pH at which the milk was heated had considerable effects on the partition of these complexes between the surface of the micelles and the solution. Heating at acidic pH leads to an attachment of all whey proteins to the casein micelles, while alkaline pH leads to formation of more soluble whey protein aggregates (Anema and Li, 2003a; Vasbinder and de Kruif, 2003). The changes in casein micelle size when heated at different pH levels are attributed to the denatured whey proteins associated with the casein micelles (Anema and Li, 2003a; 2003b).

2.4. Concentration of milk

Concentration of milk is a common unit operation in dairy industry. During concentration water is removed from the bulk and particles starts to interact more frequently with each other due to increased volume fraction (de Kruif, 1998).
The processing functionality of concentrated milk is dependent on the interactions of the casein micelles and the medium in which they are suspended. In general the suspensions of casein micelles closely follow the behaviour of colloidal hard sphere systems with respect to their viscosity (Griffin et al., 1989, Dahbi et al., 2010), diffusivity (de Kruif, 1992), voluminosity (de Kruif, 1998) and light and neutron scattering behaviour (Holt et al., 2003; Alexander et al., 2011; de Kruif et al., 2012). The diffusion coefficient of casein micelles in reconstituted milk powder follows colloidal hard sphere behaviour until a volume fraction of 0.45 (Alexander et al., 2002).

At high volume fractions, the voluminosity and the altered interactions modulate the rheological properties of the skim milk concentrates (Snoeren et al., 1982). The viscosity of milk changes with concentration in a non-linear fashion, and as concentration increases there is a change from Newtonian to non-Newtonian behaviour (Snoeren et al., 1982; Walstra, 1984; Dahbi et al., 2010). The dependence of the viscosity as a function of volume fraction can be modeled as a hard sphere behaviour (de Kruif, 1992), with a deviation at a volume fraction \( \phi > 0.53 \) (Dahbi et al., 2010). Other authors reported deviations at earlier volume fractions (0.45) (Griffin et al., 1989).

The Eilers equation is a generally accepted rheological model (Hallström and Dejmek, 1988; Snoeren et al., 1982; Karlsson et al., 2005) for concentrated milk,

\[
\eta = \eta_0 \left[ 1 + \frac{1.25\phi}{(1-\phi/\phi_{max})} \right]^2
\]  

[Eq. 2.1]

where \( \eta \) is the viscosity and \( \eta_0 \) is the viscosity of the continuous phase and \( \phi_{max} \) is the maximum packing volume fraction of the dispersed particles. According to Snoeren et al.
for milk is 0.79. However, the Eilers equation does not take into account inter-particle interactions, which will occur at higher volume fractions. It is also important to note that usually, the serum viscosity is considered as a constant in the study of the rheological behaviour of concentrated milk, without considering serum composition changes which could affect the rheological properties of the system as a whole (Hallström and Dejmek, 1988; Karlsson et al., 2005).

Recently a rheological model was proposed by Mendoza and Santamaría-Holek (2009) for hard spheres which takes into account inter particle interactions:

\[
\eta = \eta_o \left[ 1 + \left( \frac{\varphi}{1-c\varphi} \right) \right]^{-5/2} \tag{Eq. 2.2}
\]

where \(c\) represents the ratio between \([(1- \varphi_{max})/\varphi_{max}]\), and all the other parameters are defined as above. 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).

The volume fraction \(\varphi\) of casein micelles is a function of their voluminosity. In general the total volume fraction \(\varphi\) in milk is given by

\[
\varphi = C \nu \tag{Eq. 2.3}
\]

where \(C\) is the protein mass concentration (g/mL) and \(\nu\) is the protein voluminosity (Snoeren et al., 1982; and Hallström and Dejmek, 1988). Voluminosity is the effective hydrodynamic volume as defined in the Einstein expression and a value of 4.4 cm\(^3\)/g has been reported for casein micelles in skim milk (de Kruif, 1998). Voluminosity is affected
by pH, heat treatment, crowding etc. Heat treatment causes an increase in the voluminosity of the micelles (de Kruif, 1998; Walstra, 1979). In addition, a decrease in voluminosity has been reported with concentration because of crowding, with higher voluminosities with mild dilutions (Hallström and Dejmek, 1988).

Different methods can be used for measuring voluminosity, namely, microscopy, viscometry, centrifugal sedimentation, dynamic light scattering, and the values determined from different experiments have a different physical meaning. However, most studies of voluminosity of casein micelles have been carried out using centrifugal sedimentation, and this may cause compression of the hairy outer layer (Walstra, 1979; Hallström and Dejmek, 1988). Further, the values obtained from one sedimentation method are different from another using a different centrifugation speed (de Kruif, 1998).

2.4.1. Concentration of milk using Osmotic pressure

In industrial operations such as membrane filtration, evaporation and drying, the properties of micelles change, because these operations affect the ionic balance of the system and may affect the composition, in turn causing changes in the processing functionality of the proteins. Osmotic stress is an alternative process for concentrating milk and can be used for fundamental studies in the laboratory. This method is non-invasive, compared to evaporation or ultrafiltration. Osmosis is the movement of water across a selectively permeable membrane from an area of high water potential (low solute concentration) to an area of low water potential (high solute concentration). The osmotic pressure is defined to be the pressure required to prevent the net movement of solvent. It is directly related to the difference in chemical potential
(Koning et al., 1993). The osmotic pressure $\pi$, is a function of concentration and can be described by (Flory, 1953)

$$\pi = RT \left[ \frac{1}{M_n} C + \left( \frac{1}{2} - \kappa \right) \left( \frac{\nu^2}{V_s} \right) C^2 + \left( \frac{\nu^3}{3V_s} \right) C^3 \right]$$  \[Eq.2.4\]

where $M_n$ is the number average molecular weight, $C$ is the concentration, $\kappa$ is Flory-Huggins interaction parameter which is a measure of the strength of the interactions between the polymer and the solvent, $\nu$ 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. At equilibrium, the molar concentrations of the solutes in the two solutions separated by a permeable membrane are equal.

Farrer and Lips (1999) conducted a detailed study on osmotic stressing using model sodium caseinate dispersion. The range of concentration studied was 0.1-35% w/v. The concentrations reached were higher than close packing concentrations, enabling to study three different regions: a dilute, semi-dilute and a highly concentrated region. This allowed testing interpenetration interactions between the particles. The dispersions did not show hard sphere behaviour as these did not follow the Krieger-Dougherty rheological model.

More recently, osmotic stressing has been applied to model dispersions of native phosphocaseinate (Bouchoux et al., 2009a; 2009b). With this technique it was possible to obtain a wide range of casein concentrations, i.e., from 20 to 500 g/L. The dispersions were concentrated using milk permeate (milk serum) as stressing solution. This allowed maintaining the ionic composition of the serum phase constant, maintaining the integrity
of the casein micelle structure. The results were described in terms of three compression regimes: (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. By analysing the rheology of the concentrated system (Bouchoux et al., 2009b) it was concluded that when casein micelles are well separated from each other, i.e., below close packing, the dispersions behave exactly like a fluid dispersion of hard-spheres. Further, at concentrations of about 178 g/L – approaching close-packing concentrations, the elastic modulus increases rapidly and the dispersions undergo a sol-gel transition, showing a frequency independent elastic modulus.

As previously mentioned, this technique was also employed to investigate the structural changes occurring to casein micelles, using small angle x-ray scattering, at various volume fractions (Bouchoux et al., 2010). The findings revealed hard and soft regions in the casein micelles.

2.4.2. Concentration using membrane filtration

The membrane processes most frequently used in the dairy industry are microfiltration, ultrafiltration, diafiltration, nanofiltration and reverse osmosis, and they differ based on size and selectivity of the membrane. The portion retained and concentrated by the membrane is called retentate; while the fraction transmitted across the membrane is called permeate.
In the present work ultrafiltration (UF) has been employed to collect permeate from milk. UF involves the use of membranes with a molecular weight cut-off in the range 1-200 kDa and a pore size of less than 100 nm. In the dairy industry, UF is mostly used for concentration of proteins in milk with transmission of soluble components (i.e. lactose and minerals) in the permeate (Rosenberg, 1995). The permeate flux through the membrane depends mainly on the membrane type and thickness, fluid viscosity and processing parameters such as trans membrane pressure (TMP), feed concentration, temperature and fluid dynamics in the channels (Cheryan, 1998). Membrane fouling is one of the serious concerns and it reduces the flux over time. Fouling can be due to protein-protein interactions, or protein-membrane interactions, or calcium mediated electrostatic interactions between the charged membrane and proteins (Tong et al., 1989; Jimenez-Lopez et al., 2008).

Tangential flow membrane processes seem to decrease the extent of membrane fouling since the feed flow is tangential to the surface of the membrane, and the feed flow prevents the solids from residing on the membrane (Cheryan, 1998). In this research, a variant of the tangential feed flow, with improved fluid dynamics across the channels (SmartFlow Technologies, Apex, USA) was used; The CONSEP SmartFlow© line of filtration modules have flow channels designed to reduce gel layer build up and prevent fouling. This creates fluid dynamics producing equal flow rates across the membrane surface, optimizing membrane use and expanding the performance limits of traditional cross tangential flow filtration (see manufacturer application notes, for example, SmartFlow Technologies, Apex, USA, NCSRT, 2007).
2.5. Age gelation of concentrated milk

Age gelation is often observed during storage of highly heat treated and/or concentrated milk and it is a process that needs to be controlled to ensure product quality. During storage, the viscosity increases over time, indicating a structure build up and not a sudden change in state (Trinh et al., 2007; Bienvenue et al., 2003).

The important factors which influence the onset of gelation include storage temperature, total solids content, the nature of the heat treatment, seasonal milk production factors, proteolysis during storage, milk composition and initial quality (Datta and Deeth, 2003). The cause of age gelation is still being debated. It was recently hypothesized that the sol gel transition observed in UHT age gelled milk is the net result of a combination of various physico-chemical processes (Datta and Deeth, 2003). A number of observations have been made in heated age gelled milk samples: a marked increase in Maillard browning, hair-like protrusions from casein micelles, and a high extent of proteolysis (Venkatachalam et al., 1993; Datta and Deeth, 2003).

Different mechanisms may be responsible for age gelation in single strength or concentrated milk. In heated, single strength milk (UHT milk) gelation is a two-stage process where proteolysis is followed by nonenzymatic physicochemical changes (Manji and Kakuda, 1988). On the other hand, it has been hypothesized that in concentrated milk gelation occurs by non-enzymatic physicochemical processes (Manji and Kakuda, 1988).

The most agreed upon mechanism for age gelation is proteolysis, attributed to the milk enzymes namely; milk plasmin, a natural milk alkaline serine proteinase (Kelly and Foley, 1997), and proteases produced by psychotropic bacterial contaminants of raw milk.
(Kelly and Fox, 2006). The heat treatment activates the former and eliminates the later.

Milk plasmin is associated with the casein micelle and the milk fat globule membrane in milk, and is highly heat-resistant (Visser, 1981). Both active form and its precursor, plasminogen, is present in bovine milk. Indeed the activity of plasmin in milk is regulated by a complex system of activators and/or inhibitors, namely plasmin activators (PAs), and inhibitors of both plasmin and PAs which converts plasminogen, the precursor of plasmin, to plasmin during storage of milk. Considerable autolysis of plasmin happens at 5°C, while enzymes of psychotropic bacteria are significantly active during cold storage of milk (Crudden et al., 2005; Kelly et al., 2006).

Plasmin preferentially cleaves the polypeptide chains after a lysine or arginine residue. β-casein is the most susceptible protein to plasmin proteolysis, followed by αs1- and αs2-casein. κ-casein is resistant to proteolysis by plasmin. On the other hand, microbial proteases attack predominantly κ-casein to form para- κ-casein (Snoeren and van Riel, 1979), followed by extensive nonspecific hydrolysis (Law et al., 1977).

In heated milk, both plasmin and bacterial proteinases can accelerate age gelation by hydrolysing the anchor points of the β-lg and κ-casein complex from the surface of micelles and releasing it to the serum phase. However, there has been conflicting reports on the correlation between proteolysis and age gelation in concentrated milk.
2.6. Experimental methods

2.6.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Electrophoresis is defined as the migration of charged molecules in a solution through an electrical field. In Polyacrylamide Gel Electrophoresis (PAGE), proteins are solubilised and dissociated into subunits in a buffer containing SDS. Often a reducing agent, namely mercaptoethanol or dithiothreitol, is also employed to break disrupt covalent S-S bridges between/within proteins. SDS is a powerful detergent, which has a very hydrophobic end (the lipid like dodecyl part) and highly charged part (the sulfate group) (Smith, 2010). The dodecyl part interacts with hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends on interactions between hydrophobic amino acids in their core, the detergent destroys 3D structures, transforming what were globular proteins into linear molecules now coated with negatively charged SDS groups. After extensive heat in the presence of SDS, proteins become elongated and fully charged and they will move towards a positive electrode based on their size alone. It is not surprising that the largest extended molecules are generally retarded the most by polyacrylamide gels and the smallest ones the least.

Reducing agents, such as mercaptoethanol or dithiothreitol, are used to reduce disulfide bonds within a protein subunit or between subunits. Since some proteins have few or no hydrophobic residues it is also not surprising that such molecules do not run on SDS PAGE in a fashion which accurately reflects their molecular weight. Modifications such as phosphorylation and especially glycosylation can modify their migration.
There are generally two gels, the resolving gel and the stacking gel. The stacking gel is of very low acrylamide concentration and is used to form the wells into which the protein is loaded. The low acrylamide concentration ensures that the protein sample is concentrated at the front, and the run is homogeneous between samples in the same gel. The higher the acrylamide concentration the resolving gel the slower the proteins go through the gel. On lower percentage gels proteins go faster and vice versa. Ammonium persulfate is rather unstable and decays to produce free radical SO$_4$- ions, which react with the acrylamide molecules and initiate their polymerization. The polymerization occurs by opening the double bond of acrylamide; CH$_2$=CHCONH$_2$. So an acrylamide can react with another acrylamide to produce a linear polyacrylamide molecule, and the incorporation bisacrylamide, (CH$_2$=CHCONH)$_2$CH$_2$, can generate cross links between such linear molecules and the usual application rate is 1 part in 20 to 1 part in 50. The pore size of the resolving gel is selected based on the molecular mass of the proteins of interest and is varied by altering the concentration of ratio of acrylamide with N,N’-methylene-bis-acrylamide in solution. TEMED, (CH$_3$)$_2$NCH$_2$CH$_2$N(CH$_3$)$_2$, acts as a catalyst, speeding up the decay of the ammonium persulfate.

Proteins are usually separated on resolving gels that contain 4–15% acrylamide. Acrylamide concentrations of 15% may be used to separate proteins with molecular mass below 50,000. Proteins greater than 500,000 Da are often separated on gels with acrylamide concentrations below 7% (Smith, 2010).

To perform a separation, proteins in a buffer of the appropriate pH are loaded on top of the stacking gel. A dye (usually bromophenol blue) is added to the protein solution. This dye is a small molecule that migrates ahead of the proteins and is used to monitor the
progress of a separation. After an electrophoresis run, the bands on the gels are generally visualized using a protein stain, such as Coomassie brilliant blue or silver stain. Once stained, different proteins appear in the gel as bands of different thickness and densities. By means of a suitable scanner and analytical software, the protein bands can be quantified (Smith, 2010).

2.6.2. Chromatographic Techniques

Chromatography is a general term applied to a wide variety of separation techniques based on the partitioning or distribution of a sample (solute) between a moving or mobile phase and a fixed or stationary phase. It can be viewed as a series of equilibrations between mobile and stationary phase. The mobile phase is usually a liquid (liquid chromatography) or gas (gas chromatography), or a supercritical fluid (supercritical fluid chromatography) while the stationary phase may be a liquid or a solid. Separation is based on the partition (k) or distribution (D) coefficient; which is defined as the ratio of concentration of the solute in the stationary phase to the concentration of the solute in the mobile phase. By changing the stationary/mobile phase combinations, it is possible to achieve a suitable partition or distribution coefficient for most molecules to be separated. Regardless the kind of separation used, chromatography can be achieved in different assemblies (e.g. paper chromatography, thin-layer chromatography, column chromatography) (Ismail and Nielson, 2010). In column chromatography the stationary phase is enclosed in a column. The mobile phase is passed through the column in an isocratic or gradient elution under a certain pressure. Gradient elutions are often used to elute the molecules interacting to different degrees with the stationary phase (Rounds and Gregory, 2003).
In size-exclusion chromatography (SEC), molecules are separated based on their size, and other types of interactions between solutes and the stationary phase are minimized. The stationary phase consists of particles with defined pore sizes. Large solutes will then be unable to enter the pores and travel with the mobile phase, and are eluted first. On the other hand, small solutes travel also within the pores, extending their elution time. Column packing materials for SEC can be divided into two groups: semirigid, hydrophobic media like polystyrene and soft, hydrophilic gels like Sephadex or cross linked dextran. The former are usually used for the separation of polymers, such as rubbers and plastics and used with organic mobile phases. The latter soft matrixes are available in a wide range of pore sizes and are useful for the separation of water-soluble substances in the molecular weight range $1–2.5 \times 10^7$ (Ismail and Nielson, 2010). The present work employed Sephadex packing material, with the aim of separating whey protein-κ-casein aggregates formed during heating of milk at different pH levels. The S-500 Sephacryl high-resolution gel is a hydrophilic rigid allyl dextran/ bis acrylamide matrix with a fractionation range of 40 to 20,000 kDa and used to separate polysaccharides, macromolecules with extended structures, and even small plasmids. In SEC chromatography, the buffer used as mobile phase must not interact nor destabilize the packing material. The present work employed, 20 mM Bis-Tris-propane (1, 3-Bis [Tris(hydroxymethyl) methylamino] propane) has a wide buffering range, from 6 to 9.5 and fully meets these characteristics. Further it did not cause the denaturation or disruption of the aggregates under study (Donato and Dalgleish, 2006). This methodology has been successfully used to separate native and aggregated serum proteins in milk (Donato and Dalgleish, 2006; Donato et al., 2007). In this research, SEC was
used to determine the amount of heated denatured whey protein-κ casein aggregates present in concentrated milk, heated at different pH levels. The column volume was 120 mL. The fractions were collected and subsequently analyzed by SDS-PAGE.

Reverse Phase-HPLC (RP-HPLC) is a partition chromatography, where solutes are partitioned between two liquid phases according to their partition coefficients. It utilizes a nonpolar stationary phase and a polar mobile phase. Many silica-based, reversed-phase columns are commercially available where silica is the support material for the stationary phase functional groups. Differences in their chromatographic behaviour result from variation in the type of organic group bonded to the silica matrix or the chain length of organic moiety. Some commonly used reverse phase packing materials are octadecylsilyl bonded phases, with an octadecyl (C18) chain and octylsilyl bonded phase with octyl (C8) chain. Shorter hydrocarbons like butyl (C4) or phenyl groups are also employed. Reverse Phase-HPLC utilizes polar mobile phases, usually water mixed with methanol or acetonitrile. Solutes are retained due to hydrophobic interactions with the nonpolar stationary phase and are eluted in order of increasing hydrophobicity. Increasing the polarity of the mobile phase usually increases solute retention, whereas increasing non polarity of the mobile phase decreases retention and results in faster elution. So a gradient elution is practised most of the time to get a better resolution (Reuhs and Rounds, 2010).

Chymosin (rennet) specifically cleaves the Phe\textsubscript{105}-Met\textsubscript{106} bond of κ-casein and releases a hydrophilic C terminal peptide known as caseinomacropeptide (CMP). CMP is highly glycosylated with oligosaccharides that are O-linked to threonine and serine. In the present
research, 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 (Rounds and Gregory, 2003). The gradient elution is 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. Hence the detection is carried out at 214 nm.

For the age gelation study in this project, the existence of proteolysis was tested using RP-HPLC. In the past, the non-protein nitrogen in the acid-soluble fraction has served as an indicator for hydrolytic protein breakdown (Harwalkar, 1992; Datta and Deeth, 2003). In parallel, casein hydrolysis can be followed by gel electrophoresis (Srinivasan and Lucey, 2002, Bienvenue et al., 2003). These techniques are not very sensitive, and for this reason RP-HPLC using an Octadecyl (C18) bonded column was employed in this work.

Ion-exchange chromatography is another type of adsorption chromatography, in which the interactions between solute and stationary phase are primarily electrostatic in nature. The stationary phase contains fixed functional groups that charged either negatively (hence cation exchangers) or positively (hence anion exchangers). There are two ways to elute the bound solutes, either by changing the mobile-phase, for example, changing the pH or increasing the ionic strength (e.g., use NaCl) (Ismail and Neilson 2010).
In the present work cation exchange chromatography was employed as recently reported (Holland et al., 2010). The separation of the caseins was carried out using a HP SP cation exchange column (1 mL). This is a column which uses a packing material based on Sepharose™ (a tradename for a crosslinked, beaded-form of agarose). Sample preparation includes the denaturation of the proteins with urea and 2-mercaptoethanol in the sample buffer. The elution is carried out with a gradient of NaCl in a buffer containing 6 M urea - 0.2 M sodium acetate at pH 3.5.

Determination of insoluble and soluble calcium was estimated with a slight modification of new method using non-suppressed ion chromatography (Rahimi-Yazdi et al., 2010). This method allowed for large number of samples to be measured at once maintaining high accuracy and reproducibility. The method is based on the changes in conductance caused by the elution of ions. A cation column packed with silica gel with weakly acidic carboxyl acid functional groups (RCO₂⁻) group was employed. In ion chromatography a suppressor system is often used to reduce the high background conductivity of the electrolytes in the eluent, and to convert the sample ions into more conductive form. However, by careful selection of eluents, column and system, determination of cations by ion chromatography is possible without the presence of a suppressor (Rounds and Gregory, 2003). As detection is carried out by differences in conductance of the ionic forms, mineral species need to be in their ionic form when reaching the detector and the organic species should be minimised.

For conventional calcium estimation, samples are often incinerated and digested to decrease the amount of organic minerals present (Moreno-Torres et al., 2000). In this research both the conventional method and IC chromatography were employed for the
estimation of the calcium concentration in the samples. In the case of chromatographic separation, two important features were included to avoid the time consuming, labour intensive sample preparation by incineration or digestion. Firstly to decrease the interference of di- and tri-carboxylic acid present in the milk (mainly citrate) the samples were diluted, acidified and isolated by preparative ion exchange chromatography (Rahimi-Yazdi et al., 2010). Preparative ion exchange was carried out by packing a column with Amberlite IRA 958 (Fluka, Fisher) resin. This is a strongly basic anion exchange resin of acrylic copolymer with quaternary ammonium groups.

After sample preparation, a dialysis unit was employed (Metrohm Canada Ltd, Mississauga, ON, Canada) to separate high molecular weight organic material (eg. protein) from the sample, while the ions are carried by the acceptor solution of nitric acid. The samples for IC were then pumped by an autosampler to one side of the dialysis membrane. At the same time an acceptor solution (2 mM nitric acid) was continuously pumped on the other side of the membrane to collect the ions. The ion enriched acceptor solution was then injected into the column, which (with a mobile phase consisting of a solution of 1.7 mM nitric acid and 1 mM pyridine-2,6- dicarboxylic acid in isocratic solution) allowed the separation of cations such as sodium, potassium, calcium and magnesium. The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration curves prepared with individual cation standards.
2.6.3. Electron Microscopy

Two types of electron microscopy techniques are generally employed for studying food microstructure: Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The electron beam is focused using magnetic lenses in both techniques. The specimen is placed into the path of the electron beam in the TEM, while in the SEM, it is placed at the end of the focused electron beam path. The image is produced in the form of a shadow on a fluorescent screen in TEM, whereas in SEM reflected and secondary electrons are processed by an electron detector to form a three dimensional image on a monitor screen.

In this research images were obtained using a cold field-emission ultra high resolution scanning electron microscope (FESEM). This instrument shows improved details on the surface structure of casein micelles, compared to the conventional SEM. In conventional SEM with thermionic emission, the electron beam is formed by heating a tungsten hairpin, and the size of the resulting beam is thus limited by the geometry of the filament, which in turn limits resolution and magnification. In FESEM, a cold field emission source produces electrons by applying a high voltage to a very sharp point, extracting the electrons in a tunnel directly from the point without heating (Watt, 1985; Pawley, 1997). Large electric fields and ultra high vacuum are required, but the very small diameter and brightness of the beam that results, along with advances in electron optics, filters and turbo molecular vacuum pumps, greatly increase the resolution and hence possible magnification (up to 800,000) of the microscope (Hitachi High Technologies Corporation, 2003).
The samples were prepared using a self-assembled monolayer (SAM) technique as previously reported (Martin et al., 2006). This technique requires the use of polished carbon planchets. The planchets are then immersed in a solution 11-mercaptoundecanoic acid to form a SAM which provides the functional groups that will bind to casein micelles. These planchets were then incubated with few drops of concentrated milk for 45 min to allow binding between the protein and the SAM. After the reaction, the plachets were washed with buffer, to remove the unbound material, and samples were then fixed using glutaraldehyde, dehydrated with a graded series of ethanol, and critical point dried using carbon dioxide. The samples were then mounted onto SEM stubs with colloidal carbon, and stored in a desiccator at room temperature until imaging.

2.6.4. Light Scattering

Dynamic light scattering measures the Brownian motion of particles and relates this to their hydrodynamic size. The particles are illuminated with a laser, and the intensity fluctuations in the scattered light are then collected. The Brownian motion is the movement of particles due to random collision with the molecules of the liquid that surrounds the particle, hence small particles move faster and large particles move slower. The relationship between size and the translational diffusion coefficient is defined by the Stokes- Einstein Equation:

\[ d_h = kT/3\pi\eta D \]  \hspace{1cm} [Eq. 2.5]

where \( d_h \) is the hydrodynamic diameter, \( D \) the translational diffusion coefficient, \( k \) is the Boltzman’s constant, \( T \) the absolute temperature and \( \eta \) is the viscosity of the medium. The diameter obtained by DLS is the diameter of a sphere that has the same diffusion
It is important to notice that the diffusion coefficient will not only depend on the size of the particle but also by factors such as the viscosity of the medium, the surface structure and shape of the particle.

As the particles are constantly in motion, the speckle pattern will also appear to move. As the particles move around, the constructive or destructive phase addition of scattered light will result in intensity fluctuations. If we compare the intensity of signal of a particular part of the speckle pattern at one point of time (say t) to the intensity of the signal a very short time later (t+\(\delta t\)) the two signals are very strongly correlated. If we compare the original signal a little further ahead in time (t+2\(\delta t\)) there would be still reasonable comparison between the two signals, but it will not be as good as (t+\(\delta t\)). The correlation is therefore reducing with time. At a much later time, there will be no longer a correlation, as the particles are moving in random directions. DLS measurements deal with short time scales; in a typical speckle pattern the length of time it takes for the correlation to reduce to zero is in the order of 1 to 10\(\times\)10\(^{-3}\) s. The \(\delta t\) will be in the order of nanoseconds or microseconds. The fitted correlation function is then given by:

\[
g(1)(t) = e^{-\left(\frac{t}{\tau}\right)} \quad \text{[Eq. 2.6]}\]

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

\[
\tau = \frac{1}{(Dq^2)} \quad \text{[Eq. 2.7]}
\]

where D is the diffusion coefficient, and q is the scattering vector.
\( q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2} \) \hspace{1cm} [Eq. 2.8]

\( \theta \) is the scattering angle, \( n \) the refractive index of the medium, and \( \lambda \) the wavelength of the laser in vacuum. In a system of polydisperse sizes, each size generates an equation of the type shown in 2.6 and 2.7.

An extension of the traditional dynamic light scattering technique, diffusing wave spectroscopy (DWS) makes it possible to study colloidal processes such as gelation or phase separation. DWS is a light scattering technique that permits the investigation of the interparticle interactions \textit{in situ}, avoiding the necessity of extensive dilution, which may alter the “true” behaviour of the colloidal suspensions. DWS relies on many scattering events happening when the light passes through a colloidal dispersion. The light propagation through the sample is assumed to occur in a diffusive fashion. Similarly to dynamic light scattering, DWS measures the intensity fluctuations of the transmitted scattered light caused by the Brownian motion of colloidal particles.

In transmission DWS, detected intensity fluctuations can be mathematically represented by Eq. 2.9 (Weitz and Pine, 1993).

\[
G_1(t) \approx \frac{\left( L \left( \frac{l^*}{l^*} \right)^{4/3} \right)^{1/2}}{(1+\frac{8t}{3\tau}) \sinh \left[ \frac{L}{l^*} \left( \frac{6t}{\tau} \right)^{1/2} \right]^4 + 4 \left[ \frac{L}{l^*} \left( \frac{6t}{\tau} \right)^{1/2} \right]^{1/2}} \hspace{1cm} [Eq. 2.9]
\]

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 \( t \ll \tau \) and \( L/l^* > 10 \).

The parameter \( l^* \) can be obtained from the equation:

\[
\frac{I}{I_0} = \left( \frac{5l^*}{3L} \right) \left( 1 + \frac{4l^*}{3L} \right) \tag{Eq. 2.10}
\]

where \( I \) is the intensity measured and \( I_0 \) is the initial laser intensity obtained from the measurement of a latex standard of known particle size.

The exponential correlation function, \( g(1)(t) \), is described with a characteristic decay time \( \tau = \frac{1}{k_0^2 D} \), where \( k_0 \) is the wave vector \( (k_0 = 2\pi n/\lambda, \text{where } n \text{ is the refractive index of the continuous phase and } \lambda \text{ is the wavelength}) \) and \( D \) is the particle diffusion coefficient. This relation holds true only in freely diffusive systems, therefore, a direct correlation between the decay time and the diffusion coefficient would only be valid before strong interparticle interactions can be detected. Once the particle dynamics 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. 2.9 must be substituted by \( k_0^2 \langle \Delta r^2(t) \rangle /6 \), where \( \langle \Delta r^2(t) \rangle \) is the mean squared displacement.

\[
\langle \Delta r^2(t) \rangle = \frac{6}{k_0^2 \tau} t \tag{Eq. 2.11}
\]

DWS detects the mobility of scatterers over a length scale much shorter than the wavelength of the laser used to illuminate the sample. As mentioned above, each of the
numerous scattering events contributes to the complete dephasing of the light. Thus, each particle has to move a relatively short distance to collectively achieve the complete randomization of incident light.

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

$$l^* \propto (\int F(q)S(q)q^3 dq)^{-1}$$  \hspace{1cm} [Eq. 2.12]

In a highly dilute system in which particles are far from each other and interparticle interactions are negligible, $S(q) = 1$. In 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 can be attributed 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 $F(q)$.

In summary, DWS allows the 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 (Weitz et al., 1993). DWS has been successfully used to follow sol-gel transitions in milk systems (see for example, Alexander and Dalgleish, 2004; Sandra et al., 2012).
2.6.5. Rheology

Rheology is the study of deformation and flow of matter. The relationships between stress and deformation for complex systems like foods differ from Newton`s law of viscosity, which describes the shear behaviour of normal fluids and Hook`s law of elasticity, the relationship between stress and deformation for metals and other elastic materials. The type of stress, force applied per unit area, depends on the direction of the force, with respect to the impact surface. If the force is directly perpendicular to a surface, a normal stress results and the force act in parallel to the sample surface, a shear stress ($\sigma$) is experienced (Morrison, 2001; Daubert and Foegeding, 2010). The resultant deformation is called normal strain or shear strain respectively. When material is liquid, shear strain quantification is little challenging, instead shear rate ($\gamma$), degree of deformation with time, has been used.

In the present research a controlled stress rheometer, with a cone and plate geometry was used for both rotational and oscillatory measurements. The cone has an angle of 1° and its special design permits the shear rate and shear stress to remain constant for any location of sample in the fluid gap (Mezger, 2006). Rotational measurements were employed to obtain flow behaviour and viscosity data on concentrated milk, while oscillatory measurements were used for characterizing the viscoelastic components of the system at very small deformation.

For Newtonian fluids, the viscosity function is constant and called Newtonian viscosity. However, for most liquids the viscosity term is not constant, but rather a change as a
function of the shear rate, and the material is considered non-Newtonian. The apparent viscosity function is the result of the shear stress (\(\sigma\)) divided by the shear rate (\(\gamma\)):

\[ \eta = f(\gamma) = \frac{\sigma}{\gamma} \quad \text{[Eq. 2.13]} \]

where \(\eta\) is the apparent viscosity, and it is a function of the shear rate.

For the study of sol-gel transitions, small deformation rheology is employed, as the material characterization is carried out within the linear viscoelastic range of the sample. In this case, stress or strain is applied in a oscillatory, sinusoidal mode, and the response, strain or stress and the difference in the wave phase can be measured over time (Mezger, 2006; Daubert and Foegeding, 2010). Indeed, being an oscillatory measurement, when a harmonic periodic shear stress applies parallel to the surface the resultant deformation varies sinusoidally with the response shifted out of phase by a phase angle \(\delta\). The phase angle ranges from 0° (elastic response) to 90° (viscous response), and is given by the equation

\[ \delta = \omega \Delta t \quad \text{[Eq. 2.14]} \]

where \(\omega\) is the angular frequency of oscillation. Additionally, the complex modulus, \(G^*\) is calculated from both the stress (\(\sigma_A\)) and strain (\(\gamma_A\)) amplitudes with the equation:

\[ G^* = \frac{\sigma_A}{\gamma_A} \quad \text{[Eq. 2.15]} \]

with the complex modulus and the phase angle it is possible to derive material properties such as the elastic (\(G'\)) and the viscous (\(G''\)) moduli.
In a sol gel transition, at the beginning of the reaction, $G''$ is higher than $G'$ and the phase angle is high ($\delta \sim 90^\circ$). As structure begins to form, the phase angle starts to decrease, and $G'$ starts to overcome $G''$, showing a more elastic character of the gel. The crossover of $G'$ over $G''$ can mark the transition from liquid like to solid like behaviour, at this point $\tan \delta =1$ (Doublier et al., 1992; Curcio et al., 2001).
CHAPTER 3

PHYSICO-CHEMICAL PROPERTIES OF CASEIN MICELLES IN UNHEATED SKIM MILK OSMOTICALLY CONCENTRATED: INTERACTIONS AND CHANGES IN THE COMPOSITION OF THE SERUM PHASE.
3.1 Abstract

The changes in processing functionality of concentrated milk are caused by a number of factors, amongst the most important, the ionic equilibrium and the increase in the interactions between the casein micelles because of their increased volume fraction. The objective of this work was to characterize the physico-chemical properties of casein micelles as a function of their volume fraction, by using osmotic stressing as a non-invasive method to obtain concentrated milk, in the attempt to preserve the ionic balance during concentration. Osmotic concentration was carried out for 18h at 4°C, using different concentrations of polyethylene glycol dissolved in permeate as the stressing polymer. The viscosity of the concentrated milk could be predicted using established rheological models, although only when the changes occurring to the viscosity of the serum phase were taken into account. Both Eilers and Mendoza equations predicted a maximum packing volume fraction of 0.8 for the casein micelles. After concentration up to 20% protein, the casein micelles did not show a change in their size upon redilution. Light scattering measurements carried out using diffusing wave spectroscopy without dilution showed a hard sphere behaviour for the casein micelles with the characteristic of free diffusing Brownian particles up to a volume fraction of 0.3, and restricted motion at higher concentrations. Results of total and soluble calcium suggested release of colloidal calcium phosphate from the micelles at volume fractions > 0.35. This research brings new insights on the changes occurring in skim milk during concentration.
3.2 Introduction

The caseins are a family of calcium binding phosphoproteins that comprise about 80% of the protein present in milk with overall concentration of approximately 25 g L\(^{-1}\) and the majority (Fox, 2003) exist as particles of colloidal dimensions known as “casein micelles”. The remainder of the milk protein is whey proteins, which are composed mostly of β-lactoglobulin, α-lactalbumin and bovine serum albumin (Fox, 2003). The casein micelles are of great interest for colloid chemists as these represent the response of nature to the need to deliver a high level of calcium to the neonate. These micelles are highly hydrated colloids (3-4 g of water per g of protein), composed of a core of highly phosphorylated caseins (αs- and β-caseins) interacting with calcium phosphate, with a stabilizing layer of κ-casein on the surface (Dalgleish, 2011). The casein micelles contain calcium phosphate nanoclusters bound to the phosphoserine groups of the αs1, αs2, and β-caseins. This colloidal component is in equilibrium with the calcium and phosphate present in the soluble phase (Holt, 2002). The casein micelles are polydisperse in size (between 60 and 500 nm in diameter). In bovine milk, the content of κ-casein decreases as the micellar size increases, balanced by an increase in the content of β-casein, while the proportions of αs1 and αs2-casein are independent of micelle size (Dalgleish et al., 1989; Marchin et al., 2007). The micellar calcium phosphate is distributed uniformly inside the micellar particles, in clusters of about 2.5 nm in diameter (Marchin et al., 2007).

The understanding of the supramolecular structure of native casein micelles and their changes during processing still holds many challenges. This aggregated structure is very
dynamic as it responds in various ways to environmental changes as well as to the presence of the other components present in milk (Horne, 2009). In the past years there has been an increased interest in the study of the effects of concentration on the structure and processing functionality of casein micelles. With concentration, the serum composition may change as well as the interactions between the colloidal particles, perhaps affecting the structure and function of the casein micelles.

The viscosity of milk changes with concentration in a non-linear fashion (Snoeren et al., 1982); as concentration increases there is a change from Newtonian to non-Newtonian behaviour (Walstra, 1984). The rheological properties of concentrated milk prepared by either heat evaporation (Ruiz and Barbosa-Cánovas, 1997; 1998), ultrafiltration (Pignon et al., 2004, Karlsson et al., 2005) or powder reconstitution (Anema, 2009; Dahbi et al., 2010) have been investigated. In addition, a plethora of work has been reported on age gelation or renneting of ultrafiltered, concentrated milk (For eg., Karlsson et al., 2007; Bienvenue et al., 2003) or evaporated milk (Hwang et al., 2007; Harwalkar et al., 1983), because of the technological implications for a number of established dairy processes. However, little research has been carried out on the fundamentals of the interactions of casein micelles in concentrated milk. When membrane filtration is employed as a means to concentrate milk, shear effects and membrane fouling may occur, while when concentrating by evaporation, heating is applied and the serum composition may change. These differences in processing history make a study on the properties of the casein micelles during concentration quite challenging, and for this reason, knowledge of the fundamental aspects of how the physico-chemical properties of caseins evolve during concentration of milk is very
Osmotic stress is an alternative process for concentrating milk which may be considered non-invasive. This technique has already been successfully employed to characterize concentrated milk systems by other groups (Bouchoux et al. 2009a; 2009b). By using milk permeate (the serum phase of milk), it is possible to maintain the original serum environment for the casein micelles. This has important implications for the equilibrium between the colloidal and the soluble calcium, which may otherwise change, with consequences to protein-protein interactions within the casein micelle. In short, this allows the investigation of the fundamental aspects of concentrated systems as a function of volume fraction of the casein micelles, without applying shear (as in membrane filtration) or affecting the serum composition. A detailed study of the dependence of the osmotic pressure of sodium caseinate solutions as a function of concentration has been published (Farrer and Lips, 1999), encompassing the dilute, semi-dilute and highly concentrated regimes. The relative viscosity of sodium caseinate was shown to increase gradually with concentration up to about 10% (w/w) and then steeply after (Farrer and Lips, 1999). Recent work (Bouchoux et al., 2009a) explored model dispersions of phosphocaseinate over a wide range of casein concentrations (from 20 to 500 g L$^{-1}$). The results were described in terms of three compression regimes: dilute, transition and concentrated regimes. The same authors also studied in detail the rheological behaviour of the system (Bouchoux et al., 2009b), concluding that when casein micelles are below close-packing conditions, these protein particles behave like polydisperse hard-spheres. At concentrations close to close-packing (178 g L$^{-1}$), the elastic modulus increases rapidly and the system progressively shows a frequency independent elastic modulus.
The objective of this work was to extend the knowledge of the colloidal properties of casein micelles in untreated skim milk as a function of volume fraction. In addition to the determination of the rheological properties, a detailed composition analysis of the serum phase was carried out as well as a study of the colloidal properties of the casein micelles using diffusing wave spectroscopy, a light scattering technique which does not require dilution of the sample. This work will allow for a better understanding of the effects of concentration on the physico-chemical properties of the casein micelles, and may strengthen our current understanding of the structure of these colloidal particles.

3.3 Materials and Methods

3.3.1 Skim Milk and Permeate preparation

Sodium azide (0.2 g L⁻¹) was added to fresh raw milk (University of Guelph Dairy Research Station, Ponsonby, Ontario, Canada) to prevent microbial growth. Skim milk was prepared by centrifuging at 4000 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). Ultrafiltration permeate was prepared by ultrafiltration of reconstituted skim milk powder (100 g L⁻¹ solids) (Gay Lea Foods Cooperative, 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 average ionic composition of UF permeate is: ~20 mM Na⁺, ~40 mM K⁺, ~10 mM Ca²⁺, ~30 mM Cl⁻, ~10 mM phosphate, ~10 mM citrate, in agreement with previous reports (Jenness and Koops, 1962).
3.3.2 Osmotic Stress Method for Milk Concentration

Polyethylene glycol (PEG) with a molar mass of 35,000 Da (Fluka, Oakville, Ontario, Canada) was used as the stressing polymer. PEG is a flexible, water-soluble polymer, and preliminary experiments showed that it has no specific interactions with calcium. This polymer is widely used to obtain high osmotic pressures and the system is well characterized (Koning et al., 1993). All experiments were carried out by dispersing PEG in permeate (prepared as described above) containing 0.2 g L\(^{-1}\) sodium azide as a bacteriostatic. The use of permeate ensured that the ionic composition remained similar across the dialysis membrane, which was a standard regenerated cellulose (Fisher Scientific, Whitby, Ontario, Canada) with a molecular mass cut off of 6–8 kDa. This pore size ensured the exchange of water, ions, and lactose but not caseins or PEG. Before experiments, the dialysis membranes were washed in MilliQ water and conditioned in milk permeate. Milk samples (40 mL) were inserted in dialysis tubing, and immersed in a 1 L of permeate solution containing different PEG concentrations. The osmotic pressure experiment was conducted for 18 h at 4°C, to minimize sample degradation. Significant degradation may occur conducting the dialysis of unheated milk at 20°C (Bouchoux et al., 2009a), and the milk in these experiments was untreated. The pH of all dispersions remained unchanged during the experiment.

The volume fraction was calculated by assuming the voluminosity of the micelles to be constant at 4.4 mL/g (Holt, 1992), throughout the concentration range of our experiment.

3.3.3 Separation of the serum phase from concentrated samples

The concentrated milk samples were equilibrated at room temperature for 1 h before
serum separation. Preliminary experiments were used to determine a suitable centrifugation speed. The centrifugation speed chosen was the minimum required to effectively deposit the casein micelles as a firm pellet. During preliminary trials, the serum was also measured by dynamic light scattering with no further dilution, and very little scattering was detected, suggesting that a serum devoid of casein micelles was obtained at these centrifugation speed. Soluble whey proteins for the present experiment were therefore defined as those that did not sediment from the milk during ultracentrifugation at 100,000g for 1 h at 20°C in a Beckman Coulter Optima™ LE-80K ultracentrifuge with rotor type 70.1Ti (Beckman Coulter Canada Inc., Mississauga, Canada). The clear supernatant was carefully removed from the pellets using a pasteur pipette, and was given two sequential filtrations using 0.45 μm and then 0.22 μm (syringe driven filters, Fisher Sci.). It was then analysed for protein, calcium content, viscosity, refractive index and particle size. Protein analysis was carried out 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.

3.3.4. Diffusing Wave Spectroscopy (DWS)

DWS allows the investigation of the static and dynamic behaviour of colloidal particles in fairly concentrated suspensions (Weitz et al., 1993). Static properties of the samples were measured via the value of the photon transport mean free path, l*, which represents the length scale over which the direction of the light passing through a sample has been fully randomized. Turbidity was measured as the inverse of the l*. In addition, the apparent diffusion coefficient (D) was obtained by probing the colloidal mobility over a very short time scale. The D is derived from the characteristic decay time of the intensity
auto-correlation function and can be used to calculate the apparent particle radius, via Stokes-Einstein relation, when the colloidal particles are freely diffusing.

Once the particle dynamics are changed to a sub-diffusive motion (e.g., the point after which a liquid-like colloidal suspension is converted to a more gel-like state), mean squared displacement (MSD) values can be calculated to probe the system dynamics (Weitz and Pine, 1993). At very short times, when the time of the measurement is much smaller than the characteristic decay time of the system, the MSD can be written as:

$$\langle \Delta r^2 (t) \rangle \propto t^p$$  \hspace{1cm} [Eq. 3.1]

where the exponent $p$ has a value of 1 for a freely diffusing particle. In an arrested system the value of $p$ is always less than 1 (Krall and Weitz, 1998).

The sample (~1.5 mL) was poured into an optical glass cuvette (Hellma Canada Ltd., Concord, Ontario, Canada) with a 5 mm path length. The cuvette was placed in a thermostatted water bath at a temperature of 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 (Gaygadzhiev et al., 2008). When measuring the various concentrated milk samples as a function of time using DWS, it took about 20 min for the samples to equilibrate after the transfer from the pipette to the cuvette. The values shown in this work were taken after measuring for 40 min.

### 3.3.5. Particle Size Determination by Dynamic Light Scattering

The particle size of the casein micelles was measured by dynamic light scattering
(Zetasizer Nano-ZS). After concentration, the milk samples were diluted ~2000 times in filtered (0.2 µm nylon filters, Fisher scientific) milk permeate and analyzed.

### 3.3.6 Mineral Determination

Determination of insoluble and soluble calcium was carried out using non-suppressed ion chromatography (Rahimi Yazdi et al., 2010). The amount of soluble calcium was defined as the total calcium in the serum phase after centrifugation at 100,000 g (see above). The centrifugal supernatant (0.5 mL) was mixed with 200 µL of 1M HCl, and adjusted to a final volume of 50 mL in a volumetric flask with HPLC grade water. For the determination of total calcium aliquots (333 µL) of milk samples were mixed with 200 µL of 1M HCl and brought to a final volume of 1.5 mL in an Eppendorf microcentrifuge tube using HPLC water. The mixture was then centrifuged at room temperature for 15 min at 4500g (Eppendorf centrifuge, 5415D, Brinkmann Instruments Ltd., Mississauga, Ontario, Canada). The supernatant (333 µL) was then diluted to 50 mL with HPLC grade water.

Preparative chromatography was carried out to decrease the interference of di- and tri-carboxylic acids, mainly citrate, available in milk, as they interfere with the detection of cations. Preparative chromatography columns (Kontes FlexColumn Economy columns, 1.0 cm inside diameter, 20 cm length, Fisher, Nepean, Canada) were packed with Amberlite IRA 958 (Fluka, Sigma Steinhein, Germany) ion exchange resin. Resin was activated with a 100 g L⁻¹ NaCl and 20 g L⁻¹ NaOH solution at 1 mL min⁻¹ flow rate for 30 min and then rinsed with water for 6 h at 1 mL min⁻¹. The samples (50 mL) were loaded at a flow rate of 1 mL min⁻¹, the first 25 mL discarded and 2 aliquots of 12 mL
each were collected in sample vials for further analysis. The column was then rinsed with 25 mL of water before loading a new sample. Regeneration was carried out using 4 M sulphuric acid at 2 mL min⁻¹ for 75 min. The samples were refrigerated until further analysis.

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. The samples were loaded with a 838 sample processor into 833 IC liquid handling Dialysis unit (both Ω Metrohm). The instrument was operated with IC Net (v. 2.3, Metrohm). The samples were pumped by an autosampler to one side of the dialysis membrane. At the same time an acceptor solution (2 mM nitric acid) 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 (Metrosep C2-150, Ω Metrohm) and was eluted isocratically with a mobile phase consisting of a solution of 1.7 mM nitric acid and 1 mM pyridine-2,6- dicarboxylic acid. The elution was monitored with a conductivity detector and the area under each peak was quantified using calibration curves prepared with individual cation standards (TraceCERT, Fluka, Sigma Steinheim, Germany). Both column and detector temperatures were kept at 30°C.

Determination of total and soluble phosphate was carried out using Inductively Coupled Plasma Optical Emission Spectrometry at the Laboratory Services facilities of the University of Guelph.

The levels of colloidal calcium and phosphate in the samples can be estimated from the
difference between the total amount of calcium/phosphate and that measured in the centrifugal supernatant fraction.

3.3.7 Rheology Measurements

A controlled stress rheometer (Paar Physica MC 301, Anton Paar, Graz, Austria), was used to measure the viscosity of the reconstituted concentrate. The concentrated milk samples as well as the centrifugal supernatants were subjected to a shear sweep test using a cone and plate geometry, with a set gap of 0.51 mm. The temperature of the plate was controlled with water circulating from a Julabo F25-HP refrigerated and heated water bath (Julabo Labortechnik, GbmH, Germany). All measurements were made at 25°C.

To model the rheological behaviour of concentrated milk, a semi-empirical Eilers equation is usually applied (Karlsson et al. 2005; Snoeren et al., 1982).

\[
\eta = \eta_o \left[1 + \frac{1.25\varphi}{(1-\varphi/\varphi_{max})}\right]^2
\]  

[Eq. 3.2]

Where \(\eta_o\) is the viscosity of the continuous phase and \(\varphi_{max}\) is the critical packing volume fraction of the dispersed particles. For milk, the \(\varphi_{max}\) has been found to be 0.79 (Snoeren et al., 1982).

In addition to the Eilers equation, the Mendoza model for the effective viscosity of a solid sphere suspension was also applied to the experimental data. This model takes into account the inter particle interactions and hydrodynamic interactions between the colloidal particles (Mendoza and Santamaria-Holek, 2009).
\[ \eta = \eta_o \left[ 1 + \left( \frac{\phi}{1-c\phi} \right)^{-5/2} \right] \]  

[Eq. 3.3]

Where \( c \) represents the ratio between \( (1-\varphi_{max})/\varphi_{max} \) and all the other parameters are defined as above. 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).

3.4 Results and Discussion

Figure 3.1 depicts the changes in protein concentration in the milk sample as a function of PEG concentration in the permeate side after 18 h of dialysis. It is very clear that by increasing the osmotic stress level it was possible to achieve high protein concentrations while maintaining the serum composition close to that of skim milk. Using 120 g L\(^{-1}\) PEG, milk was concentrated from its original 32 g L\(^{-1}\) protein to about 250 g L\(^{-1}\) protein with a corresponding reduction in the volume of the sample.

The increase in the concentration of protein in milk will decrease the spacing between the micelles, affecting the interactions between them. At the native concentration in milk (25 g L\(^{-1}\)), the casein micelles, with a mass of approximately 2.8\(\times\)10\(^8\) Da and an average radius of 78 nm (Morris et al., 2000) are about 121 nm apart. At a concentration of 75 g L\(^{-1}\) their intraparticle distance decreases to 22 nm. At higher concentrations their interparticle distance will hence result in strong interactions. It has been previously reported that with concentration, the viscoelastic properties of milk change (Snoeren et al., 1982; Dahbi et al., 2010; Bouchoux et al., 2009b). At protein concentrations of 125 g L\(^{-1}\), corresponding to an osmotic pressure of 4.5 kPa, phase transition of native phospho-
Figure 3.1. Amount of protein in milk, after 18 h of dialysis in permeate, as a function of different PEG concentrations. Error bars indicate standard error of three independent trials.
caseinate from a sol to a gel has been observed (Bouchoux et al., 2009a). Jamming concentrations of 180 g L\(^{-1}\) have also been reported for a lactose free micellar suspension (Dahbi et al., 2010).

3.4.1. Characterisation of the serum phase of milk

To fully understand the behaviour of the different milk samples as a function of concentration, the soluble fraction was also characterized. In particular, the protein concentration, the refractive index and viscosity of the serum were measured, as they are critical to the interpretation of the light scattering and rheological properties of the concentrates. It is important to note that as the concentration of protein increased, an efficient separation of the serum phase was increasingly challenging (because the actual amount of serum decreased dramatically). Therefore only the serum fraction of samples up to 150 g L\(^{-1}\) protein could be successfully separated and analyzed.

The amount of protein recovered in the serum fraction increased gradually with concentration (Figure 3.2). It is important to note that when the serum fraction was analyzed by SDS-PAGE, loading the same amount of protein in each lane, the ratio between whey protein and caseins in the serum phase did not appear to change with concentration within the experimental error (data not shown). These results were not surprising, as both caseins and whey proteins were retained by the dialysis membrane. At the highest concentrations it appears that a higher amount of protein may be present in the serum phase: this could be protein originally occluded in the serum contained in the hairy layer around the micelles being squeezed out at high concentration factors because of the close approach of the micelles. Indeed, it has been previously reported that
**Figure 3.2.** The amount of soluble protein present in the serum phase as a function of concentration.
sedimentation may cause compression of the hairy layer (Walstra, 1979) and recent work showed that during the evaporation process water is removed preferentially from the serum phase (Liu et al., 2012).

Figure 3.3 summarizes the values of refractive index and viscosity of the centrifugal serum as a function of protein concentration in the milk. Both the index of refraction and viscosity increased, and this was caused by the increase in the concentration of soluble protein. The serum showed a Newtonian fluid behaviour in the whole range of concentration investigated.

3.4. 2. Changes in Calcium during Concentration

Determination of insoluble and soluble calcium, although of critical importance, has not been well reported in the literature as a function of concentration of casein micelles. Ca\(^{2+}\) is a structural component of the casein micelles and its equilibrium can affect the processing functionality of the casein micelles (Lucey and Horne, 2009). The colloidal calcium phosphate (CCP) is in dynamic equilibrium with the mineral components in the soluble phase, and it is not yet clear to which extent this equilibrium is affected during concentration of the micelles (Holt, 2002). It has been previously hypothesized that as the milk is already saturated with calcium phosphate, a considerable proportion of soluble calcium and phosphate may be transferred into the colloidal state during concentration by evaporation (Hardy et al., 1984; Nieuwenhuijse et al., 1988, Liu et al, 2012). This would lead to a greater amount of CCP per gram of casein in the concentrated milk as compared with normal milk.
Figure 3.3. Refractive index (■ and right-hand scale) and viscosity (▲ and left-hand scale) of the serum phase as a function of concentration of protein in the original milk.
Nevertheless, as a consequence of concentration, the pH decreases and ionic strength increases (Anema, 2009), both of these reducing the amount of calcium associated with the micelles (Snoeren et al., 1984). However, during membrane concentration of milk, calcium deposition on the membrane may occur. On the other hand, during osmotic stress using permeate the ionic concentration in the serum phase is maintained as close as possible to that of the original milk.

The amount of soluble calcium present in the centrifugal supernatant increased with concentration. The initial concentration of soluble Ca\(^{2+}\) in the skim milk was 9.8 ± 0.48 mM. 10 mM Ca\(^{2+}\) was present in the serum phase in the outer phase of the dialysis membrane as the dialysis was conducted against milk permeate.

Figure 3.4 summarizes the amount of total and soluble calcium as a function of the protein concentration. As expected, the total amount of calcium increased with concentration, as the calcium associated with the micelles continued to be retained. However, Figure 3.4A clearly shows that at >100 g L\(^{-1}\) protein (at a volume fraction >0.3) the amount of total calcium may be reaching a plateau. The amount of soluble Ca\(^{2+}\) present in the serum phase (Figure 3.4B) also increased with concentration (in spite of the dialysis equilibration) in a linear fashion, and this increase can be attributed to the calcium associated with the soluble proteins present in the soluble phase (mostly \(\alpha\)-lactalbumin), as previously shown by other authors (Rahimi Yazdi et al., 2010).

An analogous trend was observed in the case of total phosphate, the control milk had a total phosphate content of 28.1±0.2 mM and increased to 64.5±0.5 mM when the protein content of the sample was 100.24±0.2 g L\(^{-1}\). Similarly the ratio of soluble phosphate to
Figure 3.4: Total (A) and soluble (B) calcium content present in milk as a function of protein concentration. The mean and standard error of three independent trials are shown.
soluble protein showed a steady decline.

Because of the experimental design (the use of constant serum composition during osmotic stressing), this study could not confirm previous reports that at high protein concentrations an amount of colloidal calcium is released to the serum phase (Snoeren et al., 1984; Anema, 2009). At least up to 90 g L\(^{-1}\) there was a steady increase in the amount of total Ca\(^{2+}\) (Figure 3.4A) and only at that concentration the data may indicate a critical concentration where the micelles are trying to resist structural changes caused by the extraction of the water from the dispersion.

The release in colloidal calcium phosphate will help balance the osmotic gradient between the inner core of the micelles and the serum phase, and will result in an increase in negative charges within the micelles. Therefore the reaching of a plateau value at 90 g L\(^{-1}\) (Figure 3.4A) may signal the beginning of changes in composition of the calcium phosphate nanoclusters of the micelles. Indeed it has been hypothesized using X ray scattering (Bouchoux et al., 2010), that there are hard regions in the casein micelle of about of about 25 nm of diameter and containing about 7 calcium phosphate nanoclusters, and these regions resist compression even at much higher concentrations. These results would lead to the conclusion that calcium is released without disrupting the internal supramolecular structure of the casein micelles.

3.4. 3. Rheological properties of concentrated milk

Figure 3.5 illustrates the changes in viscosity of the samples concentrated by osmotic stressing, as a function of volume fraction. The inset shows the entire volume fraction measured, using a log scale for relative viscosity. A conversion from protein
Figure 3.5: Changes in viscosity measured at 300 s\(^{-1}\) of the concentrated milk as a function of volume fraction. Symbols are experimental values (mean and standard error of three independent trials). The solid line corresponds to theoretical predictions of viscosity using Mendoza Model (Eq. 3.3) for interacting colloidal hard spheres. The inset illustrates the values of viscosity measured in a wider range of volume fractions. The long and short dash lines represent Eilers model (Eq. 3.2) with a \(\varphi_{\text{max}}\) of 0.79 (Snoeren et al., 1982) and using the serum viscosity \(\eta_0\) from experimental values (Figure 3.3, long dash line) or constant serum viscosity of 0.00102 Pa s (short dash line).
concentration to volume fraction ($\varphi$) was necessary to be able to compare the experimental data with theoretical models.

Concentrated milk up to a volume fraction of 0.4 exhibited Newtonian behaviour and shear thinning behaviour was observed at higher concentrations (results not shown). This corresponded to a protein concentration of approximately 100 g L$^{-1}$. Previous researchers assumed constant serum viscosity to predict the rheological behaviour of concentrated milk (Karlsson et al., 2005; Dahbi et al., 2010); however, the Eilers equation could not fit the experimental values (short dashed line, Figure 3.5) when a constant value of viscosity (the viscosity of the initial serum or permeate) was employed in the calculations. The experimental data shown in Figure 3.5 were well predicted by the Eilers equation (long dashed line), if the value of $\eta_0$ was varied, to take into account the variation in serum viscosity (Figure 3.3). A critical packing volume fraction ($\varphi_{\text{max}}$) of 0.79 was used in these calculations in accordance with previous literature (Snoeren et al., 1982; Karlsson et al., 2005). For each volume fraction the corresponding value of serum viscosity determined experimentally was used. It is important to note that in Figure 3.5 it was assumed that the voluminosity of the micelles did not change with concentration. It has been recently demonstrated that during concentration by evaporation the water is preferentially removed from the serum than from the micelles (Liu et al., 2002).

As the Eilers equation does not take into account inter particle interactions, the experimental data were also fit to a model which takes interactions into account (Eq. 3.3) (Mendoza and Santamaria-Holek, 2009) with a $r^2$ of 0.999. In this case also, the viscosity of the concentrated micellar suspensions could be predicted by knowing the
background viscosity (Figure 3.3). The predicted values for hard sphere behaviour showed a $\phi_{\text{max}}$ of 0.81. It may also be worth pointing out that at the very low concentrations a slight deviation from the fit may be noted, and this could be due to an increase in the voluminosity of the casein micelles at the very low concentrations. This would be consistent with previous studies showing both for heated and unheated milk a higher voluminosity of the casein micelles in diluted milk (Hallstrom and Dejmek, 1988).

Nonetheless, it was possible to conclude that assuming no changes in voluminosity, the casein micelles behave as hard spheres with a crirical packing volume in untreated skim milk of 0.8. This is important, as it has been previously suggested that a decrease in the voluminosity of the casein micelles with concentration occurs (Hallström and Dejmek, 1988). Rheological data on lactose free micellar caseins showed a deviation from hard sphere behaviour at a volume fraction of 0.69 (Dahbi et al., 2010). Our results are in full agreement with those of Bouchoux et al. (2009b) who also described the casein micelles as incompressible polydisperse hard spheres, however their critical volume fraction was 0.65. This divergence in the critical packing volume may derive from their use of native phosphocaseinate material compared to the present work, where untreated skim milk was employed.

3.4. 4. Light scattering properties of concentrated milk

The turbidity parameter ($1/l^*$) as a function of the volume fraction of the various concentrated milk samples is illustrated in Figure 3.6. Increasing the concentration of casein micelles led to a corresponding gradual growth of $1/l^*$. The values of $1/l^*$ increased monotonically up to a $\phi$ of ~0.4. At higher volume fractions, the value of $1/l^*$
Figure 3.6 Changes in $1/l^*$ as a function of casein micelle volume fraction. The symbols correspond to three independent experiments. The solid line indicates the theoretical behaviour of the turbidity parameter $1/l^*$ fitted to the theory for a hard-sphere of 216nm of diameter with a refractive index of 1.38 and 1.34 as refractive index of the solvent.
no longer increased. Figure 3.6 also illustrates the theoretical behaviour for a hard sphere system (line): as the protein content in the sample increases, more scattering events happen in a given space and the light becomes randomized faster, decreasing the value of \( l^* \). At higher volume fractions there is a decrease in the \( 1/l^* \).

At low volume fractions (<0.32) the values obtained experimentally (Figure 3.6) fit well to the theoretically predicted values for a hard-sphere system (solid line, Figure 3.6) with the experimentally obtained values of size by DWS and refractive index parameters corresponding to those used before for casein micelles (Alexander et al. 2002); however, at higher volume fractions, the experimental values deviated from those calculated from theory, as only hydrodynamic interactions can no longer take into account the changes in the turbity of the system. At higher concentrations, there will be interparticle interactions besides those of hydrodynamic drag which will cause strong positional correlations between the particles and further decrease the value of \( l^* \). However, it is important to point out that refractive index contrast is also an important factor affecting the \( 1/l^* \) parameter. It can not be excluded that the micelles may undergo some internal rearrangements (due to the release of colloidal calcium phosphate, see Figure 3.4) but these changes will decrease the refractive index of the micelles. In addition, the rheological data shown in Figure 3.5 suggest that the voluminosity does not vary with concentration.

To determine if aggregation occurred in the milk during osmotic stressing concentration experiments, the size of the casein micelle was measured by dynamic light scattering after diluting the samples in permeate. Figure 3.7 illustrates the apparent
Figure 3.7 Hydrodynamic radius measured by dynamic light scattering after dilution of the samples with permeate. The x axis indicates the protein concentration of the original sample, before dilution. Values are the average of three replicates. Note that in these experiments, samples were concentrated up to 200 g L$^{-1}$ protein.
radius measured after dilution for all the concentrated milk samples. After redilution, for milk concentrated up to 20% protein (corresponding to approximately 0.65 volume fraction) the casein micellar size was constant after dilution, confirming that no aggregation occurred in these samples.

The self-diffusion coefficient of the particles was also derived from the DWS experiments, for the milk concentrated by osmotic stressing. At the low volume fractions the normalized diffusion coefficient decreased with increasing concentration (data not shown) similarly to the behaviour of a hard-sphere suspension, in agreement with previous work (Alexander et al., 2002). At the low volume fractions (<0.4) the diffusion coefficient was used to calculate the radius of the particle, with the assumption that the system was free diffusing. The values of the radius, calculated by DWS (83.34±6.14) were in full agreement with that measured by DLS (82.43±1.38).

To better understand the behaviour of the casein micelles as a function of concentration, the dynamics were also evaluated, by calculating the mean squared displacement (MSD). In other words, for free diffusing particles, there is a linear relationship between their displacement (MSD) and the time. Figure 3.8 describes the MSD obtained from the correlation functions of milk with different volume fractions. At the low volume fractions <0.32%, the particles showed a behaviour characteristic of free diffusing Brownian particles as it is clear that there is a linear relation between time and MSD. The milk with a volume fraction of 0.39 started to deviate from this behaviour, and at higher protein content, the particles are no longer free diffusing and experience restrictions in their movement. These results show for the first time that restricted motion
Figure 3.8: Time dependence of MSD of milk samples with different volume fraction. From left to right: 0.1 (control milk), 0.29, 0.39 and 0.5. To show the deviation of MSD with time, straight lines are drawn from the origin to each set of experimental results.
of casein micelles starts to occur at such low volume fractions. Similar light scattering experiments, carried out on micellar dispersions showed a hard sphere behaviour up to volume fractions of 0.54 (Dahbi et al., 2010). The decrease in the mobility of the casein micelles shown in Figure 3.8 may be related to their polydisperse nature. Indeed at a volume fraction of 0.3, the interparticle distance between the micelles (see above) is much smaller than their size, causing the small casein micelles to be caged in, surrounded by the larger ones.

3.5. Conclusions

The present work further characterized the physico-chemical properties of casein micelles in concentrated milk. Osmotic stressing is a convenient and non-destructive method to study the concentrated milk as a function of volume fraction. Up to a volume fraction of 0.6 casein micelles do not irreversibly aggregate, as when measured under diluted conditions, their size is constant.

At volume fractions <0.32 the behaviour of casein micelles can be fully modelled to that of hard spheres of similar size and refractive index. In addition, rheological properties were well described by the Eilers’ and Mendoza equations, with a critical packing volume fraction of about 0.8. This is a much higher critical volume fraction than that reported by recent literature (Dahbi et al., 2010; Bouchoux et al., 2010). The polydispersity of the casein micelles allows such high packing values, as shown in rheological experiments, and the discrepancies from previous literature derive from differences in the sample preparation (this study used fresh milk while others used reconstituted milk or phosphocaseinate powders). The rheological measurements would
also suggest that the voluminosity of the casein micelles does not vary with concentration, once again confirming that the casein micelles behave as uncompressible hard spheres up to high critical packing volume fractions.

However, hydrodynamic interactions between the micelles occur at much lower volume fractions. Diffusing wave spectroscopy data clearly indicated that when milk is concentrated to a volume fraction higher than 0.32, the behaviour of the casein micelles can no longer be predicted using only hydrodynamic effects, and that stronger interactions occur between the particles. The casein micelles are no longer free diffusing, as the interparticle distance creates caging of the small protein particles, which are surrounded by the larger casein micelles. The combination of serum composition analysis, light scattering and rheological measurements would hence suggest that in the range of concentration studied, casein micelles behave as uncompressible hard spheres.
CHAPTER 4

COLLOIDAL PROPERTIES OF HEATED CONCENTRATED MILK
4.1 Abstract

The colloidal properties of casein micelles in heated milk as a function of volume fraction are largely unknown. Heat-treatment of milk causes denaturation and unfolding of whey proteins, which then react with caseins to form a complex mixture of soluble whey protein aggregates and whey protein coated casein micelles. The initial pH of the milk before heating is an important factor determining the distribution of the complexes between the soluble and the micellar phase. It was hypothesized that the differences in the distribution of the complexes after heating would affect the colloidal properties of casein micelles. Although there were differences in the apparent diameter after heating milk at pH 6.4, 6.8 or 7.0, redilution of the concentrated milk showed no irreversible aggregation of the casein micelles with concentration. Above 70 g L\(^{-1}\) protein, there was a decrease in the mean square displacement slope, suggesting that above this concentration, the casein micelles were no longer free diffusing. The viscosity of the concentrated milk could be predicted using a rheological model for hard spheres assuming constant voluminosity; however, a more detailed study of the experimental data suggests that changes in voluminosity with heating can not be ruled out.

4.2 Introduction

Heat treatment of milk causes changes in the conformation of whey proteins. The reactive thiol groups of β-lactoglobulin (β-lg) molecules become exposed, and the proteins form disulfide links with other thiol containing proteins such as β-lg, bovine serum albumin (BSA), α-lactalbumin (α-la) or κ- and α\(\_\text{s2}\)-caseins (Corredig and Dalgleish, 1996; Anema et al., 2004; Lowe et al., 2004). The average size of the
complexes found in the serum phase after heating is \(\sim 3.5\text{-}5.5 \times 10^6\) Da, depending on the available \(\kappa\)-casein or the ratio of casein to whey protein present in the mixtures (Guyomarc’h et al., 2003a).

The heat-induced whey protein complexes are located partly on the surface of the casein micelles and partly in the serum as small dispersed particles, stabilized by \(\kappa\)-casein. Small changes in the pH of the milk prior to heating have considerable effects on the distribution of the complexes between the serum and the dispersed phase: heating at pH below that of native milk (pH 6.7) leads to a preferential attachment of whey proteins to casein micelles, while higher pH (i.e. 7.0) leads to an increased population of soluble whey protein aggregates (Anema and Li, 2003b; Vasbinder and de Kruif, 2003). Approximately 70\% of the whey proteins are associated with the casein micelles when milk is heated at a pH of 6.5, while that of normal pH is 30\% and at higher pH values there is a progressive decrease in the amount bound to the micelles (Anema and Li, 2003b). These changes have a substantial impact of the processing functionality of heated milk (Lucey et al., 1998; Kethireddipalli et al., 2010).

It has been previously reported that casein micelles closely follow the behaviour of a colloidal hard sphere systems with respect to their viscosity (Griffin et al., 1989, Dahbi et al., 2010), diffusivity (de Kruif, 1992; Alexander et al., 2002), voluminosity (de Kruif, 1998) and light and neutron scattering behaviour (Holt et al., 2003; Horne and Davidson, 1993, Alexander et al., 2011). However, the effect of milk processing history has never been explored. In reconstituted skim milk powder, the casein micelles follow the ideal behaviour of colloidal hard spheres up to a volume fraction of 0.45 (Alexander et al.,
Previous light scattering and viscosity experiments (Chapter 3) on concentrated casein micelles demonstrated that these particles can be modelled after hard spheres even at concentrations reaching close packing (0.8 volume fraction), but that they show arrested motion at a volume fraction of about 0.3. It was concluded that in unheated milk, the voluminosity of the casein micelles did not vary with concentration, up to the point of close packing. These results supported recent studies on evaporated milk that showed that with increasing volume fraction, the water is preferentially removed from the soluble phase (Liu et al., 2012). The objective of this work was to extend the understanding of the colloidal behaviour of casein micelles in concentrated milk that has been heated. This will allow for a better design of concentration processes.

To investigate the effect of the distribution of the heat induced complexes present in milk on the colloidal properties of the casein micelles, the milk was heated at 90°C for 10 min to cause extensive denaturation of the whey proteins, after adjusting the milk to three different pH levels, 6.4, 6.7 and 7.0. Concentration was carried out using osmotic stressing to alter the volume fraction of casein micelles with minimum changes in the ionic balance or shear effects. In addition to the determination of the rheological properties, a detailed investigation was carried out on the composition of the serum phases in heated concentrated samples as well as on the colloidal properties of the casein micelles using diffusing wave spectroscopy, a light scattering technique which does not require dilution of the sample.
4.3. Materials and Methods

4.3.1. Sample Preparation

Sodium azide (0.2 g L\(^{-1}\)) was added to fresh raw milk (University of Guelph Dairy Research Station, Ponsonby, Ontario, Canada) to prevent microbial growth. Skim milk and ultrafiltration permeate were prepared as described in 3.3.1.

The pH of skim milk was adjusted to 6.4 and 7.0 by slow addition of 1 M HCl or NaOH, while stirring. The pH was allowed to equilibrate for at least 2 h, minor readjustments were made during that time. The milk samples (pH 6.4, 6.7 and 7.0) were heated for 10 min at 90°C in a water bath allowing 2.5 min for the samples to reach the final temperature. After heating, the milk was immediately cooled to room temperature by immersion in an ice bath. The samples were then kept for 2 h at room temperature before further sample manipulation.

Milk was then concentrated using osmotic stressing using a 35,000 Da polyethylene glycol (PEG) as the stressing polymer, as described in 3.3.2. The osmotic pressure experiment was conducted by placing 40 mL of heated milk in the dialysis tube and immersing it in a 2 L cylinder containing 1 L of dialysis medium (0, 4, 7 or 8.5% PEG dissolved in milk permeate). The three different PEG concentrations allowed reaching three different protein volume fractions for each pH treatment. The dialysis was conducted for a fixed time of 18 h at 4°C to minimize sample degradation. The pH of all dispersion remained unchanged during the experiment.
4.3.2. Separation of the serum phase from concentrated samples

The soluble fraction was separated from the colloidal casein micelles using ultracentrifugation. The soluble whey protein fraction was defined as the fraction of whey protein that does not sediment during centrifugation at 100,000g for 1 h at 20°C. Samples were centrifuged using a Beckman Coulter Optima™ LE-80K ultracentrifuge with rotor type 70.1Ti (Beckman Coulter Canada Inc., Mississauga, Canada). The clear supernatant was carefully recovered using a Pasteur pipette and then analysed for protein, calcium content and viscosity as described in Chapter 3. Protein analysis was carried out 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.

4.3.3. Light scattering measurements

The particle size of the casein micelles was measured, after extensive dilution with permeate, using dynamic light scattering (DLS) (Zetasizer Nano-ZS). Permeate was filtered with 0.2 μm nylon filters (Fisher Scientific) and milk diluted about 2000 times immediately before analysis at room temperature.

Although DLS is very useful to determine irreversible changes to the apparent diameter of the casein micelles, this technique is limited in its ability to characterize the colloidal properties of the various milk samples as a function of concentration, as all the samples need to be diluted. Hence, transmission DWS was also employed in this study, as it allows the investigation of the static and dynamic behaviour of colloidal particles in fairly concentrated suspensions (Weitz et al., 1993). The turbidity parameter (1/l*) was employed to determine the static properties of the samples. The l*, or photon transport
mean free path, represents the length scale over which the direction of the light passing through a sample has been fully randomized. In addition, the mean squared displacement (MSD) was also derived from the correlation function obtained using DWS (Weitz and Pine, 1993). For details on the experimental set up see 3.3.4.

4.3.4. Calcium and Phosphate Determination

Determination of total and soluble (in the centrifugal supernatant) calcium and phosphate were carried out using Inductively Coupled Plasma Optical Emission Spectrometry at the Laboratory Services facilities of the University of Guelph.

4.3.5. Rheology Measurements

A controlled stress rheometer (Paar Physica MC 301, Anton Paar, Graz, Austria), was used to measure the viscosity of the concentrated milk samples as described in 3.3.7. All measurements were made at 25°C. To model the rheological behaviour of concentrated milk, the Mendoza model for the effective viscosity of a solid sphere suspension was also applied to the experimental data. This model takes into account the hydrodynamic interactions between the colloidal particles (Mendoza and Santamaria-Holek, 2009).

\[
\eta = \eta_o \left[ 1 - \left(\frac{\varphi}{1-c\varphi}\right)^{5/2}\right] \quad \text{(Eq 4.1)}
\]

Where \(\eta_o\) is the viscosity of the continuous phase, \(c\) represents the ratio between \(\frac{(1-\varphi_{max})}{\varphi_{max}}\) and \(\varphi_{max}\) is the critical packing volume fraction of the dispersed particles.

In the case of raw and heated milk, for each volume fraction the corresponding value of serum viscosity, \(\eta_o\), determined experimentally was used for calculating the relative
viscosity (see 3.3.7).

4.3.6. Analysis of the Heat-Induced Aggregates

To better evaluate the composition and distribution of the whey protein aggregates in the serum phase, the centrifuged supernatant was analyzed by size exclusion chromatography using an AKTA purifier (900 series, GE Biosciences, Baie d’Urfe, Quebec, Canada), equipped with a UV-900 detector (set to 280 nm). The supernatants were filtered through 0.45 μm filters (Millipore Corporation, Bedford, MA) and injected (1 mL) on a Pharmacia XK 16/70 column packed with a S-500 Sephacryl high resolution gel, with a molecular weight cut-off of 2000 kDa (GE Healthcare), as reported in the literature (Donato and Dalgleish, 2006). 20 mM Bis-Tris-Propane at pH 7.0, containing 0.02% sodium azide was employed as the elution buffer. Peaks were collected, freeze dried (Virtis Freeze Mobile 12SL, San Francisco, CA, USA) and analyzed by SDS-PAGE.

The freeze dried samples were dispersed in milli-Q water in appropriate ratios depending upon the initial volume fraction of the samples, and then in a 1:2 ratio of sample to sample buffer (0.5 M Tris-HCl, pH 6.8, 20 g/kg SDS, 190 g/kg glycerol, 0.5g/kg 2-mercaptoethanol, 0.1 g/kg bromophenol blue). The protein sample was then heated for 5 min at 95°C. In the case of samples analyzed under non reducing conditions, 2-mercaptoethanol was omitted from the sample buffer. For the electrophoresis, the resolving gel contained 15% acrylamide (3.3% bis-acrylamide) in 0.75 M Tris-HCl at pH 8.9, and the stacking gel contained 4% acrylamide in 0.1 M Tris-phosphate buffer at pH 6.7. The electrophoresis buffer was 0.7 M Tris-HCl, 0.45 M glycine at pH 8.3. Aliquots
of 7 μL of the prepared samples were loaded onto the gels. The electrophoretic separation was performed at 200 V for 40 min using a Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Hercules, CA).

The gels were stained with Coomassie blue in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min with shaking and were destained with two changes of 1 h of a 45% (v/v) methanol and 10% (v/v) acetic acid solution and then one change for 12 h of a 4.5% (v/v) methanol and 1% (v/v) acetic acid solution. Whey protein isolate (1%) and sodium caseinate (1%) were used as reference. The 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.

4.3.7. Statistical analysis.

All experiments were carried out in triplicate (i.e. three separate milk batches), and the average and standard errors are reported. Statistical significance was evaluated using ANOVA at p<0.05 and Tukey’s multiple comparison tests.

4.4. Results and Discussion

4.4.1. Osmotic Stressing Concentration

Figure 4.1 illustrates the protein concentration obtained with increasing levels of PEG, for unheated milk as well as milk at different pH values heated at 90°C. There was no significant effect of treatment and all the data are consistent with those previously discussed in Chapter 3.
**Figure 4.2:** Protein concentration obtained after 18 h of osmotic stressing as a function of concentration of polyethylene glycol present. Results are the average of three independent experiments, with bars representing standard error.


4.4.2. Particle Size

Table 4.1 summarizes the values of hydrodynamic radius for unheated milk and milk heated at different pH levels, measured using DLS after dilution in fresh permeate. Casein micelles were larger when heated at pH 6.4 compared to pH 7 and pH 6.7. These results were consistent with previous literature (Vasbinder and de Kruijf, 2003). This change in the size of the casein micelles has been related to the amount of denatured protein, particularly β-lg, attached to the micelles (Anema and Li, 2003a). Heating at pH 6.4 results in an increase in micellar radius of about 30 nm with 85% of the whey proteins present in the colloidal phase, while at pH 7.0 the size is smaller than that of milk heated at normal pH and only 15% of the whey protein complexes is associated with the micelles (Anema, 2007). The increased release of casein in the soluble phase during heating of milk would also contribute to the smaller size at the higher pH values (Donato et al., 2007).

Within treatment, there was no significant difference in size with concentration, indicating that in all cases, the presence of protein aggregates did not cause irreversible aggregation of casein micelles, at protein concentration up to 110 g L$^{-1}$. This was also consistent with other literature reports (Jeurnink and de Kruijf, 1993).

4.4.3. Composition of the Centrifugal Supernatants

4.4.3.1. Soluble Proteins

It has been previously shown that the amount of soluble protein present in unheated milk increases linearly with concentration by osmotic stressing (Chapter 3).
Table 4.1: Apparent hydrodynamic radius obtained from DLS for heated milk at different pH, for three concentration levels obtained by osmotic stressing. Data were obtained after diluting the suspensions in milk permeate. Values are the average with standard error of three independent heating-concentration experiments. Control samples correspond to non-concentrated milk. Different letters correspond to significant differences at P<0.05.

<table>
<thead>
<tr>
<th>Protein (g L⁻¹)</th>
<th>Hydrodynamic Radius (nm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unheated milk pH 6.7</td>
<td>pH 6.4</td>
<td>pH 7.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>82.4 ± 1.4c</td>
<td>80.1 ± 1.0cb</td>
<td>87.3 ± 1.0d</td>
<td>77.8 ± 0.8ab</td>
</tr>
<tr>
<td>40</td>
<td>82.4 ± 1.4c</td>
<td>79.3 ± 1.0cb</td>
<td>87.4 ± 0.9d</td>
<td>77.9 ± 1.1ab</td>
</tr>
<tr>
<td>70</td>
<td>82.1 ± 1.9c</td>
<td>79.6 ± 0.6cb</td>
<td>88.3 ± 0.5d</td>
<td>77.1 ± 0.6ab</td>
</tr>
<tr>
<td>110</td>
<td>81.5 ± 0.3c</td>
<td>80.2 ± 0.3c</td>
<td>88.0 ± 0.2d</td>
<td>76.2 ± 0.9a</td>
</tr>
</tbody>
</table>
Also in the case of heated milk, the amount of soluble proteins recovered in the serum fraction increased with increasing concentration (Figure 4.2); however, the soluble protein concentration was significantly different depending on the pH of the milk prior to heating. ANOVA and Tukey’s multiple comparison tests on the soluble protein content of the concentrated milk revealed a significant difference in the soluble protein between milk heated at pH 6.7 and 6.4 (p<0.05), pH 6.4 and pH 7.0 (p<0.001) and pH 6.7 and 7.0 (p<0.01) for 3x concentrated milk. Note that three different concentration trials were averaged on the x axes of Figure 4.2. The amount of protein recovered in the supernatant of heated milk at pH 7 was higher than that of unheated milk and that of heated milk at pH 6.4 was lower. This increase in soluble protein content in heated serum between pH 6.4 and 7.0 is well in agreement with previous literature reports (Donato and Dalgleish, 2006; Anema, 2007).

The proteins present in the centrifugal supernatant of milk concentrated after heating was analyzed using SDS-PAGE, loading an equal amount of protein (24±2 μg of protein) in each well (Figure 4.3). In unheated milk, whey proteins (especially β-lg and α-la) were recovered in the centrifugal supernatant, as well as a low amount of caseins, especially β-casein. The ratio of whey protein to serum caseins did not change with concentration (Figure 4.3, lanes 10 and 11). A high ratio of whey proteins to caseins was shown in these non sedimentable fractions from unheated milk (Figure 4.3, lanes 10 and 11), with no differences between the concentrated and the non concentrated samples, within the experimental error.
Figure 4.2: Amount of protein recovered in the centrifugal supernatants as a function of the protein concentration of unheated milk as well as milk heated at different pH. Values represent the average and standard deviation of three concentration trials.
Figure 4.3: SDS PAGE under reducing conditions of centrifugal supernatants: Lanes 1, 2 and 3: Heated milk at pH 6.7 (original pH) concentrated using 0, 7 and 8.5% PEG; Lanes 4, 5, 6: Heated milk at pH 6.4 concentrated using 0, 7 and 8.5% PEG; Lanes 7, 8 and 9: Heated milk at pH 7.0 concentrated using 0, 7 and 8.5% PEG; Lanes 10 and 11: unheated milk concentrated using 0 and 7% PEG. Each lane was loaded with approximately 24 µg of protein. Note that Lines 10 and 11 were run on a different gel. The arrow indicates direction of migration.
The non sedimentable fraction of heated samples is shown in Figure 4.3, lanes 1-9 for three different concentration levels. Once again, there was no difference with concentration within this pH of treatment. In all cases, within the heating pH, the polypeptide composition was similar; however, the ratio of caseins to whey proteins present in the centrifugal supernatant varied significantly between treatments (i.e. heating at different pH). It is important to note that these supernatant fractions were filtered through 0.2 μm pore size filters. Milk heated at the initial pH of 6.4 showed the highest ratio of caseins to whey proteins in the non sedimentable fraction, (Figure 4.3, lanes 4, 5 and 6), while samples heated at pH 6.7 and 7.0 showed a higher ratio of whey protein to caseins. To better quantify these differences size exclusion chromatography was combined with electrophoresis on isolated fractions.

Amongst the caseins, in addition to β-casein, intense bands of κ-casein and α₂ caseins were recovered in the centrifugal supernatant, consistent with the formation of soluble disulphide linked whey protein aggregates. The ratio of β-casein to κ-casein seemed to be the highest in non sedimentable fractions of milk heated at pH 6.4, and the lowest in the fractions of milk heated at pH 7.0. In addition, high molecular weight proteins (Bovine serum albumin, Immunoglobulins) were also absent in the serum phase of milk heated at pH 6.4. These results were fully consistent with previous reports (Vasbinder and de Kruif, 2003; Donato and Dalgleish, 2006; Anema, 2007).

To better understand the differences in the composition of the centrifugal supernatants depending on the heating pH and the concentration, the sera were separated using size exclusion chromatography. The chromatography profiles for the heat treated milk and the
same milk concentrated 3x are shown in Figure 4.4. The chromatograms contained 5 main peaks, in full agreement with previous literature (Guyomarc’h et al., 2003a; 2003b). The first peak, eluting at approximately 50 min (Figure 4.4, Peak 1) has been shown to contain very little protein (Parker et al., 2005). A second peak eluting at about 62 min (Figure 4.4, Peak 2), quite evident in milk heated at pH 6.7 and 7.0, has been described before as the peak of elution of heat induced complexes of κ-casein and whey proteins (Guyomarc’h et al., 2003; Parker et al., 2005). This peak, in control milk heated at an initial pH of 6.4 was not present, and a peak of elution started around 62 min (identified in Figure 4.4 as Peak 3). Earlier work on single strength milk identified this peak as a unresolved peak containing whey proteins with a small amount of soluble caseins (Donato and Dalgleish, 2006). The fourth peak eluting around 80 min and a peak at 110 min (Figure 4.4, Peak 4 and 5) have been previously attributed to residual unaggregated milk proteins and non protein components, respectively. The peak 5 did not vary with pH of heating (Guyomarc’h et al., 2003) or concentration.

The chromatography profiles obtained for non concentrated milk agreed with previous reports (Guyomarc’h et al., 2003): There was a significantly larger amount of soluble complexes present in milk heated at pH 7.0 compared to what recovered in milk heated at the natural pH of milk or milk heated at pH 6.4. A different elution was shown for the unsedimentable fraction of milk heated at pH 6.4, consistent with the presence of smaller aggregates. While in single strength milk peaks 3 and 4 were not well resolved, the differences in the composition of the peaks were evident. The differences in elution between supernatants of milk heated at the initial pH of 7 and 6.7 from 6.4 further supported the data shown in Figure 4.3, suggesting different soluble complexes.
Figure 4.4: Chromatographic elution of centrifugal supernatants for milk heated at an initial pH of 6.4, 6.7 and 7.0. Profiles were obtained from control unconcentrated milk (black lines), heated milk concentrated 3x (grey line). Chromatograms are representative of three independent lines.
It was then possible to conclude that in milk heated at an initial pH of 6.4 smaller aggregates were present (eluting later in the chromatography), and these aggregates contained a high ratio of caseins to whey proteins.

In general, as expected, the peak area increased with concentration, but the chromatography profiles at the different pH levels were very similar to their control counterparts. At high concentration (approximately 100 gL\(^{-1}\), 3x concentrated milk) the area of the aggregate peaks were not significantly different for pH 6.7 and pH 7.0 heated milk, but was much lower for milk heated at an initial pH of 6.4 (Figure 4.4).

To confirm the composition of the eluted peaks, the fractions were collected and analyzed by SDS-PAGE. The samples were diluted back to the original volume fraction (i.e. 3x was diluted back to 1x), so that direct comparisons could be carried out between the bands. Hence, different bands intensities signified differences in polypeptide composition of the fractions. Representative electrophoretic patterns for the fractions eluted from control heated milk (dialysed against 0% PEG) and concentrated milk (3x concentrated, dialysed against 8.5% PEG) analyzed under non-reducing and reducing conditions are shown in Figures 4.5 and 4.6, respectively.

Under non reducing conditions, there seemed to be differences in the composition of the residual non aggregated whey proteins, within a concentration, with pH and within a pH, with concentration (see lanes 3, 5 and 7 of Figure 4.5A and B). In this case, for milk at pH 7 for example, there was more residual non aggregated whey protein at 1x concentration than at 3 times concentration (Lanes 3 Figure 4.5A and B). This trend was also observed for milk heated at pH 6.4 and 6.7. \(\beta\)-casein seemed to be present in peak 4...
Figure 4.5: SDS PAGE carried out under non-reducing conditions for fractions eluted from centrifugal supernatants of control unconcentrated (A) and 3x concentrated milk (B). Lanes 1 and 2 milk protein standards (whey protein isolate and sodium caseinate). Lanes 3 and 4, peaks 4 and 2 for milk at pH 7.0; Lanes 5 and 6, peaks 4 and 3 milk at pH 6.4; Lanes 7 and 8, peaks 4 and 2 for milk at pH 6.7. The arrow indicates direction of migration. For details on peak assignment see Figure 4.4.
**Figure 4.6:** SDS PAGE under reducing conditions for fractions eluted from centrifugal supernatants of control heated and not concentrated (A) and 3x concentrated milk (B). Lanes 1 and 2, second and fourth peak for supernatant of milk heated at pH 6.7; Lanes 3 and 4, third and fourth peak for supernatant of milk heated at pH 6.4; Lanes 5 and 6, second and fourth peak for supernatant of milk heated at pH 7.0. Lanes 7 and 8 whey protein isolate and sodium caseinate standards.
of the eluted centrifugal supernatant at all pH levels. In the case of the soluble aggregate fractions (peak 2 of Figure 4.4 for milk at pH 6.7 and 7 and peak 3 for milk at pH 6.4) Figure 4.5 shows that most of the protein in the fractions was disulfide linked. A peculiar behaviour was noted for milk heated at the initial pH of 6.4, depending on concentration. At 1x concentration, the aggregated peak eluting as peak 3 in Figure 4.4 contained a substantial amount of caseins (see lane 6 Figure 4.5A). On the other hand, at a higher concentration this peak showed much less caseins present, suggesting the presence of disulfide bridges also in this fraction (see lane 6 Figure 4.5B). This result would suggest that at higher concentration the composition of the aggregates in milk heated at pH 6.4 drastically changed from being mostly soluble caseins, to disulfide linked whey proteins and casein aggregates. In all cases, there were more high molecular weight peptides present in the supernatant of control unconcentrated milk compared to concentrated milk (compare Figure 4.5 A and B). This may indicate the presence of oligomers of whey proteins in the residual unaggregated fraction (peak 4) in single strength milk, but not for milk concentrated 3x.

Figure 4.6 shows the composition of the eluted peaks after SDS PAGE under reducing conditions. Once again, the differences in the composition of the aggregates as a function of the initial pH of the milk were quite evident. In the eluted fractions from supernatant of milk heated at pH 6.7 and 7.0, the large soluble aggregate peak (peak 2) was resolved into 3 major bands corresponding to κ-casein, β-lg and α-la, in full agreement with the literature (Guyomarc’h et al., 2003; Parker et al., 2005). It is clear that in the case of milk concentrated 3x, a different proportion of high molecular weight proteins (mainly bovine serum albumin, immunoglobulins and lactoferrin) was present in the eluted peaks.
However, in general it was concluded the composition of aggregates did not change with concentration, but there were differences in the population of disulfide bridged aggregates (when comparing Figure 4.6 to Figure 4.5). In the case of peak 4, together with the residual whey protein, in all cases, some β-casein was recovered, as already shown in Figure 4.5. The composition of the fractions eluted from the serum of milk heated at pH 6.4 was profoundly different. The large soluble complexes peak (eluting later, at 80 min and identified as peak 3 in Figure 4.4) contained caseins with little whey proteins present. By comparing these results with those of Figure 4.5, it could be concluded that this peak contained β-casein as well as disulfide linked κ- and αs2- caseins with small amounts of whey proteins. This results was consistent with the understanding that most of the whey protein complexes are associated with the colloidal fraction of the casein micelles. The fourth peak, of residual unaggregated protein, contained β-casein and α-la, with very little β-lg. In this case, from Figure 4.6 A and B it was clear that there was notably more protein in the supernatant of concentrated milk compared to the control heated samples, even after redilution. The results obtained in this work suggested for the first time, at high concentration that the composition of the non sedimentable fraction may change with concentration and pH of heating, and more research should be carried out to further elucidate this variation, to be able to better exploit the functional properties of heated concentrated milk.

4.4.3.2. Changes in the calcium during concentration

The dynamic equilibrium between the insoluble and colloidal form of calcium and the soluble form (Lucey and Horne, 2009) is critical to the functional properties of milk. It
has been previously reported that a considerable proportion of soluble calcium and phosphate may be transferred into the colloidal state during concentration by evaporation (Hardy et al., 1984; Nieuwenhuijse et al., 1988, Liu et al., 2012). Previous work using osmotic stressing as method of concentration reported values of total calcium and phosphate for unheated milk of 30.8±1.3 and 28.1±0.2 mM, respectively for non concentrated milk. These values increased to 67.4±1.3 and 64.5±0.5 mM, total calcium and phosphate, respectively, when the protein content of the sample was 100.2±0.2 g L\(^{-1}\).

It has been reported that changes in pH will affect the amount of calcium associated with the micelles (Snoeren et al., 1984), but no information is available on the changes occurring to total and soluble calcium and phosphate for milk heated at different pH values and concentrated by osmotic stressing. It may be hypothesized that with concentration, a different release of calcium and phosphate may be occurring in the serum phase. Figure 4.7 illustrates the amount of total calcium and phosphate present in milk heated and concentrated up to 3x the original protein concentration. The total calcium and phosphate increased with concentration, similarly to what observed for unheated milk (Figure 4.7). In the case of calcium, there seemed to be a lower value of total calcium after concentrating 3x heated milk with an initial pH of 6.4 (Figure 4.7A). This may suggest a release of calcium in the serum phase (in this case in the osmotic stressing solution) at high volume fractions.

Figure 4.8 summarizes the values of soluble calcium and phosphate in samples after concentration and after heating milk at different pH. There were no apparent differences
Figure 4.7: Total calcium (A) and phosphate (B) ions present in heated milk at pH 6.7 (●), 6.4 (▲) and 7.0 (■) compared with that of unheated milk (○) as a function of protein concentration. Values are the mean and standard error of three independent trials.
Figure 4.8: The amount of calcium (A) and phosphate (B) present in the centrifugal supernatants of heated milk at pH 6.7 (●), 6.4 (▲) and 7.0 (■) as a function of protein concentration. For comparison the values of raw milk (○) are also given. The mean and standard error of three independent trials are shown.
in the amount of soluble calcium and phosphate present in the heated samples, within the experimental error. However in the case of pH 6.4, there seemed to be a significantly higher amount of phosphate in the serum phase of milk concentrated 3x (Figure 4.8B). This result could be related to the higher amount of caseins present in the serum phase (see Figure 4.3).

4.4.4. Effect of concentration on voluminosity as derived from rheological measurements.

It is understood that the milk viscosity increases with concentration because of the increased interactions between the casein micelles. This behaviour can be well modelled after the behaviour of a colloidal hard sphere system (Karlsson et al., 2005; Bouchoux et al., 2009b; Dahbi et al., 2010). With increasing interactions between the casein micelles, a shear thinning behaviour is observed in concentrated milk (Karlsson et al., 2005; Dahbi et al., 2010), and this indicates a transition between a regime where the particles are free diffusing at low-shear rates to a regime where their motions are determined by hydrodynamics and interparticle interactions, presumably due to compression and interpenetration of the hairy layers.

As previously demonstrated (Chapter 3) the viscosity of unheated concentrated milk can be modelled theoretically to that of hard spheres (Equation 4.1). When considering the changes occurring to the viscosity of the serum phase (derived experimentally), a close packing volume fraction of 0.8 is derived for casein micelles in concentrated milk (Chapter 3). Figure 4.9 summarizes the changes occurring to the relative viscosity of milk heated at different pH levels as a function of concentration. The values are compared
with those of raw milk measured at a shear rate of 300 s\(^{-1}\).

It is important to point out that, as for unheated milk, also in the case of heated milk it was assumed a similar value of voluminosity for all concentrated milk. For each volume fraction the relative viscosity was determined using the value of experimentally determined serum viscosity. Although there seemed to be a slight deviation at the low volume fractions, overall the Mendoza model for raw milk, with a \(\varphi_{\text{max}}\) of 0.8 and exponent 2.5, fit reasonably well with the experimental data also in the case of heated milk. The theoretical hard sphere model (Mendoza and Santamaria-Holek, 2009) for raw milk in the same range of concentration was used as a comparison (see Chapter 3 for full curve and detail). A correlation coefficient of 0.97 was obtained while examining the relative viscosity values of raw milk within the volume fractions used in this study, while such value was much higher (0.99) for a larger range of concentration (see Chapter 3). Similarly for heated milk, the regressions with the theoretical models resulted in regression values of 0.97, 0.92 and 0.98 for pH 6.4, 6.7 and 7.0 respectively. In this case also, experimental data of serum viscosity were employed in the calculations of the relative viscosity. These values of viscosity were statistically equivalent to those for untreated milk. The slight deviation from the hard sphere behaviour can be easily explained by small changes in the voluminosity of the casein micelles, or, although more unlikely, changes in particles’ shape.

Indeed, the Mendoza model can be used for predicting the viscosity of heated milk by changing the exponent values, and best fits can be obtained when an exponent of 3.7 was used. This exponent correlates the shape of particles; while higher values are used for polydisperse and ellipsoid shaped particle systems.
Figure 4.9: Viscosity measured at 300 s\(^{-1}\) for unheated milk (o) and heated milk with an initial pH of 6.7 (●), 6.4 (▲) and pH 7.0 (■), as a function of protein concentration. The solid line corresponds to the theoretical predictions for raw milk using Mendoza equation with a maximum packing volume fraction of 0.8 and an exponent of 2.5 (\(r^2=0.999\)) (see Chapter 3 for a full curve with higher volume fractions). Values are the average of three independent experiments of concentration. Bars represent standard error.
However, microscopy images of heated casein micelles have previously shown that their overall sphericity is maintained during heating (McMahon and McManus, 1998).

The casein micelles in heated milk show slightly larger average apparent diameter than unheated milk, due to association of denatured whey protein complexes to the micellar surface. This may reflect in slightly changes in their voluminosity, with differences depending on the initial pH of heating. Indeed, a change in voluminosity with heating has been proposed before (Walstra, 1979; Hallstorm and Dejmek, 1988), but with limitations due to the type of analysis performed. Sedimentation methods may not be suitable for measuring voluminosity as the centrifugation may compress the hairy layer of the casein micelles and underestimate the voluminosity (Hallstorm and Dejmek, 1988).

Since Mendoza model was successful in predicting the rheology of both heated and unheated system (Figure 4.9) it could be argued that casein micelles would behave rheologically as hard spheres with similar packing voluminosities, in spite of the presence of associated whey protein aggregates in the already rugged surface of the casein micelles. The presence of heat induced aggregates in solution would further contribute to the polydispersity of the system.

4.4.5. Light Scattering Properties

During concentration, the number of colloidal particles per unit volume increases with concomitant increase in the scattering events per unit volume which result in increased turbidity. Figure 4.10 illustrates the changes in the turbidity parameter measured by transmission DWS as a function of protein concentration for various concentrated milk samples.
Figure 4.10: Changes in $1/l^*$ as a function of protein concentration. The open symbols represent unheated milk. The filled symbols represent the heated milk at an initial pH of 6.4(▲), 6.7(●) and 7.0 (■). Results are the average of three independent measurements; bars represent standard error.
In general, the turbidity increased at the low protein concentration. The concentration effect will, however, at a certain point be strongly influenced by interparticle interactions which will cause strong positional correlations between the particles and decrease the value of l* after reaching a maximum value. It has been previously demonstrated that the value of l* of casein micelles is well predicted by hard sphere theory up to a concentration of about 3x (Chapter 3; Sandra et al. 2011).

Turbidity values for heated milk at pH 6.7 and 7.0 were very similar to those for unheated milk (Figure 4.10). There seemed to be no difference in the light scattering behaviour of these particles with concentration, but in the case of heated milk at the initial pH of 6.4, where the values of turbidity seemed to reach a maximum at an earlier protein concentration. A similar result has been previously reported (Jeurnink, 1992), and it was probably caused by the change in the hydrodynamic diameter of the casein micelles (Table 4.1).

The changes induced by heating to the casein micelles and the presence of whey protein aggregates may affect the dynamic mobility of the casein micelles during concentration. To better characterise the system as a function of concentration and pH of heat treatment, values of mean square displacement (MSD) were obtained from the characteristic decay time of the intensity auto-correlation function. For a free diffusing Brownian particle a linear relation exists between MSD and time. The onset of arrested motion is characterised by the deviation from this linear relationship. Figure 4.11 describes the variation of the slope of MSD values versus time, for concentrated milk at different protein concentration. In general the particle dynamics of heated concentrated milk were very similar to raw concentrated milk.
Figure 4.11: Slope of log MSD-log time as a function of concentration. Unheated milk (○) or milk heated at an initial pH of 6.7 (●), 6.4 (▲) and 7.0 (■). Values are the average of three separate concentration experiments (hence the variability also on the x axis).
Up to a protein concentration of 70 g L$^{-1}$ (corresponding to 2.2x concentrated milk) the MSD slope was close to 1, regardless of heating treatment, and with no differences with the heating pH, indicating that no matter if the whey protein aggregates were associated with the micelles or in the soluble phase, the casein micelles were free diffusing. Thereafter, a sub-diffusive motion was observed, indicating that particle motion was restricted due to the inter particle interactions between the colloidal micelles or between the micelles and newly formed aggregates. In all cases, unheated milk or heat treated, the casein micelles were now close enough to one another to affect their average diffusion behaviour. This change in the short term diffusion with concentration indicates that the casein micelles started to be caged in by the near neighbour particles at much lower concentrations than those reported as critical packing concentration using rheology. These results are shown for the first time in the literature. In the case of heating at pH 6.4, adherence of denatured WP to the casein micelle surface provide an increase in the hydrodynamic diameter which may further restrict the movement of the casein micelles.

4.5. Conclusions

Heating of milk causes changes to the colloidal properties of milk. Depending on the initial pH of the milk, a different distribution of colloidal particles is present in the dispersion. At pH 6.4, heat induced whey protein aggregates are associated with the casein micelle, and with concentration, there is an increase of non sedimentable caseins. On the other hand, at pH 7.0 most of the whey protein complexes are present in the serum phase, and the casein micelles show smaller voluminosity than in the case of milk heated at pH 6.4 and 6.7.
Osmotic stressing has been employed to study the effect of heat treatment at different pH levels as a function of concentration. The total and soluble calcium and phosphate increased with concentration very similarly as for unheated milk. This concentration technique allowed for a careful characterization of the colloidal properties of heat treated casein micelles. It was possible to show that the rheological and that light scattering properties do not seem to show differences when comparing heated milk with unheated milk.

Turbidity values of pH 6.4 were higher than that of normal pH and pH 7.0 and these results were attributed to the association of denatured whey proteins with the casein micelles. Further the particle dynamics showed arrested motion above 70 mg L\(^{-1}\) protein concentration and was comparable to the results reported for unheated milk. Further redilution experiments indicated that there is no permanent association between micelles in heated samples i.e. the particle size and volume fraction did not increase due to permanent micellar aggregation, supporting previous reports (Jeurnink and de Kruif, 1993).

This research brings new insights on the interactions occurring in milk proteins during concentration, and on the effects of processing history on structure-function of casein micelles.
CHAPTER 5

RENNET INDUCED GELATION OF CONCENTRATED MILK IN THE PRESENCE OF SODIUM CASEINATE: DIFFERENCES BETWEEN MILK CONCENTRATION USING ULTRAFILTRATION AND OSMOTIC STRESSING.
5.1. ABSTRACT

Concentration of milk is a common unit operation in the dairy industry. With reduction of water the particles interact more frequently with each other, and the functionality of the casein micelles may depend on the interactions occurring during concentration. The objective of this research was to investigate the effect of concentration on the renneting properties of the casein micelles, by comparing two concentration methods, ultrafiltration and osmotic stressing. To evaluate possible differences in the rearrangements of the casein micelles during concentration, renneting properties were evaluated with or without the addition of soluble caseins, added either before or after concentration. The results demonstrated that the two concentration methods were not equivalent, and the differences are attributable to the shear and mixing occurring during membrane filtration.

5.2. INTRODUCTION

Evaporation and membrane filtration are the most common methods of concentration in dairy technology and their effects on the colloidal properties of the casein micelles have been widely studied (Ferrer et al., 2012; Karlsson et al., 2007, Dahbi et al., 2010). Membrane separation processes have been used as unit operations to standardize milk and change its composition for further processing. The physico-chemical properties of the milk and its serum can be carefully controlled by choosing appropriately the membrane selectivity. However, the integrity of the micelles after ultrafiltration, whereby only lactose and soluble components are transmitted and the protein is retained in the retentate, is still under debate (Srilaorkul et al., 1991; Singh, 2007; Ferrer et al., 2012; Alexander et al., 2011).
The changes in the processing functionality of concentrated milk are caused by a number of factors, amongst the most important, the ionic equilibrium, especially the changes in soluble and colloidal calcium (Srilaorkul et al., 1991; Alexander et al., 2011). The increased interactions between the casein micelles due to their increased volume fraction may also play a major role (Bienvenue et al., 2003). The functional properties of concentrated milk obtained by membrane processing have been widely studied, in particular for cheese making applications (St-Gelais et al., 1998; Waungana et al., 1998; Henning et al., 2004). In addition, it has been shown that casein micelles change their size distribution during ultrafiltration and diafiltration (Srilaorkul et al., 1991; McKenna, 2000; Singh, 2007). However, little proof is available that rearrangements occur to the structure of casein micelles during ultrafiltration, as composition does not seem to be affected, although some reports are available on the changes in calcium phosphate equilibrium as well as size distribution (Singh, 2007; Ferrer et al., 2012). However, in most studies, ultrafiltration is combined with diafiltration, which drastically changes the serum composition of the concentrated milk.

Rennet induced gelation is an important functionality of the casein micelles and it can be employed to probe changes to their surface. For example, it has been shown that the interactions between small molecular weight emulsifiers such as polysorbates and casein micelles affect their secondary stage of rennet induced gelation (Ion Titapiccolo et al., 2010). In addition, small changes in the calcium concentration can adversely affect the aggregation (Choi et al., 2007).

During renneting, the enzyme chymosin specifically cleaves the protective layer of κ-casein from the surface of the casein micelles; this reaction results in the loss of steric
stabilisation leaving hydrophobic and calcium sensitive patches on the surface of the particles (Dalgleish, 1992; de Kruif, 1992). When more than 90% of the CMP has been removed, the secondary phase of rennet aggregation occurs (Dalgleish, 1992, Sandra et al., 2007) moderated by free Ca$^{2+}$ (Lucey, 2009). The modification of the surface of the micelle is particularly critical to the secondary phase of rennet aggregation. In general, it is understood that sodium caseinate or whey protein complexes impair the secondary stage of the casein aggregation (Gaygadzhiev et al., 2012, Kethireddipalli et al., 2011).

It has been extensively demonstrated that during concentration the casein micelles behave as hard spheres (Dahbi et al., 2010, Bouchoux et al., 2009b, Chapters 3 and 4). In the present work we hypothesized that membrane filtration causes rearrangements to the supramolecular structure of the casein micelles. To test this hypothesis, milk was concentrated using ultrafiltration or osmotic stressing, and the renneting properties were then tested. Osmotic stress is an alternative process for concentrating milk which is considered to be non-invasive. This technique has already been successfully employed to characterize concentrated milk systems (Bouchoux et al., 2009a; 2009b; Farrer and Lips, 1999). By dialysing milk against milk permeate containing a stressing polymer, it is possible to maintain the original serum environment for the casein micelles and to obtain concentrated milk with a composition equivalent to that of ultrafiltered milk. In short, this allows the investigation of the fundamental aspects of concentrated systems as a function of volume fraction of the casein micelles, without applying shear (as in membrane filtration) or affecting the serum composition. A detailed study of the dependence of the osmotic pressure as a function of concentration for unheated milk can be found in Chapter 3.
To further test possible rearrangements, the renneting properties of equivalent milk concentrates were measured in milk containing additional soluble caseins. The presence of sodium caseinate causes inhibition of the aggregation of the casein micelles (Gaygadzhiev et al., 2011). Similar amounts of sodium caseinate were added before and after concentration by either ultrafiltration or osmotic stressing. Changes in the rennet gelation may suggest rearrangements occurring to the micelles during concentration. A combination of chromatographic, rheological, microscopic and light scattering techniques was used to describe the changes.

5.3. MATERIALS AND METHODS

5.3.1. Skim milk and Permeate Preparation

Skim milk and permeate were prepared as described in detail in 3.3.1.

5.3.2. Concentrated Milk Preparation

Milk was concentrated using osmotic stressing as described in 3.3.2. All experiments were carried out by dispersing poly ethylene glycol (PEG) (Fluka, Oakville Ontario) at the rate of 80g L\(^{-1}\) in permeate containing 0.2g L\(^{-1}\) sodium azide a bacteriostatic agent. The use of permeate will ensure that the chemical potential of all ions is similar across the membrane. A standard regenerated cellulose dialysis membrane (Spectra/Por 1, Fisher Scientific, Whitby, ON, Canada) with a molecular mass cut-off of 6–8 kDa was employed in this study. This pore size ensured the exchange of water, ions, and lactose but not caseins or PEG. The PEG did not interact with calcium ions. The osmotic pressure experiment was conducted at 4°C for 18 h to minimize sample degradation.
Control concentrated milk was prepared by skim milk placed in the reservoir containing a stressing solution composed of 80 g L\(^{-1}\) PEG and milk permeate. At the end of osmotic stressing using PEG, a volume fraction of 0.3±0.03 (corresponding to approximately 3x concentration) was obtained.

Milk was also concentrated by ultrafiltration (UF), 3x the original volume, based on volume reduction. Ultrafiltration was carried out using a laboratory scale membrane unit (PLGC10k regenerated cellulose cartridge, Millipore Corp., Bedford, MA).

Concentrated milk was also prepared with additional soluble casein. In both osmotic stressing and ultrafiltration, sodium caseinate (NaCas) (2 g L\(^{-1}\)) was added to skim milk and stirred for 2 h at room temperature. This concentration ratio has been shown to inhibit the formation of rennet gel (Gaygadzhiev et al., 2012). This sample was then subjected to osmotic concentration or ultrafiltration as mentioned before. NaCas was also added after concentration to the control concentrated milk samples, using a similar ratio (6 g L\(^{-1}\)) and stirred for 2 h at room temperature. It is important to note that both control concentrated milk samples had lower amounts of protein compared to the samples with added NaCas (added before and added after).

### 5.3.3. Separation of centrifugal supernatant from concentrated samples

Milk samples were centrifuged at 100,000g for 1 h at 20\(^\circ\)C in a Beckman Coulter Optima\(^{\text{TM}}\) LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, Canada) to separate the casein micelles from the serum fraction, as described in detail in 3.3.3. The supernatant was carefully recovered using a pasteur pipette, and then filtered twice using 0.45 µm and 0.22 µm filters.
5.3.4. Ion exchange chromatography

The soluble fraction was analyzed for caseins using ion exchange chromatography equipped with an AKTA purifier (900 series, GE Biosciences, Baie d’Urfe, Quebec, Canada) and a UV-900 detector (set at 280 nm) was used for the analysis using a previously published method (Holland et al., 2010). In brief, a sample buffer consisting of 0.2 M sodium acetate and 6 M urea at pH 3.5 was prepared, and 5 mL of aliquots of supernatant were added to 5 mL of sample buffer (0.2 M sodium acetate and 6 M urea buffer at pH 3.5), and 3.599 g of urea and 0.013 g of sodium acetate were then added. The samples were stirred for 1 h, the pH was adjusted to 7.0, and β-mercaptoethanol (0.150 μL) was added. The samples were again stirred for 1 h and the pH was adjusted to 3.5 with 1 M HCl and stirred for 5 min. The total volume was then fixed to 15 mL (if needed) by the addition of MilliQ water (Millipore, Bedford, MA) and stirred for 5 min. The samples were then filtered through a 0.45-μm syringe filter (Millex GV, Millipore) before injection and 500 μL were injected in a 1-mL cation exchange HP SP column (GE Biosciences) eluting at 1 mL/min with 0.2 M sodium acetate, 6 M urea buffer at pH 3.5 using a gradient of NaCl as previously reported (Holland et al., 2010).

5.3.5. Rennet gelation

The rennet used was Hansen’s Chymax ®Ultra (750 International Milk Clotting Units (IMCU) mL⁻¹, diluted 1:10 in Milli-Q water, and then diluted once more 1:10 before use. These diluted rennet solutions were then added to the milk samples at the rate of 0.03 IMCU mL⁻¹ milk.

The extent of caseinomacropeptide released during the rennet proteolysis was measured
using RP-HPLC, using established methods (Lopez-Fandino et al., 1993) with minor modifications. The milk was incubated at 30°C. At appropriate times, 4 mL of 3% percholoric acid was mixed to stop the reaction, and then the samples were stored in the refrigerator overnight. After equilibration to room temperature, the supernatants were collected after centrifugation at 4500g for 15 min using an Eppendorf 5415D centrifuge (Brinkmann Instruments, Ltd., Mississauga, Canada) and were filtered through 0.45 μm Millex-GV filter units (Millipore, Bedford, MA, USA). The HPLC analysis was carried out as described elsewhere (Ion Titapiccolo et al., 2010; Gaygadzhiev et al., 2012). The area under the peaks detected at 210 nm was calculated and the total amount of CMP was expressed relative to the maximum amount (defined as the value at plateau) of CMP released from the same concentrated milk sample. Total peak areas were integrated using the ChromQuest software from approximately 6 to 40 min on the chromatograms.

The preceding stages of gelation were followed using transmission DWS. This technique allows the investigation of the static and dynamic behaviour of colloidal particles in concentrated suspensions by measuring scattering intensity originated essentially only from multiple scattering events that involve many colloidal particles (Weitz and Pine, 1993). The measurements were carried out as described in 3.3.4.

The sample with rennet (~1.5 mL) was poured into an optical glass cuvette (Hellma Canada Ltd., Concord, Canada) specified with a 5 mm path length. The cuvette was placed in a water bath at a temperature of 30°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).
The development of the gel was also measured using a controlled stress rheometer, Paar Physica MC 301 (Anton Paar, Graz, Austria), using a cone and plate geometry, with a set gap of 0.51 mm. The temperature of the plate was controlled with water circulating from a Julabo F25-HP refrigerated and heated water bath (Julabo Labortechnik, GmbH, Germany). All measurements were made at 30ºC. The equipment was run at controlled strain mode, 1% strain, 1 Hz frequency. Mineral oil was used to prevent the evaporation of the samples. The gelation point was defined as the time at which the first noticeable inflection in the value of storage modulus occurs.

The measurements of DWS and rheology were made simultaneously on two fractions of the same renneted milk. Samples of concentrated milk were equilibrated at 30ºC for at least 15 min prior to rennet addition (at a ratio of 0.03 IMCU per ml milk and stirred for 30 s). The samples were then simultaneously loaded into the rheometer and transmission DWS equipment. The DWS and rheology measurements were started simultaneously at approximately 3 min after rennet addition.

5.3.6. Separation of centrifugal supernatant from renneted samples

Immediately after rennet addition, the samples were transferred in Beckman Coulter centrifugal tubes (Beckman Coulter Canada Inc., Mississauga, Canada) and incubated at 30 ºC for 150 min in a water bath. At the end of incubation period, the samples were centrifuged at 100,000g for 1 h at 30ºC in a Beckman Coulter Optima™ LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, Canada) to separate the casein micelles from the serum fraction, as described in detail in 3.3.3. The supernatant was carefully recovered using a pasteur pipette, and then filtered.
twice using 0.45 μm and 0.22 μm filters.

5.3.7. Calcium measurements

The supernatant thus separated under 5.3.3 was analysed for soluble calcium. Determination of soluble calcium in all samples was carried out using non-suppressed ion chromatography (Rahimi Yazdi et al., 2010) and as described in 3.3.6. The amount of soluble calcium in the concentrated milk was defined as the total calcium in the serum phase after centrifugation at 100,000 g (see 3.3.6).

5.3.8. Particle Size Determination

The particle size of the casein micelles was measured by dynamic light scattering (Zetasizer Nano-ZS). After concentration, the milk samples were diluted ~2000 times in filtered (0.2 μm nylon filters (Fisher scientific) milk permeate and analyzed.

5.3.9. Statistical Analysis

All experiments were carried out in triplicate (i.e. three separate milk batches), and the average and standard errors are reported. Statistical significance was evaluated using ANOVA at p<0.05.
5.4. RESULTS AND DISCUSSION

5.4.1. Characterisation of concentrated milk

5.4.1.1. Composition of the serum phase

Milk was concentrated to a comparable volume fraction (0.30±0.03) using osmotic stressing or ultrafiltration, the latter without the use of diafiltration (addition of water during filtration). In the samples with added sodium caseinate (either before or after concentration), it was important to determine if this addition resulted in an increase in the amount of non sedimentable caseins. It is important to note that it has been previously shown that under these conditions NaCas does not precipitate during ultracentrifugation (HadjSadok et al., 2008).

The centrifugal supernatants were separated by ultracentrifugation and the casein composition was analyzed using ion exchange chromatography (Figure 5.1). This method allows elution of the different caseins in separate peaks. The elution chromatograms of the soluble phase of milk concentrated by osmotic stressing and ultrafiltration showed 5 main peaks, in accordance to previous reports (Holland et al., 2010). There was a significantly higher amount of soluble caseins in milk concentrated using osmotic stressing compared to that concentrated by ultrafiltration. This was particularly noted in the peaks eluting at 8.4 mL and after 17 mL, corresponding to β-casein and to the αs-caseins, respectively. In addition, in the case of milk containing NaCas, these peaks were higher, as more non sedimentable casein was recovered after centrifugation. Note that all peaks were higher than those of a 0.6% sodium caseinate control.
Figure 5.1: Ion exchange chromatography elution peak of centrifugal supernatants of milk concentrated by osmotic stressing (A) and ultrafiltration (B). The profile of 0.6% NaCas solution is also shown (thin dash line). Control milk concentrated 3x, and milk with added NaCas, before or after concentration. Data are representative of three separate experiments.
In both concentrated milk, the peaks were statistically larger in samples where the NaCas was added after concentration, indicating a greater amount of non sedimentable caseins in these samples, suggesting that when NaCas was added before concentration a significant amount was transferred to the colloidal phase. The peak areas for the three treatments (control, added after and added before) were significantly different from each other (P<0.001) in both ultrafiltration and osmotic stressed samples.

The differences in the amount of non sedimentable caseins between Figure 5.1A and 5.1B may reflect the differences in the process of concentration. While osmotic stressing is considered a mild treatment of concentration, the continuous shear and transmembrane pressure applied to milk during ultrafiltration may lead to more rearrangements, or may form a larger amount of small micelles, creating additional surfaces that will need to be stabilized. It is also important to note that the results in Figure 5.1 suggested that in both means of concentration, addition of soluble casein before concentration increased the recovery of casein in the colloidal phase, as the recovery in the centrifugal supernatant was less than in the case of the casein added after concentration.

The levels of soluble calcium present in the supernatants of different types of concentrated milk are given in Table 5.1. There were no significant differences in soluble calcium levels for 3x concentrated control milk, irrespective of the method of concentration. The level of soluble calcium (10 mM) were similar to that of skim milk reported by the literature (Lucey and Horne, 2009), confirming that both ultrafiltration and osmotic stressing maintained the ionic environment as close as possible to that of native casein micelles. On the other hand, milk concentrated by osmotic stressing with added NaCas showed significantly higher levels of serum calcium (P<0.01) in
Table 5.1: Amount of soluble calcium recovered in the centrifugal supernatant of 3x concentrated milk with or without sodium caseinate added. Milk was concentrated by osmotic stressing or ultrafiltration. Values are the average of three independent experiments, with standard error. Letters indicate statistical differences (p<0.05) between treatments.

<table>
<thead>
<tr>
<th>Concentrated milk</th>
<th>Osmotic Stress</th>
<th>Ultrafiltration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Calcium (mM)</td>
<td>Calcium (mM)</td>
</tr>
<tr>
<td>control</td>
<td>10.2±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.35±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCas added before</td>
<td>12.5±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.5±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCas added after</td>
<td>12.5±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.35±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
comparison with ultrafiltered milk, regardless of whether the caseins were added before or after heating. This discrepancy can only in part attributed to the higher amount of soluble casein, as shown in Figure 5.1. Being highly phosphorylated, soluble caseins will bind calcium. It is important to point out that of the soluble calcium present in milk, free ionic calcium, \( \text{Ca}^{2+} \), constitutes only a small portion (2 mM) of the total calcium in skim milk (Neville, 2005). The ionic calcium plays a major role in the rennet reaction, modulating both the enzymatic activity as well as the secondary stage of rennet coagulation, as the destabilised micelles aggregate only in the presence of free \( \text{Ca}^{2+} \) (Lucey, 2009).

### 5.4.1.2. Casein micelles dynamics

Using light scattering the properties of the casein micelles in the two concentrated systems was characterized. The values of mean square displacement as a function of time for the 3x concentrated milk, as well as the milk samples with added sodium caseinate are shown in Figure 5.2.

A linear relationship exists between the particles’ mean square displacement (MSD) and time, when the particles are free diffusing (Krall and Weitz, 1998). In Figure 5.2 the MSD values as a function of time are shown for concentrated milk with and without NaCas added, and straight lines are drawn to illustrate deviations from linearity. Both concentrates were similar in protein composition and ionic strength. In the case of ultrafiltered milk, in all cases, casein micelles showed free diffusive Brownian motion. On the other hand, milk concentrated by osmotic stressing as well as the same milk with sodium caseinate added before concentration, showed a deviation from this linear
**Figure 5.2**: The mean square displacement values for milk concentrated using osmotic stressing (OS) or ultrafiltration (UF). Samples containing sodium caseinate added before or after concentration are also shown. Straight lines are drawn to guide the eye.
relationship. These results are in full agreement with the data shown in Chapter 3, whereby it was shown that at volume fractions above 0.32 the particle dynamics changed to arrested sub-diffusive motion when milk was concentrated by osmotic stressing. The hydrodynamic and steric interactions occurring between the micelles are disrupted by shear and mixing in the process of ultrafiltration.

As shown in Figure 5.1, in ultrafiltered milk the added soluble casein seemed to be more associated to the colloidal phase than in osmotically concentrated milk. The presence of a larger amount of non sedimentable protein in the osmotically concentrated milk might have caused the additional colloidal interactions. Recently, it has been shown that soluble casein can aggregate to form non sedimentable small particles of 12 nm diameter made up of 15 casein molecules in the presence of calcium (HadjSadok et al., 2008; Pitkowski et al., 2009). These non sedimentable particles may further contribute to the interactions occurring in osmotic stressed milk. It may be important to point out that when sodium caseinate was added after concentration, the gentle mixing may have disrupted the structure and affected the mobility of the casein micelles.

To better characterise the colloidal properties of the casein micelles in the concentrated samples, the diffusion coefficient and transmission parameter of the casein micelles were derived from DWS measurements, which are carried out without dilution. These results are summarized in Table 5.2, together with the apparent diameter derived from DLS after extensive dilution in milk permeate. Confirming what shown in Figure 5.2, statistical analysis of the values of diffusion coefficient demonstrated a significantly lower diffusion coefficient for casein micelles in milk concentrated by osmotic stressing compared to ultrafiltration. The larger hydrodynamic drag in osmotic stressed milk may
Table 5.2 Values of diffusion coefficient, transmission parameter and size of casein micelles for milk concentrated by osmotic stressing and ultrafiltration, with or without sodium caseinate added (before or after concentration). Values are the average of three independent experiments, with standard error. Letters indicate statistical differences (p<0.05) between treatments.

<table>
<thead>
<tr>
<th></th>
<th>Diffusion Coefficient</th>
<th>I/I*</th>
<th>Diameter</th>
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<tbody>
<tr>
<td></td>
<td>10^{-12} (m^2 s^{-1})</td>
<td>(mm^{-1})</td>
<td>(nm)</td>
</tr>
<tr>
<td>Osmotic stress</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.71±0.03^a</td>
<td>1.2±0.03^b</td>
<td>2.9±0.1^a</td>
</tr>
<tr>
<td>Added before</td>
<td>0.65±0.03^a</td>
<td>1.2±0.03^b</td>
<td>2.7±0.0^a</td>
</tr>
<tr>
<td>Added after</td>
<td>0.65±0.07^a</td>
<td>1.0±0.03^b</td>
<td>2.4±0.1^b</td>
</tr>
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</table>
be caused by the high amount of soluble casein in the serum phase, or by the quiescent nature of the process of concentration using osmotic stressing. Within a treatment, there was no difference with and without NaCas added. When rediluted in milk serum (permeate), the size of the micelles was statistically equivalent, regardless of the amount of NaCas, the mode of addition and method of concentration, indicating that no aggregation occurred.

The osmotically concentrated milk had a higher turbidity parameter (1/l*) than the ultrafiltered milk. The l*, or photon transport mean free path, represents the length scale over which the direction of the light passing through a sample has been fully randomized. For free diffusing scatterers, completely uncorrelated spatially, the l* value depends on particle size, shape, particle concentration and refractive index contrast between the scatterers and the serum phase (Alexander and Dalgleish, 2007). This change in the 1/l* was most probably caused by a change in the interparticle positioning, as size and volume fraction were equivalent, and a change in refractive index contrast due to soluble caseins in the serum phase would cause a decrease in the turbidity parameter 1/l*. This is noted in the case of osmotic stressed milk, where the addition of sodium caseinate caused a decrease in the 1/l* value. This was not the case for ultrafiltered milk, once again pointing out the difference in the interactions dynamics of the caseins in the differently processed milk samples.

5.4.2. Rennet gelation

Rennet-induced gelation of the casein micelles in concentrated milk was followed using DWS and rheology. Diffusing wave spectroscopy parameters better describe the changes
occurring in the preceding stages of the sol-gel transitions, while rheological measurements are better suited to follow the development of the gel structure.

Figure 5.3 describes the development of the turbidity parameter, $1/l^*$ and apparent radius as a function of time from rennet addition for milk concentrated by osmotic stressing and ultrafiltration, with and without addition of sodium caseinate. The point at which there was a rapid increase in radius indicated the aggregation time of the casein micelles. Figure 5.3A clearly shows that in the case of osmotic stressing the addition of soluble caseins to milk after concentration significantly hindered the aggregation of the casein micelles. This was not the case for milk concentrated by ultrafiltration. Ultrafiltered concentrated milk samples with soluble caseins added after gelled similar to that of control. The addition of soluble casein before concentration did not show any significant differences in the early stages of aggregation compared to concentrated milk controls, regardless of the mode of concentration.

The early changes in $1/l^*$ during renneting (at about 40 min) indicated changes in spatial correlation between the micelles before full aggregation commences, as discussed in earlier literature (Sandra et al., 2007, Alexander and Dalgleish, 2004). After the increase around 40 min, the $1/l^*$ value reached a plateau at the point of gelation. The steep increase around 40 min indicated an increase in the interactions between the casein micelles. This increase corresponds to about 80% of CMP release, regardless of the casein micelles volume fraction (Sandra et al., 2011). In agreement with the radius data, the milk sample with added sodium caseinate after concentration by osmotic stressing showed a different behaviour for the $1/l^*$ parameter, without reaching a plateau within experimental time (Figure 5.3A).
**Figure 5.3:** Development of turbidity parameter (1/l*), filled symbols, and the apparent radius, open symbols, during renneting of milk concentrated using osmotic stressing (A) or ultrafiltration (B). The error bars represent the standard error of turbidity parameter recorded during the experiment.
To better identify the differences in structure formation between the differently treated samples, rheology was also employed in this study. The average gelation time and final G’ after 120 min of gelation UF and osmotically stressed samples are summarised in Table 5.3. Figure 5.4 shows the development of the elastic modulus as well as tan δ (where δ is the phase angle). In 3x concentrated milk there was a significant difference in the time of gelation, as defined as the beginning of the steep increase in the elastic modulus, G’. The time of gelation for control milk concentrated by ultrafiltration was 70.1±3 min, significantly higher than in the case of osmotically concentrated milk. Interestingly, this value was also in agreement with the aggregation time measured by DWS (Figure 5.3).

This discrepancy in the time of gelation between the two controls 3x milk samples may reflect the difference in the mobility of the casein micelles. Additionally, the UF samples resulted in stiffer gels 2 h after the gelation point. The osmotically concentrated control gelled at a later time, and the value of G’ after 2 h from the gel point was 505±56 Pa. The tan δ behaviour for these samples was typical of rennet induced gels, with a steep decrease of the tan δ, which reached a plateau with a final value of 0.26 regardless of the mode of concentration.

In the case of concentrated milk with sodium caseinate added before concentration, the samples showed gelation at a statistically similar time to that of the corresponding concentrated milk. In this case, the value of elastic modulus after 2 h of gelation was significantly lower than in the control.
Table 5.3: Rheological parameters measured during rennet induced gelation. Time of gelation was defined as the point of steep increase in the value of G’. The value of G’ measured 2 h after gelation is also indicated. Values are the average of three independent experiments, with standard error. Letters indicate statistical difference (P<0.05) between treatments.

<table>
<thead>
<tr>
<th>Sample Details</th>
<th>Osmotic stressing</th>
<th>Ultrafiltration</th>
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<tr>
<td></td>
<td>Gel point (min)</td>
<td>G’ after 2 h (Pa)</td>
</tr>
<tr>
<td>3x concentrated control</td>
<td>100±2(^b)</td>
<td>505±56(^d)</td>
</tr>
<tr>
<td>NaCas added before</td>
<td>103±3(^b)</td>
<td>382±41(^c)</td>
</tr>
<tr>
<td>NaCas added after</td>
<td>n.d.</td>
<td>n.d.</td>
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**Figure 5.4:** The development of $G'$ (filled symbols) and tan $\delta$ (empty symbols) during renneting of milk concentrated by osmotic stressing (A) or ultrafiltration (B). Control (●), NaCas added before (▲) and NaCas added after (■). Values are for one representative run, averages and deviations are in Table 5.3.
The tan δ behaviour in these samples, regardless of the mode of concentration, was quite different, as a value of tan δ = 1 was reached earlier and remained around this value until a steep increase in the G’ occurred later. A higher tan δ indicates higher flexibility and rearrangement in the intra-micellar structure (Van Vliet et al., 1991) and during renneting rearrangements happen at all levels from microscopic, mesoscopic to macroscopic scale (Mellema et al., 2002). From Figure 5.1 it was clear that a part of added NaCas had moved to the colloidal phase. The newly incorporated NaCas brings more calcium to the colloidal phase, the highest being UF as its soluble phase is partly devoid of any added caseinates. This inflection before the point of gelation might be due to the internal rearrangements occurring to the casein micelles because of the increased calcium levels within the structure. Hence, the gelation point was defined differently and is considered as the point where noticeable and consistent increase in the value of G’ was first observed. However, it is worth noticing that this behaviour has never been noted for rennet-induced casein gels, and it indicates a difference in the gelation behaviour of the micelles, which needs further study.

When milk was concentrated by osmotic stressing and then sodium caseinate was added after concentration (Figure 5.4A), gelation did not occur, confirming the light scattering results shown in Figure 5.3A. On the other hand, this was not the case for the ultrafiltered milk. In this case, even after gelation, the time of gelation did not differ from control (see Table 5.3). It is worth noting that in the case of sodium caseinate added after, the final G’ value was in the same order of magnitude as for control milk, and the tan δ behaviour resembled that of control milk.
The increase in the G’ after gelation reflects the ongoing fusion of micelles, which results in an increase in the number of bonds between aggregated particles (Lucey, 2009).

The G’ value for UF samples where NaCas added after concentration contradicts the results obtained for osmotically concentrated samples provided the ionic strength, amount of rennet added and other environmental parameters were similar. There were differences in the amount of calcium recovered in the supernatants of the differently concentrated milk with added caseinate, but the values were higher in osmotically stressed milk. It could be hypothesized that the difference in behaviour depends on the level of unsedimentable caseins recovered (Figure 5.1).

The gelation experiments clearly suggested that the method of concentration has a long lasting effect on casein micelles. In the case of osmotic stressing, there was also a discrepancy in the time of gelation measured by rheology and the aggregation time measured by DWS. This is peculiar, as usually there is agreement between DWS and rheology measurements in terms of gelation points both for single strength and concentrated milk (Hemar et al., 2004; Sandra et al., 2007, Sandra et al., 2011).

To test if the lack of gelation in the osmotic stressing samples was due to a change in the accessibility of the rennet enzyme to casein micelles, the release of caseinomacropeptide was tested in the samples concentrated by osmotic stressing (Figure 5.5). The study of the first stage of the rennet reaction demonstrated that in all the samples concentrated 3x by osmotic stressing, the release of caseinomacropeptide was not significantly different from that of the control concentrated milk.
**Figure 5.5:** The total integrated peak areas of caseinomacropeptide released after the addition of rennet for concentrated milk by osmotic stressing (●), and the same milk with sodium caseinate added before (▲) or after (■) concentration. The error bars represent standard error (n = 3). The development of the elastic modulus (G') during rennet-induced aggregation for the 3x milk concentrated by osmotic stressing is also indicated (○, right hand axis) as reference.
The area measured from HPLC elution of the serum phase during renneting was higher in the case of sodium caseinate, because of the higher amount of protein. However, in all cases, the extent of the hydrolysis was sufficient for gelation to occur (Zoon et al., 1988; Dalgleish, 1992; Sandra et al., 2011). In spite of the nearly complete hydrolysis of κ-casein from the micelles at less than 80 min, the milk with NaCas added after did not gel (Figure 5.3 and 5.4).

It has been previously demonstrated for single strength milk that the presence of soluble caseins hinder the rennet induced gelation of casein micelles by shielding the hydrophobic patches on the particles (Gaygadzhiev et al., 2012). To further evaluate the fate of soluble caseins in the concentrated milk after renneting, the supernatants were separated using centrifugation (see methods) after 150 min from the addition of rennet. The ion exchange chromatographic elutions for the samples concentrated by osmotic stressing and ultrafiltration are shown in Figure 5.6. Peaks were assigned as for Figure 5.1. All elution profiles were similar, irrespective of the mode of concentration and presence of NaCas were similar. As previously shown for single strength milk, although soluble caseins may be present before renneting, after κ-casein hydrolysis these proteins will be associated with the colloidal phase. This behaviour occurred regardless of their distribution prior to gelation: the amount of non sedimentable caseins differed depending on the mode of concentration or addition of sodium caseinate.
Figure 5.6: Elution profiles for non sedimentable proteins before and after renneting of milk concentrated by osmotic stressing (A) or ultrafiltration (B). For peaks assignments see Figure 5.1.
5.5. CONCLUSIONS

The shear and mixing occurring during ultrafiltration cause modifications to the casein micelles. There were clear differences in the dynamics of the casein micelles concentrated by osmotic stressing compared to ultrafiltration, in spite of the similarities in composition. Although both methods of concentration preserved the amount of soluble calcium present in the original serum, milk concentrated by osmotic stressing had a significantly higher level of non sedimentable caseins compared to milk concentrated by ultrafiltration.

By adding sodium caseinate to the concentrated milk, and comparing the renneting behaviour of the samples, it was clearly shown that the casein micelles prepared with the two methods show a different functionality. When similar amounts of soluble caseins were added to milk concentrated by ultrafiltration compared to osmotic stressing, considered a gentler technique. It appeared as in the case of ultrafiltered milk, the majority of the soluble caseins added were transferred to the colloidal phase. It could be hypothesized that during membrane filtration, rearrangements of the micelles may occur, and smaller particles may be produced, resulting in the incorporation of the additional sodium caseinate on the surface or in the porous internal structure of these protein particles. These types of rearrangements have been described before for casein micelles during cooling, whereby β-casein is released into the serum phase, and re-associates when temperature is raised (Creamer et al, 1977).

The differences in the behaviour with renneting between samples containing sodium caseinate added before or after concentration also clearly points to the dynamic nature of
casein micelles. The addition of sodium caseinate before gelation in both cases did not interfere in the rennet induced gelation, while it clearly interfered with the reactions in osmotic stressed samples when added after concentration.

This research brings new insights on the rearrangements that may occur to casein micelles during concentration, with important consequences for a better understanding of membrane filtration processes. In addition, it brings evidence for some of the differences in composition and functionality of caseins concentrated between ultrafiltration and osmotic stressing.
CHAPTER 6

SODIUM CASEINATE HINDERS RENNET GELATION OF CONCENTRATED MILK: THE ROLE OF SOLUBLE CASEINS AND CALCIUM IN THE INHIBITION
6.1. Abstract

Previous work (Gaygadhiev et al., 2012; Chapter 5) demonstrated that the presence of soluble caseins in the non sedimentable fraction of milk can affect the renneting functionality of the casein micelles. The objective of this research was to further probe the role of soluble caseins on the impaired rennetability of casein micelles in milk concentrated by osmotic stressing. Upon redilution of concentrated milk in milk serum (permeate) the gelation of the casein micelles was hindered, because of the presence of soluble caseins inhibiting aggregation of the rennet altered micelles. It was clearly demonstrated that the additional calcium may overcome the inhibitory effect of soluble caseins by bridging between rennet altered casein micelles and forming casein aggregates of colloidal dimensions, no longer able to interact with the renneted micelles.

6.2. Introduction

Concentration of milk is accompanied by a number of changes that alter the processing functionality of casein micelles. Among these, the most important factors are the increased interactions due to high volume fractions (see Chapter 4), the changes in soluble and colloidal calcium (Ferrer et al., 2012, Alexander et al., 2011) and the increase in the non sedimentable proteins (Chapter 5). The microstructural properties of casein gels are not only related to the internal and interfacial integrity of the casein micelles, but also on the composition of the serum phase, as ionic calcium is critical to the formation of a stiff gel (Sandra et al, 2012).
Renneting is an important functional property of casein micelles. During this reaction, chymosin (rennet) specifically probes the surface of casein micelles and cleaves the protective layer of these hydrocolloids. The release of the κ-casein caseinomacropeptide results in the loss of steric and electrostatic stabilisation, forming hydrophobic and calcium sensitive patches on the surface of the micelles (Dalgleish, 1992; de Kruif, 1992). When more than 90% of κ-casein is hydrolyzed (Dalgleish, 1992), the secondary phase of rennet aggregation occurs, and the micelles aggregate in the presence of free Ca²⁺ (Lucey, 2009). The aggregation does not occur in the absence of sufficient calcium ions (Sandra et al, 2012; Martin et al., 2010).

Recent research showed that the presence of additional soluble caseins impairs the rennet aggregation of casein micelles in single strength milk (Gaygadhiev et al., 2012). In addition, a similar behaviour was shown for 3x concentrated milk with sodium caseinate added after concentration (Chapter 5). Concentration was carried out using osmotic stressing, a non invasive concentration method that maintained the ionic composition present in the serum of the original milk.

The current study aimed at a better understanding of the role of soluble caseins and calcium in the rennet induced gelation of concentrated milk. Recently, it has been shown that soluble casein can aggregate to form non sedimentable small particles of 12 nm diameter made up of 15 casein molecules in the presence of calcium (HadjSadok et al., 2008; Pitkowski et al., 2008) and free calcium is an important element mediating the secondary stage of aggregation (Lucey 2009). Therefore, it was hypothesized that by restoring the ionic calcium in the serum phase it may be possible to restore the
aggregation behaviour of the casein micelles. In this project, this hypothesis was tested by adding rennet to milk after addition of calcium or after dilution of concentrated milk in the original milk serum.

6.3. MATERIALS AND METHODS

6.3.1. Skim milk, concentrated milk and permeate Preparation

Skim milk and permeate were prepared as described in detail in Chapter 5.

6.3.2. Analysis of centrifugal supernatant from concentrated samples

Milk samples were centrifuged at 100,000g for 1 h at 20°C in a Beckman Coulter Optima™ LE-80K ultracentrifuge as detailed in Chapter 5. The composition of soluble caseins was then tested using ion exchange chromatography (Chapter 5).

6.3.3. Rennet gelation

Renneting experiments were followed using DWS and Rheology as described in Chapter 5.

6.3.4. Calcium measurements

Different concentrations of sodium caseinate (NaCas), ranging from 0-10 g L⁻¹ were dissolved in milk permeate and then stirred for 2 h. The samples were then filtered using a Prep/Scale™-TEF 1ft² cartridge ultrafiltration unit (10 KDa cut-off Regenerated cellulose, Millipore, Ontario, Canada). The soluble caseins were retained in the retentate while the permeate contained protein free serum. The permeate was then subjected to calcium analysis to determine the amount of free calcium and it was then subtracted from
the total calcium, present in the solution before UF, to obtain the calcium bound to the proteins.

Determination of soluble calcium in all samples was carried out using non-suppressed ion chromatography (Rahimi Yazdi et al., 2010) and as described in paragraph 3.3.6.

6.3.5. Statistical Analysis

All experiments were carried out in triplicate (i.e. three separate milk batches), and the average and standard errors are reported. Statistical significance was evaluated using ANOVA at p<0.05.

6.4. Results and Discussion

6.4.1. Effect of addition of calcium to rennet gelation of concentrated milk

As previously shown in Figures 5.3 and 5.4, the addition of 0.6% sodium caseinate to 3x concentrated milk inhibited the aggregation of the casein micelles. The secondary stage of gelation is mediated by ionic calcium and its absence may prevent the aggregation of destabilised micelles (Lucey, 2009). It is a customary practice to add CaCl$_2$ during cheese making to aid coagulation and form a firmer gel network.

Milk concentrated 3x by osmotic stressing had a serum calcium concentration of 10.23±0.50 mM, similar to that of skim milk reported by the literature (Lucey and Horne, 2009). Although the ratio of casein micelles to soluble calcium was lower than that of skim milk, due to the 3x volume fraction of the micelles, concentrated milk formed stiff gels after addition of rennet (Figure 5.4). On the other hand, milk concentrated by
osmotic stressing with added NaCas showed significantly higher levels of serum calcium, 12.48±0.50 mM (P<0.01) regardless whether the caseins were added before or after heating. While milk with soluble caseins added before concentration gelled, that with the caseins added after concentration did not. It may be possible to conclude that the amount of calcium free to react with the micelles may not be an important factor. However, a substantial amount of non sedimentable caseins was recovered in all the samples (Figure 5.1), with the highest in the milk with sodium caseinate added after concentration.

The amount of soluble caseins may interact with the micelles, but it may also be responsible for chelating calcium in milk. Being highly phosphorylated, soluble caseins will bind calcium and this will affect the ionic equilibrium of the concentrated milk. To better evaluate the amount of free calcium associated with soluble caseins, different levels of NaCas were added to milk permeate and the amount of calcium transmitted by the filter was measured. Figure 6.1 describes the amount of free calcium present in the milk permeate when different levels of NaCas was added. It was concluded that the addition of 0.6% NaCas to the serum phase would result in further chelation of 3.34 mM calcium.

This concentration of calcium (3.34 mM) in the form of CaCl$_2$ was then added in the dialysis medium during osmotic stressing concentration. It has been previously shown that this mode of calcium addition is equivalent to the direct addition of CaCl$_2$ to milk (Sandra et al., 2012). After concentration with added calcium in the serum, sodium caseinate was added to 3x milk. Figure 6.2 show the changes occurring to light scattering and rheological parameters measured during renneting of concentrated milk.
Figure 6.1: Amount of free calcium in the serum phase as a function of sodium caseinate added to permeate.
Figure 6.2: The development of light scattering (A) and rheology (B) parameters for milk concentrated 3x by osmotic stressing subjected to rennet aggregation. Milk was concentrated 3x (control) and sodium caseinate was added before or after concentration. CaCl$_2$ was added in the dialysis medium during concentration and sodium caseinate. A: Turbidity parameter (filled symbols), radius (open symbols). The error bars represent the standard error of turbidity parameter recorded during the experiment. B: G’ (filled symbols) and tan δ (empty symbols).
As shown in Chapter 5, when sodium caseinate was added before concentration, milk gelation was comparable to that of control concentrated milk. This behaviour was also observed for milk concentrated by ultrafiltration. While the concentrated milk with NaCas added after, did not show gelation, the same milk with additional CaCl$_2$ showed a faster gelation (75.5±1.0 min, average of three replicate experiments) than control milk, with a value of the elastic modulus at 2 h from the gelation point of 968±56 Pa. Both of these parameters were significantly different from the one obtained for the control milk, but similar to those obtained for milk that was concentrated by ultrafiltration (see Table 5.3). The changes in radius measured by DWS (Figure 6.2A) also show that in all cases, the casein micelles underwent aggregation at about 70 min, similarly to the control, apart from the case of milk containing sodium caseinate added after. The value of 1/$l^*$ was not fully equivalent to that of control milk, albeit similar in the shape of the curve, with a plateau reached around the gelation point.

It may be possible to conclude that the addition of calcium caused the aggregation of the soluble caseins and their association with the colloidal phase without impairing the aggregation of the casein micelles. In addition, with added calcium, more charges were neutralized on the surface of the casein micelles and more calcium bridges formed between casein particles. Due to the similarity in the rheological behaviour with renneting between the calcium added osmotic stressed samples and those concentrated by ultrafiltration, it may be possible to hypothesize that during concentration soluble caseins may be released together with calcium, and calcium associates with these soluble caseins, forming casein particles.
6.4.2. Rennet gelation of casein micelles rediluted in serum.

To restore the calcium levels present in the original milk and further study the renneting behaviour of the casein micelles after concentration, the milk concentrated by osmotic stressing was diluted back to its original volume fraction, with its corresponding milk permeate. These experiments were conducted with concentrated milk with and without sodium caseinate added either before or after concentration. None of the samples showed development of a gel modulus when measured by rheology. Figure 6.3 illustrates the changes in the light scattering parameters $1/l^*$ and radius for these samples. There were very limited aggregation in all samples after redilution in permeate. The renneting functionality of casein micelles was irrecoverably altered by concentrating the milk, and this was regardless of the presence of additional sodium caseinate. These results would lead to the conclusion that the presence of soluble caseins in these samples alters the renneting behaviour with a different mechanism than calcium chelation. These results are in agreement with earlier findings (Gaygadzhiev et al., 2012). The plausible mechanism being NaCas may get attached to the hydrophobic patches or hot spots of the renneted micelles and prevent the aggregation by long range hydrophobic repulsions.

Figure 6.4 shows the elution using ion exchange chromatography of the non sedimentable proteins present in the concentrated samples after redilution in permeate.
Figure 6.3: The development of turbidity parameter and radius of 1x diluted osmotically stressed samples. The filled symbols represent turbidity parameter and the open symbols represent radius. The error bars represent the standard error of turbidity parameter recorded during the experiment.
**Figure 6.4:** The soluble protein profile of diluted milk samples (osmotically stressed control 1x, added after 1x, added before 1x) and skim milk control (solid black solid line).
All the rediluted samples showed higher peaks for caseins than the serum phase of the original milk, confirming that upon redilution, the casein micelles show release of caseins in the serum phase. The centrifugal supernatant contained higher amounts of β-casein, but also of αs-caseins. This implies the formation of smaller casein particles, “minimicelles” in milk after redilution. It is important to point out that the amount of whey proteins in the concentrated milk after redilution was similar to that of that present in the serum phase of skim milk.

6.5. CONCLUSIONS

The altered renneting functionality of casein micelles after concentration can be attributed to the presence of an increased amount of non sedimentable caseins. This may in part affect the amount of ionic calcium present in the serum; however it is possible to conclude that the limited aggregation cannot be attributed to calcium chelation. There is a threshold limit for monomeric proteins the micelles can hold without affecting the rennet functionality of casein micelles. In the presence of calcium, caseins form small colloidal particles. At high enough concentration of calcium, these larger colloidal structures will co-precipitate with the casein aggregates during renneting. When excess amount of monomeric proteins is present, the aggregation is hindered by associating to the casein micelles and providing additional steric repulsion to rennet-altered casein micelles.
Chapter 7

AGE GELATION OF CONCENTRATED MILK
7.1. Abstract

The objective of this work was to study the mechanisms leading to the gelation of concentrated milk. Skim milk proteins were concentrated 6x the original volume using osmotic stressing, a non invasive concentration method, maintaining the serum composition as close as possible to that of natural milk. To determine the effect of heat induced whey protein aggregates, untreated milk was compared to milk heated at 90°C for 10 min prior to concentration. Within 9 days of storage at 4°C, the apparent viscosity increased markedly for both unheated and heated concentrated milk. After extensive dilution in milk serum, unheated milk showed an increase in the apparent diameter of the casein micelles over time. This was not the case for heated milk. Peptide analysis in unheated samples showed the appearance of a number of peptides during storage. In the presence of protease inhibitors, aggregation did not occur in unheated milk. On the other hand, in heated milk, the size of the micelles still changed even in the presence of protease inhibitors. The results demonstrated that the presence of heat induced whey protein aggregates plays a major role in increasing the viscosity of concentrated milk during storage. This work clearly identified the role played by proteases and heat denatured whey proteins in the age gelation of concentrated milk.

7.2. Introduction

Age gelation of concentrated milk is a challenge in the dairy industry. In concentrated milk, the apparent viscosity increases during storage, and may limit the shelf life of products. A number of factors have been identified as responsible to the changes in the rheological properties of concentrated milk, including the nature of the heat treatment,
proteolysis during storage, milk composition and quality, seasonal milk production factors and storage temperature (Datta and Deeth, 2003).

In milk, age gelation has been described as an enzyme mediated reaction (Payens, 1978; Snoeren and van Riel, 1979; Manji and Kakuda, 1988) or a combination of physico-chemical reactions including whey protein interactions with casein micelles, hydrolysis of κ-casein during storage, sulphide-disulfide reactions, changes in the overall charge of the complexes, dissociation of caseins from the micelles, and changes in salt equilibria (Andrews, 1983; de Koning et al., 1985).

It has been previously proposed that in heated, single strength milk, gelation during prolonged storage can be attributed to both enzymatic and non enzymatic physico-chemical changes; while in concentrated milk the enzymatic changes are less evident (Manji and Kakuda, 1988). It is now generally accepted that age gelation in heated milk is a two stage process (Venkatachalam et al., 1993; Mc Mahon, 1995; Datta and Deeth, 2003; Crudden et al., 2005). During the first stage, there is a dissociation of heat denatured β-lg and κ-casein complexes into the serum phase, partly caused by the breakdown of multiple anchor sites on casein micelles by proteolytic enzymes. Aggregation then occurs as these complexes bridge between casein micelles forming a weak gel. In addition to the high degree of proteolysis, a marked increase in Maillard browning and surface modifications of the casein micelles seem to be occurring in age gelled milk samples (Venkatachalam et al., 1993). The increase in viscosity has been taken as an indication of the build up of irreversible complexes during age thickening (Bienvenue et al., 2003; Trinh et al., 2007).
Age gelation has been attributed to proteolytic enzymes such as plasmin (Kohlmann et al., 1988; Kelly and Foley, 1997) as well as proteases originating from psychotropic bacteria present in raw milk (Kelly and Fox, 2006). Milk plasmin, natural milk alkaline serine protease, is associated with the casein micelle and the milk fat globule membrane in milk (Visser, 1981). The activity of plasmin in milk is regulated by a complex system of activators and/or inhibitors which converts plasminogen, the precursor of plasmin, to plasmin during storage of milk (Weber and Nielsen, 1991; Politis, 1996). Plasminogen, Plasmin activators or Plasmin are only partly denatured with heat treatment, while the inhibitors are heat labile. For this reason, the activity of plasmin has been shown to increase after heating, due to heat inactivation of the inhibitors (Richardson, 1983).

Plasminogen is more heat-stable than plasmin and plasmin activators are only slightly inactivated by UHT processing conditions (Cauvin et al., 1999). Additionally elevated plasmin activity is observed in late lactation milk and mastitic milk (Srinivasan and Lucey, 2002). Considerable autolysis of plasmin occurs at 5°C; however, during refrigerated storage psychotropic bacteria are significantly active (Crudden et al., 2005; Kelly et al., 2006) causing an increase in proteases in the milk, which induce considerable proteolysis leading to age gelation (Kelly and Fox, 2006).

Plasmin is a trypsin-like enzyme, which preferentially cleaves the polypeptide chain after a lysine or arginine residue. The protein in milk most susceptible to plaminolysis is β-casein (3 times more susceptible than αs1-casein). The reaction produces γ-caseins and proteose peptones from β-casein (Andrews, 1983). In addition, λ-caseins from αs2-casein and to a lesser extent, αs1-casein are produced during plaminolysis (Le Bars and Gripon, 1989; O’Flaherty, 1997). κ-casein is resistant to plaminolysis because of the presence of
carbohydrate moieties (Bastian and Brown, 1996; Doi et al., 1979). The microbial proteases attack predominantly κ-casein to form para-κ-casein (Snoeren and van Riel, 1979), followed by extensive nonspecific hydrolysis (Law et al., 1977).

The partial hydrolysis may cause a change in the physico-chemical properties of the micelles, for example, a decrease in the overall charge or steric stabilization of the protein particles, leading to aggregation (Crudden et al., 2005). The type of enzymes responsible for age gelation may be distinguished by investigating the peptide profiles, as bacterial proteases produce less hydrophobic peptides than native milk plasmin (Datta and Deeth, 2003).

Studies on concentrated milk have shown an increase in both apparent viscosity and yield stress for concentrated skim milk after 4 h of storage at 50°C (Bienvenue et al., 2003). Similar studies on reconstituted milk concentrates reported higher apparent viscosity after 100 min of storage at 65°C (Trinh et al., 2007). Storage temperatures above ambient are usually employed for accelerated storage studies. Fewer results are available on the age gelation of concentrated milk stored at refrigeration temperatures.

Recently, the physico-chemical properties of milk concentrated by osmotic stressing have been reported (Chapter 3 and 4). This concentration method does not require heat, and allows to carefully controlling the composition of the serum phase, while reaching high protein volume fractions. The present work investigated age gelation in milk concentrated using osmotic stressing.

In raw milk both plasmin and the proteolytic enzymes produced by psychotrophic bacteria can play a significant role in triggering the age gelation. The heat treatment of
milk destroys the psychotropic bacteria and some of the inhibitors of plasmin. In the current work, unheated and heated milk samples were concentrated 6x using osmotic stressing, and stored at 4°C. Their viscosity was measured over time. Protease inhibitors were also added to determine the role played by proteolysis in inducing age gelation. A combination of particle size analysis, Field Emission-Scanning Electron Micrographs (FE-SEM) and rheological analysis was employed to follow the development of age gelation.

7.3. Materials and Methods

7.3.1. Skim milk, concentrated milk and permeate preparation

Fresh milk was obtained from the University of Guelph experimental station (University of Guelph Dairy Research Station, Ponsonby, Ontario, Canada) and sodium azide (0.2 g L⁻¹) was added to prevent microbial growth. Skim milk was prepared as described in 3.3.1. Ultrafiltration permeate was prepared by ultrafiltration of reconstituted skim milk powder (100 g L⁻¹ solids) (Gay Lea Foods Cooperative, Guelph, Ontario, Canada) by passing it through an Polyethersulfone OPTISEP® Filter module (Smartflow Technologies, Apex, NC, USA) with 10 kDa molecular mass cutoff at ambient temperatures.

Milk was concentrated to 6x the original volume fraction using osmotic stressing as described in Chapter 3. A portion of the milk was heated for 10 min at 90 °C in a water bath allowing 2.5 min for the samples to reach the final temperature. After the heat treatment, the milk samples were immediately cooled to room temperature by immersion in an ice bath. After heating, milk was concentrated using osmotic stressing. The samples
were equilibrated for 2 h at room temperature after heat treatment before concentration. Concentration was carried out by placing a dialysis bag containing 40 mL of unheated or heated milk in a reservoir containing 1L of stressing solution composed of 95 g L\(^{-1}\) PEG dispersed in milk permeate. The samples were kept in the stressing polymer solution for 18 h at refrigeration temperature, to obtain 6x concentrated milk, as explained in detail in Chapter 3.

A protease inhibitor cocktail (catalogue number P 8340, Sigma Aldrich, St. Louis, USA) was added to a portion of the milk samples. This mixture was suspended in dimethyl sulfoxide and was added at a 1:100 (v/v) ratio of mixture to initial skim milk. The inhibitor was added after concentration because of the low molecular size of the inhibitors. To the control samples similar amounts of dimethyl sulfoxide were added. The inhibitor cocktail had a broad specificity for the inhibition of serine, cysteine, aspartic proteases and aminopeptidases (Sigma P8340, Sigma Aldrich product information, 2010). The mixture contained 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin.

The details of inhibitions by each of the components are known. Chymosin, is inhibited by pepstatin, a generic inhibitor for aspartic proteases such as pepsin, cathepsin D and many microbial aspartic proteases (Shakeel-Ur-Rehman et al., 1998). The small molecule AEBSF inhibits serine proteases (trypsin, chymotrypsin and plasmin). Aprotinin, a polypeptide with a molecular weight of 6512 Da is a classic member of the Kunitz-type serine protease inhibitors (Sigma P8340, Sigma Aldrich product information, 2010). Bestatin is a competitive inhibitor for aminopeptidases, which are categorized under unidentified milk proteases (Kelly et al., 2006). E-64 is an epoxide which can irreversibly
inhibit a wide range of cysteine peptidases namely cathepsins. Leupeptin is a competitive inhibitor and its inhibition maybe overcome by an excess of substrate. It shows a wide spectrum of inhibition towards cysteine (cathepsin B) and serine peptidases (trypsin, plasmin), as explained by manufacturer (Sigma Aldrich product information, 2010).

All samples were stored at refrigeration temperature (4°C) for 9 days.

7.3.2. Particle Size Determination by Dynamic Light Scattering

The particle size of the casein micelles was measured by dynamic light scattering (Zetasizer Nano-ZS). After concentration, the milk samples were diluted ~2000 times in filtered (0.2 μm nylon filters, Fisher scientific) milk permeate and analyzed.

7.3.3. Rheology Measurements

A controlled stress rheometer (Paar Physica MC 301, Anton Paar, Graz, Austria), was used to measure the viscosity of the reconstituted concentrate. The milk samples were subjected to a shear sweep test from 0.1 to 300 s⁻¹, using a cone and plate geometry, with a set gap of 0.51 mm. The temperature of the plate was controlled with water circulating from a Julabo F25-HP refrigerated and heated water bath (Julabo Labortechnik, GbmH, Germany). All measurements were made at 4°C.

7.3.4. Analysis of the serum phase

Milk samples were centrifuged at 100,000g for 1 h at 20°C in a Beckman Coulter Optima™ LE-80K ultracentrifuge with rotor type 70.1Ti (Beckman Coulter Canada Inc., Mississauga, Canada) to separate the casein micelles from the serum fraction as described
in detail in Chapter 3, session 3.3.3. The supernatant was carefully recovered using a pasteur pipette, and was given two sequential filtrations using 0.45 μm and then 0.22 μm (syringe driven filters, Fisher Sci., Ontario, Canada) and then analysed for peptides and minerals.

The clear filtered supernatant (20 μL) was injected into a reverse-phase HPLC column (C18 Nova-Pak 4μm, 3.9x150 mm, Waters, MA. USA) with a C18 guard column (GE healthcare Lifesciences). The analysis was carried out with a SpectraSystem HPLC (Thermo Fisher Scientific, Mississauga, ON, Canada) consisting of a degasser, a P4000 pump, an auto injector (AS3500) and a UV 2000 detector (Thermo Electron Corporation, San Jose, CA). Elution was carried out using 0.01g L⁻¹ (v/v) trifluoracetic acid containing 0.198 g L⁻¹ acetonitrile for 40 min, followed by a rapid increase (in 1 min) to 9.8 g L⁻¹ for 7 min. The area under the peaks detected at 214 nm was calculated using ChromQuest software (ChromQuest 4.1. Thermo Electron).

Peaks of interest were manually collected and analyzed using Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) at the Advanced Analysis Centre of the University of Guelph. In addition, mineral composition was determined using Inductively Coupled Plasma Optical Emission Spectrometry at the Laboratory Services facilities of the University of Guelph.

7.3.5. Field Emission Scanning Electron Microscopy

Scanning electron microscopy was conducted to observe the changes occurring to the structure of the casein micelles during storage. The samples were prepared using a published method (Martin et al., 2006). Briefly, clean and dry polished carbon planchets
(Canemco Inc., St. Laurent, Que., Canada) were placed in 2 mM 11-mercapto-
undecanoic acid (11-MUA, Sigma Aldrich, St. Louis, MO, USA) for 18 h to form a self
assembled monolayer. The functional groups were then modified using equal volumes of
0.1 M N-hydroxy succinimide (NHS, Fluka, Steinheim) and 0.4 M 1-Ethyl-(3dimethyl
aminopropyl) carbodiimide hydrochloride (EDS, Sigma Aldrich, St. Louis, MO, USA).
Few drops of the concentrated milk were then deposited on the polished carbon surface
and incubated for 45 min. The planchets were then rinsed with 20 mM imidazole buffer
(pH 7.0) containing 5 mM calcium chloride. The buffer was then exchanged with the
fixative, 0.15 g L⁻¹ glutaraldehyde solution for 30 min. The sample was rinsed with Milli-
Q water and then dehydrated using a graded ethanol series: 70, 90 and 100% ethanol
before critical point drying using solid carbon dioxide. The samples were then mounted
onto SEM stubs with colloidal carbon and stored in a desiccator at room temperature until
imaging.

Images were obtained using an Hitachi S4800 FESEM (Tokyo, Japan). Imaging was
conducted at Hitachi Canada (Rexdale, Ontario). Acceleration voltage was kept constant
at 2.0 kV. The samples were not coated. Images were acquired digitally using Quartz PCI
software (Vancouver, BC, Canada).

7.3.6. Statistical Analysis

All experiments were conducted in triplicate (i.e. three separate milk batches), and the
average and standard errors are reported. Statistical significance was evaluated using
ANOVA at p<0.05.
7.4. Results and Discussion

7.4.1. Rheology

Figure 7.1 illustrates the apparent viscosity of concentrated milk, heated and unheated right after concentration and after 9 days of storage. The values are shown as a function of shear rate, to illustrate their shear thinning behaviour. This behaviour is commonly reported for concentrated milk (Snoeren et al., 1984; Ruitz and Barbosa-Canovas, 1998; Bienvenue et al., 2003; Trinh et al., 2007) and it has been attributed to the rapid breakdown of structure and the realignment of casein micelles in the direction of flow.

To evaluate differences in viscosity, statistical analysis was performed on the apparent viscosity measured at 200 s⁻¹. There was no significant difference between the initial viscosity at time 0 between unheated and heated milk. After storage, the values of apparent viscosity were higher for both unheated and heated milk, although the extent of the increase was not different between heated and unheated milk. An increase in apparent viscosity for milk concentrates have been reported before for milk stored at 50°C for 4 h (Bienvenue et al., 2003). In this case, it was shown that the structural build up was irreversible. Similar results have been also report by others after storage of concentrated milk for 100 min at 65°C (Trinh et al., 2007). However, these accelerated storage studies may promote enzymatic and chemical modifications due to the high storage temperatures.

After 9 days of storage at refrigerated temperatures, unheated milk containing the protease inhibitors did not show a change in the apparent viscosity and the flow behaviour was superimposed to that of freshly concentrated unheated milk (Figure 7.1B and Table 7.1).
Figure 7.1: Apparent viscosity as a function of shear rate for unheated (empty symbols) and heated (filled symbols) concentrated milk stored for 0 day (○, ●) and 9 days (Δ, ▲) without (A) or with protease inhibitor added (B).
**Table 7.1:** Values of apparent viscosity measured at 200 s\(^{-1}\) for the fresh concentrated unheated and heated milk, and the corresponding changes in viscosity after 9 days of storage (as a difference from the initial viscosity), for milk with and without protease inhibitors. Values are the average and standard deviation for three separate replicates. Different superscript letters indicate statistically significant differences at p<0.05

<table>
<thead>
<tr>
<th></th>
<th>Initial viscosity (Pa s)</th>
<th>Changes in viscosity after 9 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No protease inhibitors</td>
</tr>
<tr>
<td>unheated</td>
<td>0.06 ± 0.02(^{a})</td>
<td>0.052±0.019(^{b})</td>
</tr>
<tr>
<td>heated</td>
<td>0.030 ± 0.002(^{a})</td>
<td>0.036±0.008(^{b})</td>
</tr>
</tbody>
</table>
In heated milk also the addition of the inhibitors seemed to affect the age gelation. There was a very small, but significant change in viscosity in heated milk, indicating that in this case, although the inhibitors strongly hindered the formation of an aggregate, other factors (i.e. heat induced whey protein complexes) play a role in age gelation.

7.4.2. Particle size

The average apparent diameter of casein micelles was measured after dilution in permeate, and results are summarized in Figure 7.2. In all cases, the milk remained monomodal in the distribution of particle size, with a shift in a larger diameter (Figure 7.3). Overall, there was a significant difference between heated and unheated milk, with casein micelles in fresh heated milk having a larger diameter than those in unheated milk. This is consistent with previous reports (Bienvenue et al., 2003). Heating induces the formation of whey protein aggregates, which associate to the colloidal phase of the micelles (Vasbinder and de Kruif, 2003). In untreated milk, the average diameter of the casein micelles appeared to increase with storage time; however, after 9 days the large standard deviation did not allow to find a statistical significance. In addition, Figure 7.2 also indicates that in the presence of the inhibitor cocktail, unheated milk did not show such an increase in size but even at 9 days the size of the micelles was comparable to that of the unheated milk.

The heated concentrated milk showed a different behaviour for radius during refrigerated storage (Figure 7.2). After 9 days of storage, both milk with and without protease inhibitors showed a moderate increase in the hydrodynamic radius.
**Figure 7.2:** Average particle size of casein micelles the unheated concentrated milk (A) and heated (B) samples with (●) or without (Δ) protease inhibitor during storage. Values are the average of three replicates with standard deviation.
In the case of heated milk, statistical analysis showed that there was little difference between the heated milk with or without inhibitor (ANOVA P value was 0.0534), while the effect of storage period, for both systems was highly significant (P value<0.01).

Figure 7.3 summarizes the changes in the intensity distribution measured by DLS. In the case of untreated milk, although the distributions seemed to continue to be monomodal, from the 6\textsuperscript{th} day onwards there was the appearance of larger aggregates, these large aggregates contributed to the large standard deviation of unheated milk without protease inhibitor. There were no noticeable changes in the distribution in the case of unheated milk with protease inhibitor, once again confirming that the presence of the protease inhibitor cocktail in unheated concentrated milk hindered age gelation at cold temperatures. In heated milk, there was no difference in the size distribution after 9 days of storage at refrigerated temperatures (Figure 7.3), although the size seemed to shift to a slightly larger diameter over time (Figure 7.2). The results presented in Figure 7.2 and 7.3 may suggest that in heated milk rearrangements occur to the proteins during cold storage, with an increase in the association of the whey protein complexes with the casein micelles over time. This was noted in the increase in the size of the micelles. It was concluded that in addition to the effect of proteases, in the case of heated concentrated milk whey protein complexes also play a role in the age induced gelation. The contribution of whey protein complexes in age gelation of heated milk has been suggested before (Datta and Deeth, 2003; Crudden et al., 2005), however, in this case, the authors hypothesised that gelation was mediated by the removal of β-lactoglobulin and κ-casein complexes from the micelles and aggregation of these complexes in the serum to form a space filling gel. SDS-PAGE electrophoresis of the centrifugal
Figure 7.3: The intensity-size distribution measured by DLS for unheated (A) and heated (B) concentrated milk during storage, for milk with or without protease inhibitors. Representative distributions are shown for initial milk (day 0), solid line, for 9 days without (broken line) or with protease inhibitor added (dotted line).
supernatant after 9 days of storage did not show a change in the amount of whey proteins or κ-casein complexes (data not shown). The slight increase in the size of the micelles showed in Figure 7.2 would suggest additional aggregation of the complexes with the caseins during storage.

The present results are only in part consistent with the literature. Bienvenue et al., (2003) investigated the age thickening of skim milk concentrate (45% total solids) and observed a bimodal intensity size distribution with a second peak between 1 and 5 µm; however, the samples were kept at 50°C for 8 h. Previous literature also discussed age gelation as induced by the formation of initial flocs, acting as activators of the formation of larger aggregates (Clark, 1992). The present work is in agreement with recent reports (Trinh et al., 2007) suggesting that irreversible aggregation occurs slowly and is present even before the onset of gelation. Indeed, the onset point of gelation should be considered as the natural end point of age gelation (Walstra et al., 1999).

7.4.3. Peptide analysis

The peptide profiles present in the soluble phase of milk during after storage were analyzed as shown in Figure 7.4 for unheated and heated concentrated milk. The absorbance was significantly higher for unheated than for heated milk, suggesting a higher amount of peptides present in unheated milk.

The peptide profiles of unheated milk concentrated (Figure 7.4A) and containing inhibitors were similar to those of the initial milk, even after 9 days of storage, with the exception of a peak eluting at 13 min – a peak characteristic of the protease inhibitor preparation.
Figure 7.4: The RP-HPLC peptide profile of supernatants of unheated (A) and heated (B) concentrated milk, analyzed the same day of preparation (solid black line), after 9 days of storage without (broken line) or without protease inhibitor added (dotted line). Chromatographs are representative of three independent replicates.
Unheated milk after 9 days of storage exhibited significantly different peaks from the initial milk, and these peaks were collected and analysed using MALDI-TOF mass spectroscopy to identify the range of molecular weight for the peptides increasing significantly during storage. The signal m/z under positive ionisation mode obtained by MALDI-TOF MS is given in Table 7.2. A combination of mainly small (2-6 kDa) and medium molecular weight peptides (12 kDa) were observed, suggesting the presence of proteose peptones. The present observations were consistent with previous reports. For example, using HPLC/electrospray ionization mass spectrometry, it was shown that aged pasteurized milk has different molecular weight proteose peptones around 12 kDa (De Noni et al., 2007). In a separate study, small peptides between 2 and 4.4 kDa were shown in stored UHT milk (Meltretter et al., 2008).

Figure 7.4B represents the peptide profile of concentrated heated milk after 9 days of storage either with or without inhibitor cocktail. The peptide analysis of heated milk did not yield any difference between 0 day or 9 day or samples treated with the cocktail of protease inhibitors. This result confirmed the reports of Table 7.1 and size changes in Figure 7.2 that indicated a much lower extent of proteolysis in these aged samples, compared to unheated milk. In addition to partial inhibition of proteases by heat, a possible reason for the limited proteolysis in heated milk could be autolysis of plasmin at refrigerated temperatures. It has been reported that considerable plasmin autolysis occurs at 5°C as well as little activation of plaminogen (Cruden et al., 2005). It could be concluded that raw milk contained a number of heat labile proteolytic enzymes derived from psychotrophic bacteria capable of producing proteolytic enzymes under refrigerated conditions.
Table 7.2: Molecular weight estimated using MALDI-TOF MS from the major fractions eluted from RP-HPLC of the supernatant of unheated milk after 9 days of refrigerated storage. For peak assignment see Figure 7.4.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Molecular weight of major peptides present in the fractions collected by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.41</td>
<td>2443&lt;br&gt;2523</td>
</tr>
<tr>
<td>14.97</td>
<td>2582&lt;br&gt;2507</td>
</tr>
<tr>
<td>20.12</td>
<td>3234&lt;br&gt;2424&lt;br&gt;3314</td>
</tr>
<tr>
<td>22.95</td>
<td>4273&lt;br&gt;6566&lt;br&gt;6486&lt;br&gt;6793&lt;br&gt;13322</td>
</tr>
<tr>
<td>24.45</td>
<td>8102&lt;br&gt;8870&lt;br&gt;12824</td>
</tr>
</tbody>
</table>
7.4.4. FE-SEM of casein micelles

FE-SEM images of unheated concentrated milk fresh and after 9 days of storage, with and without protease inhibitors are shown in Figure 7.5. These are the representative images of three independent trials. The micellar structure was consistent with previous observations using a similar technique (Dalgleish et al., 2004). In general, the individual micelles were spherical in nature. During storage, an increase in extensions was observed, with a tendency for a higher number of connections with neighbouring micelles (Figure 7.5 C, D). These increased interactions may be at the basis of the age gelation observed for unheated milk, reflected in change in viscosity as well as an increase in size distribution of the micelles. The presence of peptide inhibitor seemed to limit the formation of such appendages (Figure 7.5 E and F). It is important to note that in the case of protease inhibitor, there was a larger scattering of the background, most probably due to the contamination of the inhibiting substances.

The casein micelles in heated concentrated milk freshly prepared (Figure 7.6 A, B) showed a different morphology compared to those of unheated, concentrated milk. These particles showed a rough surface compared to those shown in Figure 7.5. The difference in the appearance of the micelles may be due to the presence of interacting whey proteins. The average size of the heat induced complexes present in heated milk is approximately ~3.5-5.5 million Da, depending on the available κ-casein or the casein: WP ratio in the mixtures (Guyomarc'h et al., 2003a).
Figure 7.5: Electron micrograph of the casein micelle of raw concentrated milk 0 day (A and B), 9 day (C and D) and with PI 9 day (E and F) using field-emission scanning electron microscopy. Samples were prepared on a carbon planchet and fixed as described in the text. No coating techniques were employed. The scale bars and accelerating voltage are given in the figure itself.
Figure 7.6: Electron micrograph of the casein micelle of heated concentrated milk 0 day (A and B), 9 day (C and D) and with PI on 9 day (E and F) using field-emission scanning electron microscopy. The scale bars and accelerating voltage are given.
The microstructures of the casein micelles appeared very different after 9 days of storage of concentrated milk. Heated milk stored for 9 d showed a very distinguishable spherical shape with the absence of surface extensions, especially when compared to the samples freshly prepared (Figure 7.6 C and D). It was postulated that the changes affected the surface charge of the casein micelles. Indeed, in the heated milk stored for 9 d an increase in the secondary emission of electrons was noticed during the imaging. Hydrolysis by plasmin reduced the zeta-potential of the casein micelles from -19 to -16 mV (Cruden et al., 2005).

An atypical behaviour was observed for heated concentrated milk treated with the inhibitor cocktail (Figure 7.6 E, F). A marked reduction in the scattering intensity from the casein micelles was noted, and an increased in the scattering from the background was observed, similarly than for unheated milk.

7.4.5. Mineral Analysis

The detailed mineral analysis of the soluble phase of different types of concentrated milk is given in Table 7.3. Changes in salt equilibira were considered to be one of the triggering mechanisms of age gelation. Mineral analysis was carried out to see if the soluble phase was changing. Any instability in the colloid phase will directly reflect the mineral equilibrium of the soluble phase. The amount of calcium, phosphate and sodium did not vary significantly among different samples. Since the dialysis was conducted against milk permeate, the levels of soluble calcium, phosphate and sodium were similar in the concentrated milk samples.
Table 7.3: The amount of minerals present in the soluble phase of different samples of milk by ICP. Different superscript letters indicate statistically significant differences at p<0.05.

<table>
<thead>
<tr>
<th>Concentrated milk</th>
<th>Calcium (mg L⁻¹)</th>
<th>Phosphates (mg L⁻¹)</th>
<th>Sodium (mg L⁻¹)</th>
<th>Sulphur (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk -0day</td>
<td>415±15ᵃ</td>
<td>520±30ᵇ</td>
<td>560±20ᶜ</td>
<td>21500±150⁰ᵈ</td>
</tr>
<tr>
<td>Raw milk -9 days</td>
<td>410±15ᵃ</td>
<td>505±5ᵇ</td>
<td>585±5ᶜ</td>
<td>20500±150⁰ᵈ</td>
</tr>
<tr>
<td>Raw milk with PI</td>
<td>410±20ᵃ</td>
<td>515±35ᵇ</td>
<td>585±45ᶜ</td>
<td>27000±300⁰ᵈ</td>
</tr>
<tr>
<td>Heated milk -0 day</td>
<td>400±10ᵃ</td>
<td>545±15ᵇ</td>
<td>575±15ᶜ</td>
<td>19500±50⁰ᵈ</td>
</tr>
<tr>
<td>Heated milk -9 days</td>
<td>410±10ᵃ</td>
<td>565±5ᵇ</td>
<td>585±5ᶜ</td>
<td>19000±100⁰ᵈ</td>
</tr>
<tr>
<td>Heated milk with PI</td>
<td>415±5ᵃ</td>
<td>580±5ᵇ</td>
<td>620±10ᶜ</td>
<td>24500±50⁰ᵈ</td>
</tr>
</tbody>
</table>
No correlation was found between the changes in soluble calcium content and the onset of gelation (Cano-Ruiz and Richter, 1998).

7.5. Conclusion

The mechanism of age gelation in raw milk and heated milk appeared to be different. Proteolysis was the predominant factor in raw milk while in heated milk the dissociation of the heat induced complexes or drainage of the surface manifested the reaction. In general age gelation is mediated by a number of complex factors, the predominant one could be proteolysis, however the role of other factors cannot be ruled out as the commencement of age gelation is the result of collective and synergetic efforts of physicochemical as well as biochemical processes.

The temperature of storage is a significant effect on promoting age thickening. At temperatures higher than ambient storage temp, Maillard browning plays a significant factor; likewise storage at refrigeration temp can promote the proliferation of psychotropic bacteria with a concomitant increase in proteolytic enzyme production. Since heated milk failed to show any irreversible structure development during refrigerated storage, we could conclude that once processed, milk should be kept cold throughout all stages of the supply chain and process as quickly as further unit operations allow, minimising the hydrolysis of casein by plasmin and hence preserve the functionality of the milk proteins.
7.6. Acknowledgements

The support of Hitachi Canada (Rexdale, Ontario, Canada) is acknowledged in this work, as well as the help of Dr. Alexandra Smith (University of Guelph).
CHAPTER 8
OVERALL CONCLUSIONS

In the past, the colloidal behaviour and physico-chemical properties of casein micelles have been investigated as a function of volume fraction using different systems, namely reconstitution, evaporation, membrane processing, and so on. In this research, the author has successfully attempted to characterize this process under native conditions by employing osmotic stressing as a non-invasive method to obtain concentrated milk, while ensuring the maintenance of the ionic balance during concentration.

In heated and unheated milk, the increase in viscosity with volume fraction was well predicted using a hard sphere behaviour model suggesting that the casein micelles behave as uncompressible hard spheres with similar voluminosity, if the viscosity of the serum phase and particle particle interactions are taken into account in the calculations. In the present study, we were able to demonstrate that, when milk is concentrated three times, the behaviour of the casein micelles can no longer be predicted using only hydrodynamic effects because of stronger interparticle interactions. Thereafter, the casein micelles are no longer free diffusing, as the interparticle distance limits the mobility of the larger micelles and causes caging of the smaller particles. However, there were no discernible permanent associations between casein micelles, confirming that the casein micelles behave as non-compressible hard spheres, up to high critical packing volume fractions. These findings contribute to a more fundamental knowledge on the behaviour of casein micelles in concentrated milk systems.
The behaviour of heated milk is ruled by the presence of heat induced aggregates and their partition between the soluble phase and colloidal phase. The heat treatment and concentration, affect the shape and voluminosity of the casein micelles. A decline in voluminosity is observed with increase in concentration.

Most of the research concerning membrane processes has assumed that membrane processing is non-invasive to casein micelles. Our research approach allowed us to demonstrate that ultrafiltration at 3x in the presence of added soluble caseins resulted in protein particles with a different renneting functionality from the same particles prepared by osmotic stressing. It was concluded that shear and mixing play an important role in the rearrangements of casein micelles in concentrated milk. During concentration, the casein micelles behave as dynamic colloids that interact with their environment during processing.

We have proven beyond doubt that monomeric caseins, when present in excess, tend to impair the renneting functionality of casein micelles by a complex mechanism not only involving chelation of Ca\(^{2+}\) but also providing steric hindernace by adhering to the hydrophobic patches of the renneted micelles. These findings bear far reaching implications and explicitly explain the altered renneting bahviour of commercially processed micellar casein powders.

The last chapter describes our study on the role of proteases in age gelation of milk by inhibiting protein degradation in 6x concentrated, heated, as well as raw milk. Peptide analysis combined with MALDI-TOF-MS of interested fractions of age-gelled raw milk samples exhibited the presence of a large number of small peptides and proteose
peptone fractions. On the contrary, protease inhibitors less effective in heated milk, as whey protein aggregates play a major role in causing age gelation at cold temperatures.

From this research, it was possible discern the behavior of casein micelles as a function of concentration, only by careful interpretation of chemical and physical properties of the systems. Against conventional wisdom, we have shown that casein micelles may undergo rearrangements during membrane concentration. These findings culminate in to a novel and better understanding on various aspects of physical chemistry of casein micelles, the effects of concentration of milk processing functionality, processing history on structure-function of casein micelles, the interactions occurring in milk proteins during concentration, and the possible use of casein micelles as functional delivery systems.
CHAPTER 9

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