Physico-Chemical Properties of Casein Micelles Concentrated by Membrane Filtration and Their Behavior in Acid Gelation

by

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Membrane filtration is a widespread unit operation in dairy technology. In this study we investigated how ultrafiltration (UF) and diafiltration (DF) may affect the physico-chemical properties of the casein micelles and their functionality related to acid gelation. By concentrating by UF, the volume fraction of protein increased while maintaining the serum composition similar to that of the original milk. By using UF in combination with DF, the mineral equilibrium was modified.

UF and DF retentates showed different behavior during heating, with the formation of different populations of non sedimentable complexes. Various combinations of heating temperature and time were investigated. Calcium solubilization as a function of pH was also affected, as demonstrated by measurements of buffering capacity and soluble calcium. The area of the maximum peak in buffering capacity observed at pH ~5.1, which is related to the presence of colloidal calcium phosphate, was significantly affected by casein volume fraction, but did not increase proportionally to casein concentration, even in the UF milk. This suggested the release of calcium phosphate even during concentration by UF. It was hypothesized that the changes in calcium equilibrium during UF and DF, as well as the formation of different types of soluble aggregates, during membrane
filtration as well as after heating, may have profound consequences on the acid gelation behaviour of the caseins. Hence, in a second part of the thesis, UF and DF retentates were subjected to heating at 80 °C for 15 min, and then gelation was induced using glucono-delta-lactone. The gelation pH, measured by rheology and diffusing wave spectroscopy, significantly increased with the extent of concentration (p<0.05, as measured by ANOVA). The increase was not only due to a reduction in the interparticle distance, but most importantly, because of the changes in the soluble fraction, and in particular the presence of the non-sedimentable aggregates. DF milk gelled at a significantly higher pH than UF milk. Furthermore, at a 2× concentration, the gel firmness at pH 4.6 was higher for DF milk than for UF milk. This was not the case for 4× concentrated milk, possibly because of limited rearrangements of the casein micelles and heat induced complexes at this high concentration. This work clearly demonstrated for the first time that UF and DF processes cause substantial changes in the composition of the soluble fraction, and modify the acid induced aggregation behaviour of concentrated milk.
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CHAPTER 1
GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

Concentration by membrane filtration has shown great potential in the dairy industry as a gentle processing technology. In particular, ultrafiltration (UF) has become an essential unit operation because it can simultaneously concentrate milk proteins and desalt or reduce the soluble components, obtaining high protein retentates (Spriggs et al., 1976; Spangler et al., 1991). Although UF is commonly applied in the manufacture of dairy products, the changes in processing functionality of caseins in retentates are not fully known, especially at high volume fractions. During membrane filtration, water is often added to improve membrane performance and achieve higher ratios of protein to solids. However, no data is available on the extent of the changes occurring during diafiltration on the properties of the retentates. A better understanding of these changes and their impact on the functionality of milk proteins will result in improved products and processes.

Milk proteins are the major components of milk and primary source of nutrition, and are usually distinguished into caseins and whey proteins; these two groups constituting 80% and 20% of the total protein, respectively. The main proteins contained in the serum are β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins (Walstra et al., 1984; Kinsella et al., 1989). Caseins, containing αS1-, αS2-, β- and κ-casein, are highly phosphorylated proteins which interact with calcium phosphate and assemble in stable, highly hydrated and polydispersed structures called casein micelles (Dalgleish, 2011). These proteins are often referred to as rheomorphic, as
they adapt their structures to the changes in environmental conditions (Dalgleish, 2011). To be able to predict the functionality and properties of casein micelles during processing, the physical and chemical changes occurring to these colloidal particles need to be well defined.

Recent studies have suggested that the renneting functionality and some physico-chemical properties of casein micelles are modified when UF concentration reaches high volume reductions (Ferrer et al., 2011). However, it is still not clear which processing factors are the most critical. This research will focus not only on the effect of the concentration by UF, but also DF.

Even though the casein micelles are very stable in their native state, they can be readily destabilized by lowering the pH. A better understanding of the behaviour of acidified concentrated milk is important, as the principles would apply to a number of dairy products. It is well-known that the acid-induced gelation of skim milk is caused by the destabilization of the surface as well as the interior of the casein micelles. Hence the composition of the serum phase changes during acidification. Although much is known about acid induced gelation of skim milk, only a few aspects are understood when concentrated milk is employed. With concentration, the total balance of soluble and colloidal calcium changes as well as the solubilization behaviour during acidification (Hardy et al., 1984). In the case of milk concentrated by UF, there is a higher ratio of protein to solids, as well as colloidal to soluble calcium and phosphate ions, with increased buffering capacity of the retentates (Bastian et al., 1991). The release of colloidal calcium and the decrease in proteins’ charge repulsion are critical to the
formation of acid milk gels. This research will test the hypothesis that volume fraction of milk and the process of concentration does not affect the aggregation behavior of the milk.

It has been previously shown that although the concentration of colloidal calcium increases with the volume fraction of the casein micelles, this increase is not proportional to the concentration factor of the milk (Le Graët and Gaucheron, 1999). It is also possible that the proportion of casein to colloidal calcium may change during UF, especially when the process is extended to high volume fractions or when DF is applied (Renner and Abd-El-Salam, 1991; McKenna, 2000; Ferrer et al., 2011).

The present work will attempt to answer a number of relevant questions when processing UF and DF retentates: does the dilution of the serum phase with water during DF affect the integrity of casein micelles, and does the change in the composition of the soluble phase together with concentration affect the colloidal phase of the micelles and their acid gelation behaviour? It is possible to hypothesize that the process of DF during membrane filtration may disrupt the casein micelles, causing an increase of soluble protein in the serum phase, and a difference in the soluble aggregates (in terms of the composition, size and type) involved in the formation of acid-induced gels.

This research will test whether concentrated milk processed by UF has heating and gelation behavior properties similar to those of concentrated milk processed by UF and DF. In brief, this research will test the hypothesis that concentration has no impact on the aggregation of the proteins during heating, and that concentration does not affect acid gelation of the casein micelles. In addition, when at the same volume fraction, milk proteins concentrated by UF and DF should show similar heat and acid aggregation behavior.
It has been demonstrated (Anema, 2009a) that heating of milk causes a decrease of calcium in its soluble phase. With increasing heating temperature there is an increasingly rapid transfer of the soluble calcium to the colloidal phase with a concomitant decrease in milk pH. As colloidal calcium content is linked to the buffering capacity of milk samples, it is possible to hypothesize that heating of concentrated milk will affect the casein micelles’ buffering capacity. In addition, since the soluble heat induced protein aggregates and their salt balance are critical to imparting texture to acid gels, this research will also focus on the changes in composition of the serum phase. To date, there is no comprehensive study of the effect of protein concentration by UF and/or DF on heat induced whey protein aggregation. Understanding the influence of heating and concentration is of major importance in the manufacturing of dairy products.

In this thesis, Chapter 3 will test the hypothesis that concentration does not modify the extent of whey protein denaturation, and does not change the composition or the size of the heat induced aggregates, and that the mode of concentration (UF and/or DF) does not have an impact on the heat induced reactions. The following chapter, Chapter 4, will describe the effect of concentration on buffering capacity and calcium release. The work in this chapter will test the hypothesis that although the concentration of calcium in the colloidal phase increases with the volume fraction of the casein micelles, this increase will not affect the release rate of the calcium in the soluble phase, and that the mode of concentration (UF or UF in combination with DF) as well as heating of milk does not affect the buffering capacity and the rate of release of the calcium during acidification.

Chapter 5 will then investigate the effect of UF and DF on the acid induced aggregation of the casein micelles. We will test the hypothesis that the compositional
differences in the serum phase caused by concentration by UF or DF do not affect the gelation behavior of the milk proteins or the stiffness and microstructure of the final gels.

Various techniques were used in this study to follow the changes in processing functionality of casein micelles. Chromatographic and electrophoretic techniques were employed to analyze the composition of the serum phase, before and during acidification. In addition, light scattering, rheology and confocal microscopy were employed to observe the difference in structure formation. This multi-technique approach should result in an in-depth investigation of the changes in the acidification behavior of the dispersed micelles after concentration (by UF and DF) and heat treatment.

The overall objective of this project is to extend the knowledge of the colloidal stability and behavior of casein micelles in the concentrated milk by membrane filtration. The major novelty of this research is the comprehensive focus not only on the effect of the concentration by UF, but also by DF, on the structure-function of the casein micelles, on the composition of the soluble fraction, which after heating seems to be responsible for imparting improved texture to the acid gels, and on the overall changes in the mechanism of casein micelle aggregation induced by acidification.
CHAPTER 2
LITERATURE REVIEW AND METHODOLOGY

2.1. Skim Milk Composition

Skim milk is a colloidal suspension of casein micelles in serum containing whey proteins, lactose, organic and inorganic acids, vitamins and minerals (Fox, 1992). The typical composition of skim milk is 9.2% total solids, 3.4% protein, 4.9% lactose, and 0.8% ash (Fox, 1992). Milk proteins are important structural components in foods and they are often used as functional ingredients due to their nutritional value and physicochemical properties (Morr, 1967; Kinsella et al., 1989). Milk proteins are usually distinguished in two groups, according to their solubility at pH 4.6: caseins, constituting about 80% of the total protein, insoluble at pH 4.6, and whey proteins, soluble at this pH.

2.1.1. Whey proteins

Most proteins present in milk serum are globular proteins, containing an appreciable proportion of α-helices and β-sheets, and present as monomers or in an oligomeric form (Walstra et al., 1999). Compared with the other major milk proteins (caseins), whey proteins are non-phosphorylated, more heat-sensitive, less calcium-sensitive, and can be involved in thiol-disulfide interchanges to form aggregates (McKenzie, 1971; Fox and Mulvihill, 1982). The main proteins in the serum are β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA) and immunoglobulins (Ig) (Walstra et al., 1984; Kinsella et al., 1989).
One of the major whey proteins, $\alpha$-la, is a compact globular metalloprotein with a molecular weight of 14 kDa. It has four intramolecular disulfide bonds with no free sulfhydryl groups. Its isoelectric point is 4.8. The serum protein present at the highest concentration in bovine milk, $\beta$-lg, is also a globular protein with an isoelectric point of 5.3. Its properties tend to dominate the properties of whey protein preparations, especially the reactions occurring upon the heat treatment, because of its high relative abundance in whey. $\beta$-lg has two intramolecular disulfide bonds with one free sulfhydryl group. During heating, the protein unfolds and forms aggregates via disulfide bridges with $\kappa$-caseins, $\alpha$-la or other $\beta$-lg molecules (see section 2.2). BSA and Ig are minor proteins present in the serum. Their contributions to the physico-chemical changes in milk during processing are considered relatively minor, compared to $\alpha$-la and $\beta$-lg.

2.1.2. Casein micelles

The caseins, namely $\alpha_{s1}$, $\alpha_{s2}$, $\beta$ and $\kappa$-casein, are a family of calcium binding phosphoproteins that comprise about 80% of the protein present in bovine milk with an overall concentration of approximately 25 g/L. The majority of the caseins exist in colloidal aggregates known as “casein micelles” (Fox, 2003). In bulk milk, the micelles are polydisperse in size, with a diameter ranging from 80 to 400 nm. Casein micelles represent 10% of the total volume fraction of milk, implying that they are at a close distance from each other (Tuinier and de Kruif, 2002). Casein micelles are highly hydrated spherical particles, holding around 4 g H$_2$O per g of protein (Walstra, 1979). The dry matter in the micelle is composed of about 94% protein; the remaining 6% is composed of minerals, mainly calcium and phosphate, but also magnesium, citrate and
others in lower amounts. The mineral fraction associated with the casein micelles is in form of calcium phosphate nanoclusters. These clusters are a few nm in size and usually referred to as colloidal calcium phosphate (CCP) (Holt et al., 2003). Casein micelles are negatively charged, with a ε-potential of about -20 mV (Fox, 2003).

Over the last 50 years, several models of casein micelle structure have been proposed and many reviews have been published on this topic over the years (Farrell, 1973; Garnier, 1973; Slattery, 1976; Horne, 1998; Smyth et al., 2004; Fox and Brodkorb, 2008). In the early models, the casein micelle was viewed as a uniform structure, containing subunits often referred to as “submicelles” (Morr, 1967). Each subunit was thought to have a similar composition, and held together by salt bridges. It was known that κ-casein molecules played a critical role in stabilizing the casein micelles, with the C-terminal trains of κ-casein providing steric stabilization to these colloidal particles as a polyelectrolyte brush (de Kruif and Zhulina, 1996). In the submicelles model, the κ-casein-rich submicelles surrounded κ-casein-poor submicelles (Walstra, 1990). This model has been supported by electron microscopy images, which show a raspberry-like structure (Buchheim and Welsch, 1973). However, recent papers have criticized models heavily weighted on electron microscopy images (Holt et al., 2003; Horne, 2006; Dalgleish, 2011). Although the submicellar model is still supported by many researchers, more recent models focus on the self-assembly properties of the casein micelles and the stabilizing role of the calcium phosphate. In 2003, Holt defined the casein micelle as a “relatively uniform protein matrix containing a disordered array of calcium phosphate nanoclusters” (Holt et al., 2003). This model, originating from the interpretation of small angle neutron and x-ray scattering data, stresses the importance of the colloidal calcium
phosphate clusters and their cementing role in maintaining the structural integrity of the casein micelles. The calcium phosphate clusters are associated to the phosphoserine groups of the caseins. $\kappa$-caseins are important in limiting the growth of this protein assembly as this protein has only one phosphorylated serine to bind calcium. This model has been criticized for disregarding the importance of hydrophobic interactions in determining the self-assembly of the casein proteins in the micelles (Horne, 2006). Indeed, the association of the micelles may be modeled after polymer science theories (Horne, 1998; 2002). The dual binding model of casein micelles attributes the association of casein proteins in the micelles to two main forces: hydrophobic and electrostatic interactions. Casein molecules polymerize and self-associate due to the presence of hydrophobic regions on their chains, limited by localized electrostatic repulsion. The phosphoserine amino acids are linked via colloidal calcium phosphate bridges and their presence leads to a decrease in the electrostatic repulsion between the proteins. In this model, $\kappa$-casein acts as chain terminator, because it has only one phosphoserine residue. This is why it is mostly present on the surface of the casein micelle. Its hydrophilic, glycosylated C-terminal region protrudes into solution providing steric and electrostatic stabilization to the protein assembly (Horne, 2006).

These proposed models interpret the existing experimental evidence within the context of the casein micelle behaviour under different environment conditions. However, the models still fall short at explaining the stability and integrity of the micelle under complex processing conditions. Recently, Dalgleish (2011) proposed that the internal structure of casein micelle is organized in clusters of casein proteins linked by colloidal calcium phosphate (CCP) via their phosphoserine centers. As with the previous
model, \( \kappa \)-casein plays a role in controlling the size of the micelles and stabilizes the surface of casein micelles. The interior of the assembly is not homogeneous, but contains more dense and less dense protein regions described as “water channels” (McMahon and McManus, 1998; Dalgleish, 2011). These less dense areas constitute pathways in the micelles through which some small molecules can diffuse. 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 calcium phosphate bridging (Dalgleish, 2011). As there is an insufficient number of \( \kappa \)-casein molecules to stabilize the entire surface of the micelles (Dalgleish, 2011), \( \beta \)-casein may play a role in stabilizing the protein rich regions in the inner core of the micelle. This model explains the behaviour of casein micelles, for example, in response to renneting, changes in pH, temperature, urea addition, and removal of calcium phosphate by chelating molecules.

2.2. Thermal Treatment of Milk

Thermal treatment of milk is an essential unit operation applied to increase the products’ safety and shelf-life. At times, the changes occurring during heating improve product properties such as texture and taste. Other times, the changes are less desirable (Walstra et al., 1984). Heat treatment at temperatures above 70 °C causes irreversible denaturation of whey proteins (Anema et al., 2003). The denatured whey proteins bind covalently to \( \kappa \)-casein on the surface of casein micelles or form non-sedimentable complexes in the serum phase (Guyomarc'h et al., 2003; Donato et al., 2007a). These heat-induced complexes are key to providing texture to fermented milk products such as yogurt (Robinson and Tamime, 1993; Lucey et al., 1997a). Given the potentially
important function of the heat-induced complexes for many dairy products, it is critical to fully understand their mechanism of complex formation.

2.2.1. Heat induced whey protein complexes

With heating, whey proteins undergo changes to their conformation. They expose amino acid residues buried within the native structure, resulting in an increase in the reactivity of such groups. β-lg is present as a non-covalently linked dimer (Walstra et al., 1999; Damodaran et al., 2008) and has two internal disulfide bonds and one free thiol group. During heating, the protein dissociates, unfolds and the thiol groups become exposed, initiating disulfide interchange reactions (Fox and Mulvihill, 1982; Anema and McKenna, 1996; Walstra et al., 1999). α-la, on the other hand, contains eight cysteine groups (which are all involved in internal disulfide bonds), responsible for the reversible conformational changes occurring with heating, in the presence of calcium, if the protein is present in isolation (Swaisgood, 1982; Relkin et al., 1992). However, when α-la is present in milk or with β-lg, it becomes part of the heat-induced complexes. Additionally, it has been shown that immunoglobulin and serum albumin are easily denatured with heating (Fox and Mulvihill, 1982; Swaisgood, 1982; Walstra et al., 1984).

In contrast to the whey proteins, caseins are relatively heat stable; however, the cysteine residues of κ-casein and αs2-casein react forming disulfide bridges with whey proteins (Fox and Mulvihill, 1982; Anema and McKenna, 1996).

It is well-established that heat treatment of milk above 70 °C causes the exposure of the reactive thiol groups in β-lg that then freely form disulfide links with other thiol groups of β-lg or other whey proteins like α-la and BSA (Mulvihill and Donovan, 1987;
Corredig and Dalgleish, 1996). In skim milk, heated at 90 °C for 10 min, the soluble heat induced complexes are composed of a ratio of approximately three whey proteins to one κ-casein, but this ratio depends on the relative concentrations of these proteins in milk (Guyomarc’h et al., 2003; Donato and Dalgleish, 2006). The other caseins, αs1 or β-caseins, are not found in the soluble complexes after heating milk at its natural pH (Donato and Dalgleish, 2006).

The heat-induced complexes of whey proteins are located partly on the casein micelles’ surface and partly in the serum as small dispersed particles often referred to as soluble complexes, as they can be separated from the casein micelles by centrifugation by using field of 20,000× g and above (Anema and Li, 2000; Guyomarc’h et al., 2003; Jean et al., 2006). Those soluble complexes are formed via hydrophobic interactions and thiol/disulphide exchanges and have sizes ranging from 30 to 100 nm (Anema and Li, 2003; Guyomarc’h et al., 2003; Donato and Dalgleish, 2006; Donato et al., 2007b). At the natural pH of milk, approximately 70% of the denatured whey protein is present in the serum and the remaining is associated with the micelles (Kethireddipalli et al., 2010). With a lower pH of milk before heating, the ratio of aggregates present in the serum to those associated with the micelles decreases (Dalgleish, 1990; Dalgleish et al., 1997; Guyomarc’h et al., 2003; del Angel and Dalgleish, 2006).

Furthermore, the heat-induced interactions between whey proteins and casein micelles are dependent on temperature, time, rate of heating and pH. For example, α-la interacts with casein more efficiently than β-lg at temperatures below 90 °C. Corredig and Dalgleish (1999) studied the effect of heating temperatures ranging from 70 to 90 °C and times from 0 to 80 min on the interactions of α-la and β-lg with casein micelles. The
composition of the complexes depended on the \(\alpha\)-la concentration in milk (Corredig and Dalgleish, 1999). \(\beta\)-lg binds to a higher extent with casein micelles during short heating times, compared to \(\alpha\)-la when skim milk is subjected to heating at ultra-high temperatures (Oldfield et al., 1998a). This behaviour is attributed to the difference in heat stability of the proteins. The same authors also pointed to a temperature dependence of the mechanisms involved in the interactions, whereby at temperatures below 80 °C both hydrophobic interactions and disulfide bonds are as important, while at higher temperature, disulfide interchanges dominate (Oldfield et al., 1998a). Moreover, higher levels of \(\alpha\)-la are associated with casein micelles at lower temperatures (below 90 °C) (Oldfield et al., 1998b). On the other hand, in the temperature range studied by these authors (70–130 °C), the maximum level of \(\beta\)-lg (~55% of total \(\beta\)-lg) associated with casein micelles did not differ with temperature (Oldfield et al., 1998a; Oldfield et al., 1998b). Additionally, Cooper and others (2010) suggested that during heating of milk at various heating regimes (74–85 °C) the extent of soluble complex formation can be adjusted and that the relative proportion of casein to whey protein in the heat-induced aggregates changes with the extent of heating (Cooper et al., 2010).

### 2.2.2. Effect of concentration

The rate and degree of the heat-induced complexes formation do not only depend on the time and temperature of heating and the initial pH of the milk, but also on the concentration of milk proteins (Singh, 1995; Lucey et al., 1998). Most of studies have investigated the effect of heat on the denaturation of whey proteins in milk at their natural concentration in milk, and only a few studies are available on the influence of milk
protein concentration. It has been reported a minimum in whey protein denaturation seems to occur when the total solids of milk reach 20% (Guy et al., 1967). Other researchers also suggested that the denaturation of whey proteins (as a percentage of the total) is markedly reduced when heated at 75 or 80 °C at a higher concentration of solids (McKenna and O'Sullivan, 1971). It is important to note, at this point, that with increasing solids, the ionic strength and lactose concentration also increase (Anema, 2009a).

It is well-known that the increased concentration of soluble complexes in milk has a critical effect on the acid gelation of casein micelles, such as a greater gel stiffness and an increase in pH of gelation (Lucey et al., 1998; Donato et al., 2007a). However, the details related to the formation of these complexes in milk concentrated by membrane filtration and their consequences on the processing functionality of milk are not well defined. No data is available on the effect of both concentration and heat treatment on the formation of heat-induced complexes and their impact on acid gelation of casein micelles.

2.3. Membrane Filtration

For decades, membrane processing has successfully shown great potential in the dairy industry as a gentle technology to separate bacteria and spores from milk or to concentrate milk proteins (Spriggs et al., 1976). Unlike other industrial operations, such as evaporation and drying, membrane filtration selectively retains different components of the feed depending on the properties of the membrane without modification of the serum composition (Cheryan, 1998). The portion retained and concentrated by the membrane is called the retentate, while the fraction transmitted across the membrane is
called the permeate. A hydrostatic pressure gradient (transmembrane pressure, TMP) between the retentate and permeate is the driving force for the separation. Different types of membranes are used in food processing. Membranes can also be classified as hydrophilic and hydrophobic, and their characteristics will affect the fluxes and selectivity of the membrane. Surface hydrophobicity contributes to interactions of components with the membrane, with consequences related to pore clogging, concentration polarization or irreversible fouling (Maximous et al., 2009). Charged membranes can interact with oppositely charged molecules, influencing the selectivity and rejection of some components, ultimately affecting the permeate flux (Lewis, 1996). The relationship between hydrophobicity and charge versus membrane fouling and selectivity is also affected by ionic strength and pH (Salgın et al., 2006). Thus, the relations between filtration performance and membrane physical properties are complex, with the properties of the components present in the feed playing a key role. The membrane used in this research was hydrophilic and negatively charged (polyethersulfone, PES).

Membrane processes are distinguished as dead-end or cross flow. In dead-end filtration, the feed is passed perpendicularly to the membrane, and while the permeate passes through the membrane the retentate is being trapped by the membrane. This process quickly results in extensive fouling of the membrane. Membrane fouling is a serious concern as it reduces the flux and the selectivity of the membrane over time. Fouling may occur because of protein-protein interactions, protein-membrane interactions, or ion-mediated electrostatic interactions between the charged membrane and proteins (Tong et al., 1989; Jimenez-Lopez et al., 2008). On the other hand, in cross
flow or tangential membrane processes the feed flow is tangential to the surface of the membrane, and the feed flow prevents the solids from residing on the membrane (Lewis, 1996). In this research, a tangential feed flow membrane was employed, with a unique flow channels design to reduce gel layer buildup and prevent fouling. The system was designed to create fluid dynamics producing equal flow rates across the membrane surface, thus optimizing membrane use and expanding the performance limits of traditional cross tangential flow filtration (Cheryan, 1998).

The membrane processes most frequently used in the dairy industry are microfiltration, ultrafiltration, nanofiltration and reverse osmosis, and they differ according to the size and selectivity of the membrane. By microfiltration, membranes of large pore size ranging from 0.1 to 10 µm are able to retain bacteria, fat and some protein particles from the remaining of the milk. This process is often used to obtain extended shelf life in milk. When smaller pore sizes below 0.8 µm are employed, the ratio of casein to whey proteins can be modified, as a portion of the whey protein can be recovered in the permeate. This certainly depends on the selective permeability of the membrane as well as the processing conditions. Ultrafiltration (UF) is a pressure-driven membrane process in which membranes having molecular weight cut-off ranging from 2 to 100 kDa are used to separate macromolecules such as proteins, while small molecules (e.g. lactose and minerals) diffuse through the membrane into the permeate (Waungana et al., 1999; Anema et al., 2003; Karlsson et al., 2005; 2007). In UF, because of the small pore size of the membrane, the casein and whey proteins do not pass through the membrane and the ratio of them can be maintained the same as in the original milk, while increasing the ratio of protein to total solids. Recently, UF has become an essential unit
operation in dairy technology as an efficient tool, because it can simultaneously concentrate milk proteins and desalt/lactose-reduce protein retentates (Spriggs et al., 1976; Spangler et al., 1991). To separate particles with molecular weights ranging from 0.2 to 2 kDa, smaller pore sizes have to be used. Nanofiltration is defined as the rejection of constituents through the membrane being driven also by the diffusion characteristics and charge of the ions (Rosenberg, 1995). In reverse osmosis processes, membranes of molecular weight cutoff of approximately 0.1 kDa and very high pressures are used. Reverse osmosis allows the concentration of milk or whey by the removal of water and some minerals.

To further increase the retentate concentration and improve fluxes and permeation, diafiltration (DF) is often used. DF, the process of adding water to the concentrated retentate, reducing its viscosity, causes further permeation of small molecules, enhancing the level of protein concentration in the retentate. The present research focused on the effect of UF, with and without DF.

### 2.4. Production of Milk Protein Concentrate by Ultrafiltration

Over the past decade, milk protein concentrates (MPC) have been introduced as a new category of functional ingredients often employed in dairy formulations. MPC ingredients are produced by membrane filtration and subsequent evaporation and spray drying (Mistry and Hassan, 1991). MPC is usually identified by the percentage of protein content (e.g., MPC70). MPC has been employed in the manufacture of yogurts (Guzmán-González et al., 1999), ice cream (Alvarez et al., 2005), and emulsions (Hemar et al., 2005). Their principal application is to increase cheese-making efficiency (Novak, 1996;
Rehman et al., 2003; Žbikowska and Szerszunowicz, 2003). However, there are significant technological challenges in using MPC in traditional processes, as MPC dispersions cannot be easily dissolved by stirring at 20 °C. Their decreased solubility seems to be due to the formation of disulphide bonds from β-lg aggregates and casein complexes during processing and storage conditions (Kameswaran and Smith, 1999; de Castro-Morel, 2002; de Castro-Morel and Harper, 2003; Anema et al., 2006; Havea, 2006). The decrease in solubility is proportional to time and conditions of storage (Havea, 2006).

MPC addition to milk seems to affect the gelation behaviour of casein micelles. In a study of rennet-induced gels obtained with MPC, it was demonstrated that gels made with MPC56 were stronger than those prepared with MPC85, when tested at the same protein concentration (Kuo and Harper, 2003a; 2003b). This work clearly suggested that the functionality of the micelles is affected by processing conditions. Other researchers have studied the rheology of rennet gels made from reconstituted, standardized or ultrafiltered milk (Caron et al., 1997; Pomprasirt et al., 1998; Waungana et al., 1999). It is not yet clear how the processing functionality of casein micelles is affected by concentration, evaporation and drying.

This thesis focused on the effect of UF and DF concentration on the acid induced gelation of milk. Fresh concentrates were employed, as evaporation and drying may also affect the functionality of the micelles. The effect of concentration by UF on the acid gelation of retentates has been previously studied. It is known that concentration by UF dramatically decreases gelation time and causes a faster firming rate than non-concentrated milk (Biliaderis et al., 1992; Lucey et al., 1997b). Additionally, a recent
publication (Zander et al., 2012) showed differences in the acidification of water solutions of MPC 75, depending on the particle size distributions of the reconstituted MPC, the protein and GDL concentrations. The mechanism of acidified gels made from MPC powder seems different from that of fresh concentrates prepared by UF; however more research is needed in this area.

The application of UF for milk fortification has been widely studied (Chapman et al., 1974; Biliaderis et al., 1992). Milk concentrates by UF has been shown to produce smooth and creamy yogurts (Chapman et al., 1974). Ultrafiltered milk has been shown to be advantageous for cheese production, since the higher protein level results in higher buffering capacity and ionic strengths, which seem to favour greater cheese yields (Marshall, 1986). However, very little is understood about the effect of membrane concentration (UF and DF) on the physico-chemical properties of the casein micelles and their changes in processing functionality. Recent studies have suggested that the renneting properties of casein micelles are modified during processing of milk protein concentrates by UF, especially at high volume reductions (Ferrer et al., 2011). Moreover, during DF, when water is added and the concentration of total solids in skim milk is increased, the distance between casein micelles is closer and the ionic balance is affected, causing changes in the equilibrium of forces affecting interparticle interactions. The increase in volume fraction of casein micelles would force them to interact more frequently with each other (DeKruif, 1998). As DF becomes increasingly common in manufacturing of dairy ingredients and cheese, it is important to understand the effect of DF on the processing properties of the retentates.
2.5. Skim Milk Acidification

2.5.1 Mechanisms of acidification

Casein micelles are very stable in their native state, e.g. they can be heated to high temperatures and pressure-treated to 100 MPa without significant structural changes. However, they can be readily destabilized by specific proteolytic enzymes (i.e. rennet) or by acidification for the manufacture of milk products, such as yogurts, fresh cheese, and cottage cheese (Lucey, 2002; Donato et al., 2007a; Sandra et al., 2007). During acidification, as the pH decreases from the natural pH of 6.7, the surface charges of the casein micelle are titrated and the calcium phosphate nanoclusters associated with the phosphoserine amino acids in the interior of the micelles are progressively diffused into the serum till the complete release at pH 5.0 (Le Graët and Gaucheron, 1999). The change in pH causes the collapse of the κ-casein hairy layer, so that steric and electrostatic stabilization are diminished and the aggregation of the micelles can occur as the proteins approach their isoelectric point (pH~4.6) (Dalgleish et al., 2004; Donato et al., 2007a). During acidification, even though the hydrodynamic diameter may decrease due to the collapse of the hairy layer of κ-casein on the surface or some shrinkage of the micelle, the casein particles maintain their individuality in the gel, as the micelles are still covered by the glycosylated portion of κ-casein (DeKruif, 1998).

The mechanisms of gelation behaviour in milk have been studied by using diffusing wave spectroscopy (DWS) and rheology (Alexander and Dalgleish, 2005; Alexander et al., 2006). These studies measured the details of the aggregation of the casein micelles under undiluted conditions. In unheated milk, the aggregation occurs at pH <5.0, very close to the isoelectric point of caseins. There is a rapid change in milk turbidity in
unheated milk at the same pH as the rapid increase in the elastic modulus by rheology. The consistency of gelation pH observed by both methods suggests that intramicellar interactions are the major factor affecting the formation of the acid gel. The weak nature of the linkages formed in unheated milk gels is probably due to the collapsed hairy layer still present among casein micelles, separating these protein particles from each other (Lucey et al., 1997b; Lucey and Singh, 1998). In heated milk, on the other hand, it appears that the heat-induced complexes play a critical role in gel formation (Lucey et al., 1997b; Lucey and Singh, 1998; Lucey, 2002; Donato et al., 2007a). The gelation point is at about pH 5.3. In this case the network is much stiffer than in the case of unheated milk (Lucey and Singh, 1998; Lucey et al., 1998; Lucey et al., 1999; Alexander and Dalgleish, 2005; Donato et al., 2007a). In heated milk, the gelation pH measured by DWS and rheology do not fully overlap, and the discrepancy is due by the presence of heat-induced whey protein complexes, which destabilize at a much earlier pH than the casein micelles (Guyomarc'h et al., 2003). In the case of heated milk, after the gelation point the value of tan δ shows a decrease, and later at pH > 4.8 a slight increase before decreasing again. The peak in tan δ observed in the heated milk samples has been extensively discussed in the literature (Lucey, 2002; Horne, 2003; Ozcan-Yilsay et al., 2007), and has been attributed to the presence of whey protein aggregates rearranging to fill the voids between micelles, and to residual colloidal calcium phosphate remaining in the casein micelles, not yet fully solubilized. Solubilization continues to occur between pH 5.3 and 4.8, causing an increase in the electrostatic repulsion of the protein, before their neutralization around the isoelectric point (Lucey, 2002; Horne, 2003; Ozcan-Yilsay et al., 2007).
2.5.2 Calcium equilibrium during acidification

During acidification, the composition of the serum phase changes. Several researchers have described the behavior of minerals in milk as a function of different processing conditions (Brule et al., 1974; Covacevich and Kosikowski, 1977; Mehaia and Cheryan, 1983; Fischbach-Greene and Potter, 1986; Bastian et al., 1991). With concentration, the total balance of soluble and colloidal calcium changes and this could result in a considerable proportion of soluble calcium and phosphate being transferred between soluble and colloidal states (Hardy et al., 1984). However, to distinguish between colloidal and insoluble calcium is a challenge. It has been previously demonstrated that although the concentration of calcium in the colloidal phase increases with protein concentration, this increase is not proportional to the concentration factor of the milk (Le Graët and Gaucheron, 1999). Much less is known about the effect of concentration by UF and DF on the calcium distribution between the colloidal and soluble phase, and no information is available on the release of colloidal calcium phosphate with acidification of UF and DF milk.

During heat treatment, the equilibrium between the soluble calcium and calcium bound to casein micelles is also affected by heating and is markedly dependent on temperature and concentration (Holt et al., 1989). It was demonstrated that heating causes a decrease of calcium in the soluble phase, and it was suggested that with heating temperature there is an increasingly rapid transfer of the soluble calcium to the colloidal phase with a concomitant decrease in milk pH (Anema, 2009a). This thesis will test if heating UF and DF concentrated milk causes changes in the buffering capacity of milk,
and if these treatments will affect the release of calcium in the serum phase during acidification.

2.5.3. Effect of concentration on acid gelation

The mechanism of acid-induced gelation behaviour in skim milk has been well studied (Alexander and Dalgleish, 2005; Alexander et al., 2006; Donato et al., 2007a). However, acid coagulation of casein micelles in milk concentrated by UF and DF is less understood. When milk serum is removed, and the concentration of total solids increases, the distance between casein micelles is closer, affecting interparticle interactions. The increase in volume fraction of casein micelles would force them to interact more frequently with each other (DeKruif, 1998). It is possible to hypothesize that in a concentrated system the rate of aggregation of casein micelles would increase (DeKruif, 1998). However, this does not seem to be the case in rennet-induced aggregation, as the gelation time occurs only when almost all of the \(\kappa\)-casein has been hydrolyzed, regardless of the concentration (Sandra et al., 2011). Nevertheless, there are substantial changes to the stiffness of the gel with concentration. The acid-induced aggregation of fresh milk concentrated by UF and DF has never been systematically studied.
2.6. Methodologies

2.6.1. Protein analysis

2.6.1.1. Dumas method

The principles of the Dumas combustion method were introduced by Jean-Baptiste Dumas (1983). Samples are combusted at high temperatures (700–1000 °C) with a flow of pure oxygen. All carbon in the sample is converted to carbon dioxide during flash combustion. Nitrogen-containing components produced during combustion include N₂ and nitrogen oxides, which are reduced to nitrogen in a copper reduction column at a high temperature (600 °C). The total nitrogen (including inorganic fraction) released is carried by pure helium and quantified by gas chromatography using a thermal conductivity detector. EDTA (ethylenediamine tetra acetate) is used as the calibration standard. In the present research, total protein was determined using a Leco FP-528 (Leco Corp., St. Joseph, MI, USA) using a conversion factor of 6.38.

2.6.1.2. Protein concentration assay

A colorimetric protein assay (Bio-Rad, Mississauga, ON, Canada) was employed to determine the soluble protein concentration. The reaction is similar to that of the well-documented Lowry method (Lowry et al., 1951; Peterson, 1979), widely used in the protein biochemistry because of its simplicity and sensitivity. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin–Ciocalteau phenol reagent (phosphomolybdic-phosphotungstic acid). As with the Lowry assay, there are two steps which lead to color development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated
protein. Color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins cause a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

2.6.2. Chromatographic techniques

Chromatography techniques have been applied to separate and isolate compounds based on the partition or distribution of molecules between mobile and stationary phases. The mobile phase is usually a liquid or gas, while the stationary phase could be a liquid or solid. Separation is based on partition coefficients, defined as the ratio of the solute in the stationary phase to the solute in the mobile phase. By changing this ratio, it is possible to achieve separation. Regardless of the kind of separation used, chromatography can be achieved in different assemblies (e.g. paper chromatography, thin-layer chromatography and high performance liquid chromatography) (Ismail and Nielsen, 2010). Column liquid chromatography is employed in the present research. In column chromatography, the stationary phase is enclosed in a column and the mobile phase is passed through the column in an isocratic or gradient elution under a defined pressure and temperature. Gradient elutions are used to systematically desorb molecules interacting with the stationary phase (Rounds and Nielsen, 2003).

2.6.2.1. Size exclusion chromatography (SEC)
SEC is used to separate molecules based on their size. Usually, its application is to determine the size distribution of molecule, to isolate and fractionate the compounds of interest. Depending on the size of the molecules, there is a different diffusion or partition of the species in the gel matrix. Molecules bigger than the matrix pores do not diffuse through the pores, thus eluting early in the chromatographic run. On the other hand, small solutes travel within the pores, thus they elute later compared to larger molecules (Rounds and Nielsen, 2003).

The stationary phase consists of a column packing material which contains pores varying in size. Column packing materials can be divided in two groups: semirigid, hydrophobic media like polystyrene derivates, or soft and hydrophilic gels (e.g. Sephadex). The present work employed S-500 Sephacryl high-resolution gel, a hydrophilic, rigid allyl dextran/bis acrylamide matrix used to separate polysaccharides and other macromolecules with extended structures, with a fractionation range of 40 to 20,000 kDa. This set up has been successfully used to separate native and aggregated serum proteins in milk (Donato and Dalgleish, 2006; Donato et al., 2007b), using bis-tris (1,3-bis(tris(hydroxymethyl)methylamino) propane. This buffer has a wide buffering range and does not cause disruption of the aggregates under study (Donato and Dalgleish, 2006; Donato et al., 2007b). In this research, the fractions eluted by SEC were also collected and subsequently analyzed by SDS-PAGE.

2.6.2.2. Reverse phase – high performance liquid chromatography (RP-HPLC)

RP-HPLC results from the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. In RP, the less polar of the two liquids
is held stationary on the inert support, while the more polar solvent is used to elute the sample components. Partitioning is based on the reversible hydrophobic interaction of the molecules to the immobilized hydrophobic stationary phase. Thus, the more hydrophobic (less polar) molecules are eluted later because they interact to a higher extent with the less polar stationary phase (Rounds and Nielsen, 2003). In this research, the polar mobile phases are a mixture of acetonitrile-water with 1% trifluoroacetic acid (TFA) as an ion suppression and/or ion pairing agent. 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 nonpolarity of the mobile phase decreases retention and results in faster elution. Gradient elution is often practiced to improve resolution (Rounds and Nielsen, 2003). The gradient elution is designed to improve the separation of the different molecules in the sample, depending on their hydrophobicity. In this research, RP-HPLC was used to analyze the presence of undenatured whey proteins in the supernatant of different milk samples.

2.6.2.3. Ion chromatography

Ion chromatography (IC) is often used to measure amount of anions and cations, such as transition metals, inorganic and organic acids, amines, phenols, and surfactants, based on their conductivity or fluorescence (Weiss, 1995). Determination of insoluble and soluble calcium was critical in this work, and a new method was developed using non-suppressed IC (Rahimi-Yazdi et al., 2010). This method is based on the changes in conductance caused by the elution of ions. This method allows for large numbers of
samples to be measured at once while maintaining high accuracy and reproducibility. In the present work, a cation column (packed with silica gel with carboxyl groups) was employed.

To apply IC to detect total and soluble calcium in milk, sample preparations need to be carried out to change all the calcium forms into free ionized calcium because the measurements are based on Ca\(^{2+}\) conductance. To decrease the amount of organic material, the samples are often incinerated or digested (Moreno-Torres et al., 2000). In this research, to decrease the interference of the di- and tri-carboxylic acids present in milk (mainly citrate), the samples were diluted and directly acidified with hydrochloric acid to precipitate the proteins and dissolve the micellar calcium phosphate during sample preparation (Rahimi-Yazdi et al., 2010). Ion exchange chromatography can be described as a type of adsorption chromatography in which interactions between the solutes and the stationary phase are primarily electrostatic in nature and depend on the charged species present in the sample (Rounds and Nielsen, 2003). The ion exchange matrix contains charged functional groups. The stationary phase contains fixed functional groups that are charged either negatively (cation exchangers) or positively (anion exchangers). There are two ways to elute the bound solutes, either by changing the mobile-phase (e.g., changing the pH), or increasing the ionic strength (e.g., use NaCl) (Ismail and Nielson, 2010).

After sample preparation, a dialysis unit was employed (Metrohm Canada Ltd, Mississauga, ON, Canada) to separate high molecular weight organic material (e.g., protein) from the sample while the ions were carried by the acceptor solution of nitric acid. The samples for IC were pumped by an autosampler to one side of a dialysis membrane. At the same time, an acceptor solution (2 mmol L\(^{-1}\) 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 mmol L\(^{-1}\) nitric acid and 1 mmol L\(^{-1}\) pyridine-2, 6-dicarboxylic acid in isocratic elution) allowed for the separation of cations such as sodium, potassium, calcium and magnesium, etc. 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. Buffering capacity

The buffering capacity of milk is an important physico-chemical characteristic that corresponds to the ability of the milk to be acidified or alkalinized. The buffering capacity in milk results from the sum of the buffering capacities of each individual acid–base group including small constituents (inorganic phosphate, citrate, and organic acids) and milk proteins (caseins and whey proteins). Natural and induced variations in the composition of milk will affect this physico-chemical parameter. The buffering capacity observed for milk is related to its high colloidal calcium phosphate content and to the presence of highly phosphorylated caseins (Salaün et al., 2005). Hence, this parameter can be taken as an indication of differences in colloidal calcium phosphate and protein concentrations.

To determine the buffering capacity, base or acid solutions are added in incremental steps and the pH is measured. The titration protocol usually starts with an acidification and then alkalinisation curve (Lucey and Fox, 1993; Salaün et al., 2005). The buffering
capacity can be determined graphically by observing the amount of acid or base used as a function of pH (van Slyke, 1922). The \( \frac{dB}{dpH} \) ratio can be defined as

\[
\frac{dB}{dpH} = \frac{\text{(Volume of acid or base added)} \times \text{(Normality of acid or base)}}{\text{(Volume of sample)} \times \Delta pH}
\]

Eq. 2.1

This \( dB/dpH \) ratio can be plotted graphically against pH and allows the determination of different characteristics. In the current research, to investigate and compare the buffering capacity of each individual acid–base group in the milk induced by concentration and heating, acid-base titrations were performed as previously described by Lucey et al. (1993), with minor modifications. Buffering indices were calculated according to Salaün et al. (2005), and the buffering curves were prepared by plotting the calculated indices as a function of pH.

2.6.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique to separate proteins according to their electrophoretic mobility and identify them based on molecular weight or size. The term electrophoresis refers to the migration of charged molecules through an electric field. SDS is often used in this analysis, as this anionic detergent binds to protein, disrupts hydrophobic interactions causing secondary and non–disulfide–linked tertiary structure changes, and provides the protein with an overall negative charge. The protein then migrates to the positive electrode according to its size (Smith, 2010). Usually, a reducing agent, namely mercaptoethanol or dithiothreitol, combined with heating is also employed to disrupt covalent S–S bonds between/within proteins. Under these conditions, the protein is
reduced to its primary structure. A tracking dye such as bromophenol blue is added to the sample to follow mobility through the gel during the run (Smith, 2010).

The gel matrix is prepared with polyacrylamide, a polymer of acrylamide with N,N’-methylene–bis–acrylamide as a cross-linking agent, which provides the medium that separates the proteins based on size. The pore size of the gel can be adjusted by modifying the concentration of acrylamide. In addition, ammonium persulfate and TEMED are added to the polymeric solution, as initiators and catalyzers of the polymerization reaction. The electrophoretic gel is composed of resolving and stacking gel portions. The stacking gel with low acrylamide concentration is used to form the wells into which the protein is loaded at the top. The high acrylamide concentration remaining in the resolving gel at the bottom makes the molecules slowly travel in the gel, where the polypeptides migrate to different extents depending on their size.

Once samples are loaded on the gel, an electric field is applied across the gel causing the negatively-charged protein to migrate towards the anode. After the electrophoretic run, the bands of individual proteins identified by standards on the gels are visualized by using a protein stain, usually Coomassie blue. Once stained, different proteins appear in the gel as identical bands with different thicknesses and densities. By means of a suitable scanner and analytical software, the protein bands can be quantified.

**2.6.5. Confocal scanning laser microscopy (CSLM)**

CSLM is a technique which uses optical sectioning to obtain high-resolution images. Individual images are acquired point-by-point and reconstructed with a computer, and as the sample can be observed at different depths, it is possible to obtain a
three-dimensional reconstruction of the specimen. Image generation comprises three steps: i) the specimen is scanned with a focused laser beam deflected in the X and Y directions by two galvanometric scanners, ii) the photons emitted are detected pixel-by-pixel with a photomultiplier tube, and iii) the information from the photomultiplier is digitized and presented as a two dimensional image. In this research, CSLM (Leica TCS SP2, Wetzlar, Germany) was employed to observe the structure of the gel network formed by acidification.

CSLM enables samples to be observed with minimal preparation procedures because of its unique optical sectioning capabilities and high spatial resolution (Brooker, 1995), and it is very suitable for observing the overall microstructure of milk gels (Lucey and Singh, 1998).

2.6.6. Diffusing wave spectroscopy (DWS)

Light scattering has been used for many years in the study of colloidal systems, due to its well-understood theory. However, its utilization in more industrially realistic (for example, turbidity and gelling) samples is limited because of the need to dilute the sample to reach the single scattering regime. In recent years, DWS has been accepted as a method for studying concentrated suspensions, such as milk, aggregation and phase separation (Alexander et al., 2002; Alexander and Dalgleish, 2004; 2007). DWS is a light scattering technique that permits the investigation of the interparticle interactions in situ, avoiding the necessity of extensive dilution, which may alter the “true” behaviour of the colloidal suspensions. The basic principle of DWS is that of dynamic light scattering, which measures the intensity fluctuations of the transmitted scattered light caused by the
Brownian motion of colloidal particles. The light propagation through the sample is assumed to occur in a diffusive fashion. DWS relies on many scattering events happening when the light passes through a colloidal dispersion and the scattered light is collected after it has traversed the whole length \( L \) of the scattering cell, as shown in Figure 2.1.

In the current research, transmission DWS was used with a 532 nm laser. With this instrument geometry, the detector is placed after the sample holder and only light that goes through the sample is detected. The 350 mW VERDI laser (Coherent Inc., Santa Clara, Canada) is directed to a sample contained in a sample holder of path length \( L \) (5 mm) and the scattered light is collected and fed to the correlator via two matched photomultipliers. Data is processed with a computer program designed specifically for this setup (Mediavention, Guelph).

In DWS, the fluctuating intensity of light due to particle motion generates an autocorrelation function, which is described by:

\[
g_{(1)}(t) \approx \frac{\langle L^2 + \frac{4}{3} \rangle \sqrt{6t}}{(1 + \frac{8t}{3}) \sinh \left( \frac{L}{1^2 t} \right) + \frac{4}{3} \sqrt{6t} \cosh \left( \frac{L}{1^2 t} \right)}
\]

Eq. 2.2

where \( \tau = (Dk_o^2)^{-1} \), \( D \) is the particle diffusion coefficient, \( k_o = 2\pi n/\lambda \) is the wave vector of the light, and \( n \) is the refractive index of the medium. \( L \) is the thickness of the sample (i.e. 5 mm in the present research and \( \tau \) the decay time being measured (Weitz et al., 1993)).
**Figure 2.1** A schematic of a typical transmission (forward-scattering) DWS set-up. Original drawing is taken from the paper by Alexander and Dalgleish, 2006.
The parameter $l^*$, needed to resolve equation 2.2 is also 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:

$$\frac{I}{I_0} = \frac{\left(\frac{5l^*}{3L}\right)}{\left(1 + \frac{4l^*}{3L}\right)}$$

Eq. 2.3

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.

DWS can be used to estimate $l^*$, the photon transport mean free path (the length scale over which the direction of the scattered light has been completely randomized), which can give information on the spatial interparticle correlations and interparticle forces (Weitz et al., 1993). The changes in the physical properties of the sample as gelation progresses can be determined by analyzing the change in $l^*$; in addition, the comparison of $l^*$ values among samples may be possible to detect differences in the structure of the final gels (Wyss et al., 2001). In concentrated and optically dense suspensions, the spatial positions of the particles and their correlation may become significant. These correlations in position affect the angular distribution of the scattered light, and hence the turbidity and $l^*$. In general, $l^*$ is a function of the scattering form factor, $F(q)$, and the structure factor, $S(q)$ (Weitz et al., 1993). The $l^*$ parameter relates to the scattering properties of a particle via $F(q)$ and the interparticle correlations via $S(q)$. Any change in either of these two functions will produce a change in the measured $l^*$.

$$l^* \propto (\int F(q) \times S(q) \times q^3 dq)^{-1}$$

Eq. 2.4
Moreover, \( \hat{I} \) is necessary for the calculation of diffusion coefficients, and hence apparent particle radius through the Stokes–Einstein equation (Eq. 2.5), from the measured correlation functions of the transmitted light.

\[
d(H) = \frac{kT}{3\pi\eta D}
\]

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 changes in radius could be considered as an indication of changes in sizes of the particles, which may be related to the collapse of the \( \kappa \)-casein layer during acidification (Vasbinder et al., 2003; Dalgleish et al., 2004). But it can be taken only as an index of aggregation once gelation has started, because the approximation of non-interacting particles is no longer valid (Alexander et al., 2002). By transmission DWS it is possible to measure the changes of casein dissusivity and turbidity in undiluted milk samples (Alexander et al., 2006; Alexander and Dalgleish, 2007).

2.6.7. Rheology

Rheology is the study of deformation and flow of matter resulting from the application of force (Goodwin and Hughes, 2000). 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 (Goodwin and Hughes, 2000).

For the study of sol gel transitions and soft materials, small deformation rheology is generally employed. With this methodology it is possible to investigate viscoelastic
parameters using dynamic mechanical analysis. Controlled-stress or controlled-strain rheometers are designed to apply the stress or strain in a sinusoidal mode, and the response to the varying stress or strain can be measured over time. Stress \( (\sigma) \) is a measure of force divided by unit of area; the strain is a dimensionless quantity representing the deformation of material as a result of the applied stress. In the particular case of milk gelation, time sweep tests are the most common way to follow the sol gel transition, and eventually the formation of gel structure (Daubert and Foegeding, 2003).

In a typical time sweep test for milk gel formation using couette-type geometry, a milk sample is positioned in the interior of a cylinder. A cylindrical fixture shape, usually called a bob is suspended from a torque \( (M) \) measuring device, and is immersed in the liquid in the interior of the slightly larger cylinder. As the force applied acts in parallel to the sample surface a shear stress is experienced and the resulting deformation is a shear strain \( (\gamma) \) (Daubert and Foegeding, 2003). Being an oscillatory measurement, the bob is moved back and forth in a sinusoidally varying function. As a result both stress and strain vary 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

\[
\delta = \omega \Delta t \tag{2.6}
\]

where \( \omega \) is the frequency of oscillation. Additionally, the complex modulus is calculated from both the stress \( (\sigma_A) \) and strain \( (\gamma_A) \) amplitudes with the equation (Kissa, 1999):

\[
G^* = \frac{\sigma_A}{\gamma_A} \tag{2.7}
\]

With the complex modulus and the phase angle it is possible to characterize the viscoelastic behavior of the sample, by obtaining the elastic and the viscous modulus.
The elastic modulus $G'$, describes the elastic (solid-like) character of the sample and also describes the ability of the sample to store energy during the shear process, and is defined as

$$G' = G^* \cos \delta.$$  

Eq. 2.8

The viscous modulus describes the viscous (liquid like) character of the sample and is a measure of the energy dissipation during shear, and is defined as

$$G'' = G^* \sin \delta.$$  

Eq. 2.9

During a sol-gel transition, by measuring the phase angle it is possible to follow the changes in the viscoelastic properties as the sample changes from a liquid to a gel. At the beginning of the reaction, $G''$ is higher than $G'$ and the phase angle is high ($\delta \sim 90^\circ$) describing the viscous character of milk. As gel formation begins, the phase angle starts to decrease, and $G'$ starts to overcome $G''$, showing a more elastic character of the gel (Fox et al., 2000). Based on the changes of these parameters, the determination of the onset of gelation is possible. There has been no consensus in the literature on the definition of gelation point. While some authors define the gelation point as the time when $G'$ becomes higher than $G''$ or $\tan \delta = 1$ (at a given frequency) others prefer to use the time when $G'$ has increased to a value higher than the noise level (and this is arbitrarily set) (Fox et al., 2000; Lucey et al., 2000). For this research the gelation point was defined as the time when $\delta = 45$ or $\tan \delta = 1$.

In our experiment, small oscillatory rheological measurements (controlled stress) were carried out using an Advanced Rheometer AR 1000 (TA Instrument Ltd., New Castle, USA) with a constant strain of 0.01 and a frequency of 1 Hz. The temperature of the cup was controlled at 40 °C by circulating water from a water bath (Julabo
Labortechnik, GbmH, Germany). Data was collected and analyzed with Rheology Advance Data Analysis software (version 5.0.38) (TA Instrument Ltd., New Castle, USA). The main parameters obtained were $G'$, $G''$ and $\tan \delta$, which is related to the relaxation of bonds in the gel during deformation, and used to define of the gelation point at a specified pH during acidification.
CHAPTER 3
INFLUENCE OF HEATING TREATMENT AND MEMBRANE CONCENTRATION ON THE FORMATION OF SOLUBLE AGGREGATES

3.1. Abstract

This study investigated the effect of thermal denaturation on concentrated milk by ultrafiltration (UF) with or without diafiltration (DF) at various time-temperature combinations. The non-sedimentable protein fractions present in milk after heating were analyzed using size exclusion chromatography and their composition determined. The extent of whey protein denaturation and the composition of the soluble complexes depended not only on heating temperature and time, but also on the protein concentration. There was a greater extent of protein aggregation in concentrated milk compared to 1× milk, and there was a significant difference in the size and composition of the aggregates present in the non-sedimentable fraction depending on if milk was concentrated by UF or DF. DF milk contained a higher concentration of non sedimentable protein compared to UF milk. In all samples, κ–cas and β–lg were the main proteins present in the soluble aggregates, and higher temperatures and longer heating times resulted in greater extent of α–la denaturation. This work clearly identified differences in the extent of aggregation between milk concentrated by UF or UF and DF, and unconcentrated milk, with important consequences on the processing functionality of caseins.
3.2. Introduction

Heating is a critical step in the processing of many dairy products. Heat treatment of milk at temperatures above 70 °C causes changes in the structural and functional properties of casein micelles and the irreversible denaturation of whey proteins (α-lactalbumin and β-lactoglobulin) (Smits and van Brouwershaven, 1980; Anema et al., 2003). The denatured whey proteins bind covalently to κ-casein on the surface of casein micelles or form non-sedimentable complexes in the serum phase (Anema et al., 2003; Guyomarch et al., 2003; Donato et al., 2007a). These heat-induced complexes are key to influence the mechanisms of structure formation and provide appealing texture to fermented milk products such as yogurt (Robinson and Tamime, 1993; Lucey et al., 1997a; Lucey et al., 1998; 2001). Given the potentially important function of the heat-induced complexes for many dairy products, it is critical to fully understand their mechanism of complex formation.

During thermal processing, whey proteins undergo conformational changes, which expose the amino acid residues buried within the native structure, resulting in an increase in the reactivity of such groups. β-lactoglobulin (β-lg) is present as a non-covalently linked dimer (Walstra et al., 1999; Damodaran et al., 2008), and has two internal disulfide bonds and one free thiol group. During heating, the protein dissociates, unfolds and the thiol groups become exposed, initiating disulfide interchange reactions (Fox and Mulvihill, 1982; Anema and McKenna, 1996; Walstra et al., 1999). α-lactalbumin (α-la), on the other hand, contains eight cysteine groups (all involved in internal disulfide bonds), which are responsible for the reversible conformational changes occurring with heating in the presence of calcium if the protein is present in isolation (Swaisgood, 1982;
Relkin et al., 1992). When $\alpha$-la is present in milk or with $\beta$-lg, it becomes part of the heat-induced complexes. Additionally, it has been shown that immunoglobulin and serum albumin are easily denatured with heating (Fox and Mulvihill, 1982; Swaisgood, 1982; Walstra et al., 1984). In contrast to the whey proteins, caseins are relatively heat stable; however, the cysteine residues of $\kappa$-casein and $\alpha_{s2}$-casein react forming disulfide bridges with whey proteins (Fox and Mulvihill, 1982; Anema and McKenna, 1996).

The heat-induced complexes of whey proteins are located partly on the casein micelle surface and partly in the serum as small dispersed particles, and often referred to as soluble complexes since they can be separated from the casein micelles by centrifugation by using a field of 20,000× g and above (Anema and Li, 2000; Guyomarc'h et al., 2003; Jean et al., 2006). The soluble complexes formed during heating of skim milk are composed of a ratio of approximately three whey proteins to one $\kappa$-casein; however, the ratio depends on the relative concentrations of these proteins in milk (Guyomarc'h et al., 2003; Donato and Dalgleish, 2006). Other caseins, $\alpha_{s1}$ and $\beta$-caseins, are not found in the complexes at the normal pH of milk (Donato and Dalgleish, 2006). Those soluble complexes are formed via hydrophobic interactions and thiol/disulphide exchanges and have sizes ranging from 30 to 100 nm (Anema and Li, 2003; Guyomarc'h et al., 2003; Donato and Dalgleish, 2006; Donato et al., 2007b). At the natural pH of milk, approximately 70% of the denatured whey protein is present in the serum and the remaining is associated with the micelles (Kethireddipalli et al., 2010). With a lower pH of milk before heating, the ratio of aggregates present in the serum to those associated with the micelles decreases (Dalgleish, 1990; Dalgleish et al., 1997; Guyomarc'h et al., 2003; del Angel and Dalgleish, 2006).
The heat-induced interactions between whey proteins and casein micelles are dependent on temperature, time, rate of heating and pH. For example, it has been reported that, in skim milk, $\alpha$-la interacts with caseins more efficiently than $\beta$-lg at temperatures below 90 °C (Corredig and Dalgleish, 1999). It has also been reported that $\beta$-lg binds to a greater extent with casein micelles during short heating times, compared to $\alpha$-la when skim milk is subjected to heating at ultra-high temperatures (Oldfield et al., 1998a). This was attributed to the difference in heat stability between the proteins. A recent study suggested that the extent of soluble complex formation can be controlled by modulation of heating regimes (74–85 °C) and that the relative proportion of casein to whey protein in the heat-induced aggregates changes with the extent of heating (Cooper et al., 2010).

The rate and degree of heat-induced complex formation not only depend on the time and temperature of heating and the initial pH of the milk, but also on the concentration of milk proteins (Singh, 1995; Lucey et al., 1998). Most studies have investigated the effect of heat on the denaturation of whey proteins in milk at their natural concentration, while only a few studies used concentrated milk. It has been reported that a minimum in whey protein denaturation seems to occur when the total solids of milk reach 20% (Guy et al., 1967). Other researchers have also suggested that the denaturation of whey proteins (as a percentage of the total) is markedly reduced when heated at 75 or 80 °C at a higher concentration of solids (McKenna and O'Sullivan, 1971). However, it is important to note that in reconstituted milk powder or evaporated milk increasing the solids, ionic strength and lactose concentration also increase (Anema, 2009a), and this is not the case in milk concentrated by UF or UF with DF.
It is well-known that the increased concentration of soluble complexes in milk has a critical effect on the acid gelation of casein micelles, such as a greater gel stiffness and an increase in the pH of gelation (Lucey et al., 1998; Donato et al., 2007a). However, the details related to the formation of these complexes in milk concentrated by UF and DF have yet to be fully investigated. During concentration, changes in the structure of casein micelles may occur, and these could influence the composition and type of heat-induced complexes formed, in turn affecting the processing functionality of the milk. The current study intends to provide further understanding on the combined effects of heating and concentration on the mechanisms of formation of soluble complexes by determining the differences in composition of the non-sedimentable fraction of unheated and heated milk samples, 1× and 4× by UF and DF, after heating at temperatures of 75, 80 and 90 °C for times ranging from 0 to 30 min.

3.3. Materials and Methods

3.3.1. Sample preparation

Fresh, pasteurized skim milk (Crown Dairy Ltd., Guelph, Canada) was concentrated using a tangential flow filtration system (PUROSEP LT-2, SmartFlow Technologies, Apex, NC, USA), using an OPTISEP 3000 polyethersulphone (PES) membrane module (nominal molecular weight cutoff 10 kDa; membrane area of 0.18 m²; 0.175 mm channel height) (SmartFlow Technologies, Apex, NC, USA). UF was performed at 40 °C with 170 kPa transmembrane pressure, re-circulating the skim milk to the feed tank at a cross-flow velocity of 12 L/min. The concentration was determined
based on volume reduction by measuring the amount of permeate. Control milk (1×) or ultrafiltered milk with volume concentration ratio of 4× was obtained.

DF was carried out by adding MilliQ water (twice the retentate volume) to milk concentrated by UF. Filtration was then continued under the same conditions as above until the volume of permeate collected corresponded to the amount of water originally added. Sodium azide (0.02% w/v) was added right after filtration to control milk (1×) and the resulting retentates, named 4× UF and 4× DF, to prevent microbial growth.

3.3.2. Heating experiments

1× control, and 4× UF or DF (in aliquots of 15 mL) were placed in a water bath and heated at temperatures of 75, 80 and 90 °C for times from 0 to 30 min in a thermostatically controlled water bath, allowing 2 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, and stored for at least 1 h at ambient temperature before the analysis.

3.3.3. Separation of non-sedimentable fraction

To investigate the effect of the different treatments on the formation of soluble (non-sedimentable) complexes of casein and serum protein, samples were centrifuged at 25,000× g for 1 h at 20 °C in a Beckman Coulter Optima LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The supernatants were removed from each centrifuge tube with a syringe and then filtered using a 0.45 µm filter (Millipore Corporation, Bedford, MA, USA). Supernatants were
analyzed and stored at 4 °C for further analysis. The total protein concentration of milk was measured by using the Dumas method with a Leco FP-528 (Leco Corp., St. Joseph, MI, USA) and a conversion factor of 6.38. While the supernatant concentration was measured using the DC protein assay method (Bio-Rad, Mississauga, ON, Canada).

3.3.4. Size exclusion chromatography (SEC)

Centrifugal supernatant (1 mL) was analyzed by SEC using an ÄKTA purifier 10 system (GE Healthcare, Uppsala, Sweden) equipped with a 1 mL sample loop. The column (AA Pharmacia XK 16/70) had a packed bed height of 67 cm. The packing material was S-500 Sephacryl high-resolution gel, with a nominal fractionation range of 40–20,000 kDa (Amersham Biosciences Inc., Baie d’Urfé, Quebec, Canada). The eluted peaks were detected by absorption at 280 nm, with a flow rate of 1 mL/min and a mobile phase of 20 mM Bis-Tris-Propane at pH 7.0 containing sodium azide (0.02%). Peak integration was carried out using UNICORN 5.11 software (Amersham Biosciences Inc., Baie d’Urfé, Quebec, Canada). To be comparable to all the samples, the elution profiles were fitted and normalized by using the Sigmaplot program (SPSS Inc., Chicago, IL, USA). To determine differences in the type and composition of proteins eluted by chromatography, 10 mL fractions were collected and then freeze dried (VirTis, Gardiner, NY, USA) for further analysis. The peak areas were calculated consistently from 60 to 95 min for Peak 2 and from 95 to 120 min for Peak 3 (see Figure 3.1) by the software OriginPro 8 (OriginLab Corp., Northampton, MA, USA).

3.3.5. Reversed-phase high-performance liquid chromatography (RP-HPLC)
RP-HPLC was employed to determine the residual whey protein in the serum phase. The HPLC system consisted an AS 3000 automatic injector, P 2000 binary gradient pump, UV 2000 detector and a SN 4000 interface module, all from Thermo Instruments Canada Inc. (Mississauga, ON, Canada), and controlled by software (ChromQuest, version 4.1). The supernatants were eluted using a Nova-Pak C18 reversed phase column (150 mm length × 3.9 mm i.d., 4 µm with a C18 guard column, Waters, Mississauga, ON, Canada) with a gradient of 0.1% v/v trifluoroacetic acid (TFA) in ultrapure MilliQ water (eluent A) and acetonitrile, MilliQ water, and TFA in a ratio 900:100:1 (v/v/v) (eluent B) at 1 mL/min. The gradient program started with 2% eluent B, increasing to 43% B in 8 min, followed by a slow gradient to 44% between 8 – 17 min; 47.5 – 80% to 40 min, and to 100% immediately thereafter. The sample injection volume was 20 µL. Detection was carried out at 280 nm. To compare supernatant samples at different concentrations, the supernatants of concentrated milk were diluted back 1× immediately before injection. To confirm the peak composition, selected fractions were collected (for multiple runs of the same sample), freeze dried, and analyzed by gel electrophoresis. The peak areas were calculated by the software OriginPro 8 (OriginLab Corp., Northampton, MA, USA).

3.3.6. Protein composition of fractions

The freeze-dried samples eluted from SEC were dissolved with 1.0 mL sample buffer (1 M Tris-HCl, pH 6.8, 16 g kg⁻¹ SDS, 150 g kg⁻¹ glycerol, 0.4 g kg⁻¹ β-mercaptoethanol, 0.1 g kg⁻¹ bromophenol blue). In addition, selected fractions from RP-HPLC were also analyzed, by diluting the collected sample fractions with 0.2 mL sample
buffer. Samples were then heated for 5 min at 95 °C. The analysis was conducted under non-reducing conditions (sample buffer without the β-mercaptoethanol) or reducing conditions.

SDS-PAGE was performed using a Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC, Mississauga, ON, Canada). The resolving gel contained 15% acrylamide in 0.4 M Tris-HCl at pH 8.9, and the stacking gel contained 4% acrylamide in 0.05 M Tris-HCl buffer at pH 6.7. The electrophoresis buffer was 0.7 M Tris-HCl with 0.45 M glycine at pH 8.3. Aliquots of 5 µL of the prepared reference samples, 1% (w/w) WP isolate (WPI, Power Pro, Land O’ Lakes Dairy Proteins Group, St. Paul, MN, USA) and 1% (w/w) sodium caseinate (NaCas, Alanate-180, Fonterra Milk Products, Lemoyne, PA, USA), were loaded onto the gels. In the analysis of the SEC peaks, 20 µL fractions eluted from 1× controls were loaded, while 5 µL aliquots were used for 4 × UF and DF fractions, to be able to make direct comparisons. In the case of fractions from RP-HPLC, 20 µL were loaded into the gels. Electrophoresis was performed at 100 V and 30 mA for 120 min. The gels were then 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 followed by one change for 12 h of a 4.5% (v/v) methanol and 1% (v/v) acetic acid solution. For each supernatant sample, three bands of interest were identified corresponding to caseins (αs-casein, β-casein, and κ-casein were considered all together), α-lac, and β-lg. The gels were scanned (Bio-Rad Laboratories, Mississauga, ON, Canada), and the integrated intensities of the protein bands were analyzed using Image lab software (Bio-Rad, version 3).
3.3.7. Statistical analysis

All experiments were carried out in triplicate (i.e. three separate milk batches and concentrations by filtration), and the average and standard deviations were reported. In all of the description as followed, the profiles are the averages of those obtained from three experiments. Statistical significances were evaluated using analysis of variance (ANOVA) at p<0.05; the mean values were compared using a Tukey test, and all statistical data were processed using R software.

3.4. Results and Discussion

3.4.1. SEC profiles of centrifugal supernants

The elution profiles of the non-sedimentable fractions of unheated milk and milk heated at 75, 80 and 90 °C for 5 min are shown in Figure 3.1. The elution behaviour of milk supernatant obtained for control (1×) milk was in full agreement with that reported in previous literature, for both heated and unheated milk (Donato and Dalgleish, 2006; Cooper et al., 2010) (Figure 3.1). In all cases, three regions could be identified, as shown in Figure 3.1 as peaks 1-3. Peak 1, eluting in < 60 min, contained little to no protein. In some cases, especially for the high temperature/time combinations, protein eluted right after the excluded volume. Peak 2 was identified as the intermediate region between the excluded volume (Peak 1) and the peak corresponding to the unaggregated whey proteins (Peak 3). The intermediate peak, Peak 2 increased in size with the extent of heating, and was predominant in the samples heated at high temperatures, as well as for the 4× milk concentrates. The early elution of Peak 2 suggested that the size or the composition of the protein aggregates may have changed. Both elution time and absorbance were
significantly different between heating treatments, concentrations and mode of concentration (UF or DF) (p<0.05), as shown in Figures 3.1B, C and D.

**Figure 3.1** SEC elution profiles of the supernatant isolated from 1× control (circles), 4× UF (squares) and 4× DF (triangles), unheated (A) and heated at 75 °C (B), 80 °C (C) and 90 °C (D) for 5 min. Data are representative of three separate experiments.
In the case of unheated milk or milk heated at 75 °C, the intermediate peak (Peak 2) eluted much later, at about 75 min, and it has been previously shown to be composed of small casein micelles and non-sedimentable casein aggregates. This fraction is rich in κ-casein (Cooper et al., 2010). Peak 3 contained unaggregated whey proteins, and decreased with increased extent of heating as previously reported (Donato et al., 2007b).

In 4× concentrated milk, there was more soluble material present in the serum phase, noted by the higher intensity of the absorbance, compared to the 1× control. More importantly, 4× UF showed a significant difference in the elution of the peaks compared to 4× DF milk.

In unheated milk samples Peak 2, containing non-sedimentable caseins, was much more evident in 4× concentrated milk than in the 1× control, and the area of the peak was significantly higher for DF milk than for UF milk (measured as average of three separate experiments). These results suggest for the first time a higher extent of disruption of casein micelles during concentration using UF and DF than during concentration by UF without DF. This compositional change in the continuous phase of milk may play an important role in the formation of complexes during heating. It is also important to note that while the amount of undenatured whey protein in Peak 3 increased between 1× and 4× milk, as expected, it showed no significant difference in the total area between serum prepared by UF or UF and DF (Figure 3.1A).

In general, with heating, while the unaggregated whey protein peak decreased (Peak 3), the non-sedimentable material eluted < 100 min increased, demonstrating an increase in size and amount of heat induced aggregates in the supernatants (Figure 3.1B, C and D).
Supernatants from 4× DF retentates (triangles, Figure 3.1) showed a greater quantity of heat-induced soluble complexes (Peak 2) and less residual whey protein (Peak 3) compared to the UF retentates (square symbols, Figure 3.1) at all three temperature tested. This observation is consistent with the greater extent of casein solubilisation noted in the unheated samples for DF retentates compared to UF retentates (see Figure 3.1A). It is also important to note that DF and UF milk also differed in the salt and lactose concentrations in the serum phase. Earlier elution of Peak 2 occurred in UF retentates compared to DF retentates heated at 90 ºC (Figure 3.1D), suggesting a difference in the type and size of the aggregates present in the supernatant of heated UF or DF milk concentrates. Figure 3.2 illustrates the elution profiles of the sera of unheated and heated milk at 75, 80 and 90 ºC for 15 min. These curves are very similar to those shown in Figure 3.1 for 5 min heating.

The higher the temperature and longer the heating time, the more soluble complexes were recovered during elution and the lower the amount of residual whey protein in Peak 3. Compared to the milk heated for 5 min (Figure 3.1), the differences in elution behavior between 4× UF and DF milk samples were greater. Not only did DF milk seem to show a greater extent of soluble aggregates at any temperature, but also it showed a smaller peak of residual whey proteins. Once again, as already observed in Figure 3.1D, after heating at 90 ºC, the elution of non-sedimentable material in UF samples was much different than for DF milk, and the earlier elution of UF samples indicated the presences of larger particles.
Figure 3.2 SEC elution profiles of the sera in the milk samples, 1× control (circles), 4× UF (squares) and 4× DF (triangles), unheated (A) and heated at 75 °C (B), 80 °C (C) and 90 °C (D) for 15 min. Data are representative of three separate experiments.
Figure 3.3 SEC elution profiles of the sera in the heated milk samples, 1× control (top row), 4× UF (middle row) and 4× DF (bottom row), at heating temperatures, 75 °C (left panel), 80 °C (center panel) and 90 °C (right panel), at different times 5 (circles), 15 (squares) and 30 (triangles) min. Elutions are representative of three different replicates.
Figure 3.3 summarizes the SEC elution profiles for all centrifugal supernatant from heated milk at the three temperatures for various heating times. It was clearly demonstrated that within each sample (1×, 4× DF or 4× UF), the composition of the serum phase was affected by both heating temperature and heating time. With time, there seemed to be an increase in the amount of protein aggregates and a decrease in the residual whey protein peak (Peak 3), as well as a shift in the elution to earlier times.

To better quantify the changes in peak area for the various samples, the variation in the peak area relative to area for the unheated milk or retentates is shown in Figure 3.4 for the intermediate peak (Peak 2) as well as the unaggregated whey protein peak (Peak 3). In all cases, the area of Peak 3, attributed to the residual whey proteins, decreased with heating time. The decrease was slower for samples heated at 75 ºC compared to 80 and 90 ºC, regardless of concentration. However, there were some differences in the decrease of Peak 3 between DF and UF retentates (Figure 3.4).

Figure 3.4 also shows the changes in the area of Peak 2, containing heat induced aggregates. At every temperature, the amount (indicated as relative area %) of such aggregates increased with time of heating, but with a distinct difference in the behavior between the treatments. In 1× milk, the total area of the peak did not increase with heating at 75 ºC, while it doubled at higher temperatures (Figure 3.4). In 4× UF and DF the area for Peak 2 reached values almost 6 times those of the original peak. In addition, similarly to what observed for the residual whey protein, and the increase in the amount of aggregates was faster for heating at 90 ºC than at 80 or 75 ºC.
**Figure 3.4** The changes in elution peak area for peak 2 (top row) and peak 3 (bottom row) of supernatants from heated milk samples, 1× (left panel), 4× UF (center) and 4× DF (right panel), at heating temperature of 75 °C (circles), 80 °C (squares) and 90 °C (triangles), as a function of heating time (min). Results are the average of three independent experiments.
There was a clear difference between the peak area growth for 4× UF and 4× DF milk, once again, showing that there were differences in the composition between these two retentates, after heating.

### 3.4.2. RP-HPLC analysis of centrifugal supernatants

To better investigate the differences in the residual serum proteins, the centrifugal supernatants were also analyzed by RP-HPLC and compared to those of the control milk. Changes in RP-HPLC profile patterns of supernatants of unheated and heated samples at 75, 80 and 90 °C for 5 min are shown in Figure 3.5. The milk proteins still present in the supernatant were eluted based on differences in their hydrophobicity in the order: κ–cas, αs–cas, β–cas, α–la, and β–lg, as reported in literature (Bobe et al., 1998; Anne et al., 2003; Jean et al., 2006). In unheated milk (Figure 3.5A), there was a significantly higher amount of αs–cas in DF samples compared to UF and control samples. It is important to note that before the analysis the supernatants were diluted back to the original skim milk volume fraction. The higher amount of caseins present in the DF samples was consistent with the increase in soluble caseins in the unheated supernatants analyzed by size exclusion chromatography (Figure 3.1).

It may be possible to hypothesize that the shear effect during filtration, combined with the modifications of the ionic environment during diafiltration may affect the integrity of casein micelles, resulting in a greater amount of αs–cas and β–cas in the centrifugal supernatant of 4× DF rententates.
Figure 3.5 Changes in RP-HPLC profile patterns of supernatants for: 1x control (black), 4x UF (red) and 4x DF (blue), unheated (A) or heated at 75 (B), 80 (C) and 90 (D) °C for 5 min. Small peaks (eluted before 15 min) are caseins (κ, αs, β-cas) while whey proteins elute later (α-la and then β-lg). Representative runs.
No significant differences were noted for the $\alpha$–la and $\beta$–lg peaks in unheated samples. During heating, the $\alpha$–la peak was dramatically reduced. However, this was not the case for the second peak containing native $\beta$–lg (Figure 3.5A). The peak area for this second peak increased with heating, and it contained complexes of $\kappa$–cas, $\alpha$–la and $\beta$–lg, when analyzed by SDS-PAGE under reducing conditions (results not shown). The co-elution of the aggregates in the 4× DF and 4× UF milk did not allow for quantification of the amount of residual native $\beta$–lg.

A summary of the RP-HPLC elution of the various supernatants from heated skim milk control, 4× UF and 4× DF is shown in Figure 3.6. In all samples, the peak eluting at about 20 min, consisting of $\alpha$–la decreased with temperature and time. Hence, the decrease from the initial concentration of $\alpha$–la as a function of temperature and time of heating for 1×, 4× UF and 4× DF milk was quantified (Figure 3.7). Heating at 75 ºC showed a much larger extent of $\alpha$–la denaturation for 4× UF and 4× DF milk compared to 1× milk (Figure 3.7). This was less evident at the higher temperatures. After 30 min of heating at 75 ºC, > 65% of the original $\alpha$–la was present in 1× milk, but very little in the concentrated retentates. This result suggested differences in the composition of the whey protein aggregates in 4× UF and 4× DF retentates compared to control milk heated under the same conditions. No significant differences in the residual $\alpha$–la were noted for 4× UF and 4× DF at these temperatures.
Figure 3.6 Changes in RP-HPLC profiles of supernatants for 1× control (top row), 4× UF (center row) and 4× DF (nottom row), after heating temperatures at 75 (left hand panel), 80 (center panel) and 90 (right hand panel) °C, for 5 (black), 15 (red) and 30 (blue) min. Representative runs.
Figure 3.7 Peak area relative to the initial area for α-lactalbumin present in centrifugal supernatants of 1× control (A), 4× UF (B) and 4× DF (C), after heating at 75 (circles), 80 (squares) and 90 (triangles) °C, as a function of heating time. Results are the average of three independent experiments.
3.4.3. Protein composition of the eluted fractions

To better determine the type and composition of soluble material present in the centrifugal supernatants of unheated and heated 1× SM, 4× UF and DF samples, the various fractions eluted from SEC were collected and analyzed by SDS-PAGE, as shown in Figure 3.8. It should be noted that to be able to compare the samples to the control or to previous literature, the loading volume of control milk was 4× that of the concentrated milk. The protein contained in fractions eluted at 60–70, 70–80 and 80–90 min were analyzed (Figure 3.3), to determine differences in the composition of the aggregates eluting in Peak 2.

In the case of unheated milk (Figure 3.8, left hand side), the non-sedimentable fractions contained only small amounts of caseins and β–lg. Among the caseins, αs–cas and κ–cas were the most prominent and only in the fraction eluting close to Peak 3 (Figure 3.3). The intensity of the bands for the fractions present in the supernatants of unheated 4× UF and 4× DF was much higher than that of control samples in spite of a similar dilution ratio in the electrophoresis gel. In addition, there was a larger amount of casein eluted in the 4× UF and DF for the peak eluting at 70 min, compared to 1× control. In this case also, the main proteins were αs, and κ–cas, in addition to β–lg. The fractions in UF and DF unheated retentates showed similar composition. It is important to note that the β–lg recovered in the fraction eluted between 80 and 90 min of the unheated samples may not be complexed with caseins, but coeluting as an oligomer. No α-la was recovered in Peak 2 (the aggregate fraction) for unheated milk.
Figure 3.8 SDS-PAGE patterns (under reducing conditions) of the serum in unheated and heated samples collected from the SEC column at a 10 min interval elution time ranging from 60 to 90 min, 60-70 (A), 70-80 (B) and 80-90 (C), where the maximum values are for the presence of heated-induced complexes. Sample preparation and conditions are described in Materials and Methods. For the purpose of comparison, controls are loaded at a level of 20 µL and 5µL for 4x UF and 4x DF. The bands in the gels are identified as (i) minor WP; (ii) $\alpha$–cas; (iii) $\beta$–cas; (iv) $\kappa$–cas; (v) $\beta$–lg; (vi) $\alpha$–la.
In 1× heated milk, at all temperature/times combinations, the main proteins present in the collected fractions were κ-cas and β-lg, with a higher ratio of β-lg to κ-cas, in full agreement with what has previously been reported in the literature (Donato and Dalgleish, 2006; Donato et al., 2007a; Anema, 2008b; Donato and Guyomarc'h, 2009). κ-cas was the dominant casein present in the aggregates, although some αs-cas and β-cas were also present in 1× milk fractions eluting between 80–90 min (see also Figure 3.3) for samples heated at 75 ºC for 15 min or 80 ºC for 5 min. At the higher temperature/times combinations, the only casein present in the aggregate was κ-cas.

In the supernatant fractions of 4× UF and 4× DF milk, α-la was also present in all temperature and time combinations above 75 ºC for 15 min. In addition, the aggregates contained a larger amount of caseins. In general, the larger aggregates eluting between 60 and 70 min (see Figure 3.3) contained mainly κ-cas and β-lg, while the fractions eluted at 70 and 80 min also contained other caseins. There was a significantly lower amount of protein in each fraction in the control supernatant compared to 4× UF and 4× DF in spite of the fact that for control fractions 4× the volume was loaded, to compare with the retentate fractions.

To better evaluate possible differences in the composition of the soluble aggregates, the band intensity for the fraction eluting at 80-90 min for each sample was measured by scanning densitometry, and the average values are shown in Figure 3.9. The κ-cas to whey protein ratio was about 0.5 for all temperature/time combinations tested, apart from 1× milk control heated at 75 ºC. In other words, the non-sedimentable complexes eluted at 80-90 min were composed of a ratio of approximately two whey proteins to one κ-casein.
Figure 3.9 Changes in the area ratio measured from scanning densitometry for κ-casein to whey protein and caseins to whey proteins for the fraction isolated from SDS-PAGE eluting at 80-90 min. 1× control (left hand side), 4× UF (center) and 4× DF (right hand side), after treating at 75 (circles), 80 (squares) and 90 (triangles) as a function of heating time. Results are the average of three independent experiments.
Nonetheless, it is important to point out that more $\alpha$-la was present in the concentrated retentates compared to $1\times$ control. Similarly, the casein to whey protein ratio did not vary in all heated samples. These observations differ from previous findings (Dalgleish et al., 1997; Corredig and Dalgleish, 1999; Guyomarc'h et al., 2003; Donato and Dalgleish, 2006). The difference could be attributed to different sample preparation. Additionally, in this study the ratio was calculated based on the band intensities of samples collected from SEC elution at certain elution time instead of directly from the supernatant.

3.5. Conclusions

In conclusion, the effect of heat treatment on whey protein denaturation and soluble complex formation depended on the combination of heating temperature, length of heating time and the concentration of milk proteins. There was a difference in the type of heat-induced complexes formed during heating of $4\times$ concentrated milk with a higher extent of aggregate formation, compared to $1\times$ milk, as well as different composition, with more $\alpha$-la and caseins present in the soluble aggregate fraction. These changes in composition and size of the soluble aggregates may have profound consequences on the gelation behaviour of concentrated milk prepared by UF or UF and DF.
CHAPTER 4
CALCIUM RELEASE OF CONCENTRATED MILK BY
ULTRAFILTRATION AND DIAFILTRATION

4.1. Abstract

The present work studied the solubilization of calcium during acidification in milk concentrated by ultrafiltration (UF) and diafiltration (DF). The effect of heating milk at 80 °C for 15 min was also evaluated. In addition to measuring buffering capacity, the amount of calcium released as a function of pH was measured. The area of the maximum peak in buffering capacity observed at pH ~5.1, related to the presence of colloidal calcium phosphate, was significantly affected by casein volume fraction, but did not increase proportionally to casein concentration. In addition, a lower buffering capacity and less soluble calcium were measured in 2× DF milk compared to 2× UF milk. Heat treatment did not change the buffering capacity or calcium release in 1× and 2× concentrated milk. On the other hand, at a higher volume fraction (4×), more calcium was present in the soluble phase in heated 4× UF and 4× DF milk compared to unheated milk. This is the first comprehensive study on the effect of concentration, and distinguishing the effect of UF from DF, before and after heating, on calcium solubilization.

4.2. Introduction

Skim milk is a colloidal suspension of casein micelles and whey proteins, and contains calcium, magnesium, potassium and zinc, as well as chloride, phosphate and citrate anions (Walstra and Jenness, 1984; Holt, 2004). Calcium and phosphate are
present in colloidal form in the casein micelles, associated with phosphoserine amino acids (Walstra and Jenness, 1984; Holt, 2004). The colloidal calcium phosphate (CCP), is composed of amorphous hydrated CaHPO$_4$$\cdot$2H$_2$O, distributed throughout the micelle as nanoclusters of a few nanometers in size (Holt et al., 1998; Marchin et al., 2007). CCP plays an important role in the stability of casein micelles, and is in equilibrium with the calcium present in the serum phase (Holt, 1985). The concentration of total calcium in milk is approximately 1200 mg/L, of which 400 mg/L is in soluble form and 800 mg/L in insoluble form, at milk’s natural pH (Walstra and Jenness, 1984; Rahimi-Yazdi et al., 2010).

The distribution and equilibrium state of calcium are affected by environmental conditions such as pH and temperature, altering the processing characteristics of milk (Law and Leaver, 1998; Le Graët and Gaucheron, 1999; Dalgleish et al., 2005). For example, it has been reported that when milk is stored at 4 °C, CCP is released from casein micelles along with β-casein due to reduced hydrophobic interactions. These changes are reversible once the milk is rewarmed to room temperature (Davies and White, 1960; Downey and Murphy, 1970; Pierre and Brule, 1981; Dalgleish, 2011). On the other hand, extensive heat treatment (for example > 90 °C for several minutes) results in a decrease of soluble calcium, a transfer of the soluble calcium to the colloidal phase and a concomitant decrease in milk pH (Rose and Tessier, 1959; Pouliot et al., 1989).

The equilibrium between colloidal and soluble calcium is dependent on pH (Law and Leaver, 1998; Le Graët and Gaucheron, 1999; Dalgleish et al., 2005). At a pH around the isoelectric point of the casein, there is a collapse of κ-casein layer on the surface of the protein particles and solubilization of CCP, especially between pH 5.5 and 5.0, with
solubilization of caseins depending on temperature (Dalgleish and Law, 1988; Walstra, 1990; Holt et al., 1992; Le Graët and Gaucheron, 1999). The calcium solubilization is complete around pH 5.0 (Dalgleish and Law, 1989; Le Graët and Gaucheron, 1999), with maximum buffering capacity at pH 5.1 (Lucey et al., 1996; Salaün et al., 2005).

While the effect of pH on casein micelle structure and related calcium release in skim milk are at least partly understood, much less is known about the changes occurring in milk that has been concentrated by ultrafiltration (UF) and diafiltration (DF). When water is removed and the concentration of total solids in skim milk is increased, the decreased distance between casein micelles will force them to interact more frequently with each other (DeKruif, 1998). In addition, with concentration the total balance of soluble and sedimentable calcium changes, resulting in a considerable proportion of soluble calcium and phosphate being transferred between soluble and colloidal states (Hardy et al., 1984). Although some work has been published on the effect of concentration on calcium release during acidification, most research so far has been carried out on reconstituted milk powder or milk concentrates (Lucey et al., 1996; Le Graët and Gaucheron, 1999), therefore a comprehensive look at the effect of UF and DF concentration is needed.

Concentration of skim milk by UF changes the composition of milk by increasing the proteins and colloidal minerals in the retentate while decreasing the water, soluble minerals, lactose and non-protein nitrogen, which are transmitted through the membrane and maintained in the permeate (Mistry and Maubois, 2004). Recent studies have suggested that the renneting functionality and physical properties of casein micelles are affected when concentrating milk protein by UF, especially at high volume reductions.
(Ferrer et al., 2011). Moreover, DF, the process of adding water to the concentrated retentate, while enhancing the levels of concentration in the retentate, may further affect the integrity of the casein micelles and the release of calcium. Earlier studies have shown that dialysis of milk against water (i.e. a change in the ionic composition of the serum phase) removes free calcium and other ions from the serum phase and causes dissociation of caseins due to the loss of colloidal calcium phosphate (McSweeney and Fox, 2009).

The present study focused not only on the effect of the concentration (i.e. increase in the volume fraction of the casein micelles), but also DF (i.e. the addition of water and resulting mineral imbalance created in the serum phase), on the buffering capacity and calcium retention of casein micelles. It was hypothesized that although the concentration of calcium in the colloidal phase increases with the volume fraction of casein micelles, this increase does not affect the release rate of the calcium in the soluble phase. The mode of concentration (UF or UF in combination with DF) as well as heating was tested to determine if processing history impacts the release of the calcium.

4.3. Materials and Methods

4.3.1. Sample preparation

Fresh, pasteurized skim milk (Crown Dairy Ltd., Guelph, Canada) was concentrated using a tangential flow filtration system (PUROSEP LT-2, SmartFlow Technologies, Apex, NC, USA), as previously described in 3.3.1. Control milk (1×) or ultrafiltered milk with volume concentration ratios of 2× and 4× were obtained for study in this chapter. The resulting retentates were named 2× and 4× UF, to distinguish them from the 2× and 4× DF treatments.
1×, and 2× or 4× UF or DF (in aliquots of 15 mL) were placed in capped glass vials in a water bath at 80 °C, and heated for 15 min, allowing 2 min for the samples to reach temperature. As shown in Chapter 3, at this temperature-time combination, most of the whey proteins are denatured, and there is a large population of non-sedimentable aggregates. After heat treatment, the milk samples were immediately cooled to room temperature by immersion in an ice bath, and stored for at least 1 h at ambient temperature before further analysis.

4.3.2. Separation of non-sedimentable fraction

The non-sedimentable fraction was separated by centrifugation at 25,000× g for 1 h at 20 ºC in a Beckman Coulter Optima LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada). Details can be found in 3.3.3.

4.3.3. Buffering capacity

Acid-base titrations were performed as previously published (Lucey et al., 1996) with minor modifications. The samples (50 mL) at 25 °C were titrated from initial pH to pH 2.0 with 0.1 M HCL and back-titrated to pH 11.0 with 0.1 M NaOH, added at a rate of 0.2 mL/min. Buffering indices, the $d_B$ (acid or base)/$d\text{pH}$ ratio, were calculated according to previous literature (Salaün et al., 2005). Buffering curves were prepared by plotting the calculated indices as a function of pH and the peak areas were calculated by the software of OriginPro 8 (OriginLab Corp., Northampton, MA, USA).
4.3.4. Calcium determination by ion chromatography

The amount of calcium in all the fractions (centrifugal supernatants and retentates) was measured by non-suppressed ion chromatography (IC) (Rahimi-Yazdi et al., 2010).

The amount of calcium present in the serum after centrifugation was measured by mixing 1 mL of centrifugal supernatant with 200 µl of 1 M HCl and adjusting to a volume of 100 mL with HPLC water. For the determination of total calcium, 666 µL of milk or retentate, 400 µL of 1 M HCl and 266 µL of HPLC water were mixed in a 1.5 mL Eppendorf microcentrifuge tube. The samples were centrifuged at room temperature for 15 min at 4,500× g (Brinkmann Instruments, Ltd., Mississauga, Canada) to precipitate the proteins. The supernatant (1.333 mL) was then diluted to 100 mL with HPLC water. Samples were analyzed with IC within 48 h.

Chromatography was carried out using an 861 Advanced Compact IC (Metrohm Ltd., Herisau, Switzerland), composed of an injection valve, a high pressure pump, and a conductivity detector. Samples were eluted at 0.9 mL/min isocratically with a mobile phase consisting of 1.7 mM nitric acid and 1 mM pyridine-2, 6-dicarboxylic acid in an 838 sample processor. To accept the cations from the sample solution, the 833 IC Liquid Handling Dialysis Unit pumped a 2 mM nitric acid solution through one side of the dialysis cell while the other side of the cell was fed with the sample. The nitric acid solution (20 µl) containing the sample cation was then injected in the column (Metrosep C2-150, Metrohm). Both column and detector temperatures were kept at 30 °C. Calcium standard solutions (1 to 10 mgL⁻¹) were prepared from 1 gL⁻¹ concentrated standards (TraceCERT, Fluka, Sigma Steinheim, Germany). The amount of insoluble calcium in
milk was calculated as the difference between the total amount of calcium and that measured in the centrifugal supernatant fractions.

4.3.5. Calcium release during acidification

To investigate the calcium dissociated from casein micelles as a function of pH, different amounts of glucono delta lactone (GDL), ranging from 1 to 1.3% (w/w) were added to milk samples. After incubation of the samples at 40 °C for 4 h, the values of pH were measured by AR 15 pH meter (Fisher Scientific, Mississauga, Ontario). Samples were then immediately centrifuged (see above) and the calcium was measured by chromatography.

To obtain similar acidification conditions, different GDL concentrations (1.3, 1.8 and 3.0% (w/w)) were employed for control, 2× UF/DF and 4× UF/DF milk samples, respectively.

4.3.6. Statistical analysis

All experiments were carried out in triplicate (i.e. three separate milk batches and concentrations by filtration) as described in 3.3.7.

4.4. Results and Discussion

4.4.1. Buffering capacity

Milk contains many constituents, including salts, organic acids and proteins, all contributing to its buffering capacity (Lucey and Fox, 1993; Lucey et al., 1996; Salaün et al., 2005). To measure the buffering capacity in milk, the samples are titrated from the initial pH (approximately 6.7) to pH 2.0 with 0.1 M HCl and then back titrated to pH 11.0
with 0.1 M NaOH, as shown in Figure 4.1. Buffering curves for 1× skim milk were similar to those reported in the literature (Lucey and Fox, 1993). When milk is titrated with acid from its natural pH to pH 2.0 (Figure 4.1A), the buffering capacity shows a peak at about pH 5.1. At a pH between 3 and 4, the acidic amino acids present in milk proteins (caseins and whey proteins) are then titrated. At pH around 5.1, the “free” inorganic and organic phosphates associate with H⁺, and this buffering peak has been attributed to solubilized CCP and the formation of free phosphoserine residues (Salaün et al., 2005). This peak was expected to grow with a greater volume fraction of casein in the 2× and 4× retentate samples. Figure 4.1B shows the subsequent alkalinisation. After a wide peak for the acidic amino acids, the curves showed a peak at about pH 6, attributed to the precipitation of calcium phosphate due to neutralization of HPO₄²⁻ and H₂PO₄⁻ (van Dijk, 1990; Lucey and Fox, 1993; Lucey et al., 1993b; Lucey et al., 1996). With a further increase in pH another steep increase in the buffering capacity is measured above pH 9, associated to the presence of basic amino acid and possibly carbonate ions (Salaün et al., 2005). The changes in the buffering peaks at pH 5.1 and 6 in milk concentrated by UF and DF were evaluated to determine possible differences in the amount of colloidal calcium and phosphate present in the casein micelles. The changes in peak areas for the pH 5.1 and pH 6 peaks are shown in Figure 4.1C and 4.1D, respectively. Figure 4.1 shows the changes in buffering capacity for control milk, as well as milk concentrated 2× and 4× by UF and DF, before and after heating. It was hypothesized that if DF did not cause a significant change in the CCP distribution in the casein micelles, UF and DF milk with similar volume fractions would show similar buffering capacity curves.
Figure 4.1 Buffering curves of milk samples, 1× control (circles), 2× UF (squares), 4× UF (diamonds), unheated (filled symbols) and heated (empty symbols), acidified from the initial pH to 2.0 with HCl (A) and then backtitrated to pH 11.0 with NaOH (B). The areas of the peaks (see arrows) related to CCP are also plotted as a function of casein concentration for acidification (peak at pH 5.1) (C) and back titration (peak at pH around 6) (D). Curves are representative of three separate replicate experiments. Error bars represent standard deviations, and lines are drawn to guide the eye and indicate linear increase.
Any changes in the calcium and phosphate equilibrium with heat treatment may show a change in the buffering capacity of milk.

All samples showed the behaviour similar to that of skim milk; however, there was a statistically significant shift in the maximum of the acidification peak from pH 5.1 to 4.8, for the 4× milk concentrated by UF, compared to control skim milk. This result is consistent with previous reports on the effect of calcium and phosphate release in concentrated milk: it is necessary to decrease the pH to a lower value to induce solubilisation of colloidal calcium in concentrated milk (Le Graët and Gaucheron, 1999).

The results for 2× and 4× milk concentrates were in agreement with a previous study on the buffering capacity of a 5× UF concentrated milk (Srilaorkul et al., 1989), and the buffering capacity peaks were larger as a function of concentration, because of the greater level of protein content compared to skim milk. The alkalization curves (Figure 4.1B) showed similar trends as those shown for acidification, with a shift of buffering capacity at pH 6 to earlier pH values in the 4× concentrated milk samples, and a larger area for greater milk concentration.

To further investigate the changes in buffering capacity as a function of casein concentration (as CCP is related to the concentration of micellar casein), the areas for the two peaks related to the presence of CCP in milk were quantified, as shown in Figures 4.1C and 4.1D. Although it is known that whey proteins also contribute to the buffering capacity of milk, their change was not considered as important as the caseins in comparing the treatments. There was a clear relationship between the total peak area in the buffering capacity experiments and the concentration of caseins. The peak area for the buffering peak at pH 5.1 during acidification and at pH 6 during alkalization in 2×
concentrated milk was proportional to the concentration, i.e. lower than expected, in full agreement with previous literature (Brule et al., 1974; Covacevich and Kosikowski, 1979; Mistry and Kosikowski, 1985; Srilaorkul et al., 1989; St-Gelais et al., 1992). However, in the case of 4× UF milk (both heated and unheated) the area was lower than that expected. These results could be explained by a change in the proportion of casein to colloidal calcium and phosphate during concentration by UF, even without diafiltration, confirming previous observations (Renner and Abd-El-Salam, 1991; McKenna, 2000; Ferrer et al., 2011).

Figure 4.1 also depicts the buffering capacity of the same concentrates, after heating. It has been previously reported that the changes of buffering capacity can be affected not only by milk composition, but also heat treatment (Lucey et al., 1993a; Salaün et al., 2005). In this work, there were no statistically significant changes in the peak pH with heating. The discrepancy may derive from the differences in the heat treatment condition between the studies, as higher temperature/time combinations have been used in other studies, and in the use of reconstituted powder instead of fresh milk. Furthermore, the peak areas for 1× and 2× concentrated milk were not significantly affected by heating treatment (Figure 4.1C and 4.1D). However, there was a statically significant difference in the 4× milk concentrated by UF with heating. At this concentration, several different biochemical changes may have occurred during heating of 4× milk concentrated by UF, such as whey protein denaturation, heat-induced complex formation and calcium binding, and modification of the structure and composition of micellar calcium phosphate (Gaucheron et al., 1996; Guyomarc’h et al., 2003; Donato and Guyomarc’h, 2009).
Figure 4.2 illustrates the differences in buffering capacity between retentates concentrated by UF or UF combined with DF. In general, at both concentrations, the maximum buffering peak at pH 5 was significantly higher for UF concentrates compared to DF concentrates. No further pH shift was noted in the curves. These results would suggest that during DF some CCP is solubilized from the casein micelles, confirming recent findings (Alexander et al., 2011). It is important to note that earlier studies have shown that dialysis of milk against water (i.e. a change in the ionic composition of the serum phase) removes free calcium and other ions from the serum phase and causes dissociation of caseins due to the loss of CCP (Abd El Salam et al., 1982; McSweeney and Fox, 2009). It is hence possible to suggest that the process of DF during membrane filtration may cause some disruption of the micellar structure.

As previously shown in the case of UF milk (Figure 4.1), there were no significant differences in the buffering capacity of DF milk after heat treatment (Figure 4.2), further supporting the conclusion that heating milk at 80 °C for 15 min did not perturb the CCP equilibrium of the casein micelles.

4.4.2. Acid-induced solubilisation of calcium

To follow the details of the solubilisation of calcium during acidification in the UF and DF samples, the amount of calcium present in the non-sedimentable fraction was measured during acidification with GDL. It is well-known that during acidification charge neutralization occurs, and there is a gradual solubilization of CCP from the interior of the casein micelles (Dalgleish and Law, 1988; Walstra, 1990; Holt et al., 1992; Le Graët and Gaucheron, 1999).
Figure 4.2 Buffering capacity (titration only to pH 2) for milk concentrated 2× (A) and 4× (B) by UF (squares) and DF (triangles). Unheated (filled symbols) and heated samples (empty symbols) are shown. Curves are representative of three replicate samples.
The amount of calcium recovered in the centrifugal supernatant for 1×, 2× and 4× milk concentrated by UF (unheated and heated) is shown in Figure 4.3. In agreement with what has been previously reported on reconstituted casein micelles (Le Graët and Gaucheron, 1999), the solubilization of colloidal calcium occurs continuously during acidification. The solubilization increased around pH 5.5, and in the case of 1× milk, it seemed to reach a plateau at pH 4.6. In general, this behaviour was similar in all samples; however, in concentrated samples, there was a lag phase before the concentration of calcium in the supernatant started to increase at a pH of about 5.8. After pH 5.5, there was a higher concentration of calcium in the non-sedimentable fraction (Figure 4.3) due to the higher volume fraction of casein present in 2× and 4× concentrated milk compared to control milk.

Figure 4.3 also shows the effect of heat treatment of concentrated milk on the release of calcium with acidification. Heating causes denaturation of whey proteins and formation of complexes of whey proteins with casein micelles or soluble whey protein/κ-casein aggregates (Corredig and Dalgleish, 1996; Singh et al., 1996; Dalgleish et al., 1997; Guyomarc'h et al., 2003; Alexander and Dalgleish, 2005; Donato et al., 2007a; Donato et al., 2007b; Anema, 2008b; Donato and Guyomarc'h, 2009). The behaviour of heated samples was similar to that of unheated samples, for control milk. In concentrated milk, there were no differences at pH > 5.5 with heating. In addition, no significant differences were noted for milk concentrated 2×. On the other hand, more calcium was released at pH < 5.5 in heated than unheated 4× concentrated milk. These observations may suggest that heating has a significant effect on the structural arrangements of casein micelles concentrated to high volume fractions by UF,
Figure 4.3 Amount of calcium recovered in the centrifugal supernatant of 1× control (circles), 2× UF (squares), 4× UF (diamonds) milk, unheated (filled symbols) and heated (empty symbols) as a function of pH. Values are the average of two independent experiments, and bars represent standard deviation.
and the greater release may be attributed to a combination of heat-induced complexes and casein micelle dissociation with pH.

Figure 4.4 illustrates the percentage of calcium solubilized as a function of casein concentration for the UF milk samples at four different pH values between 6 and 4.6. Acidification was conducted using GDL. The total calcium solubilized at various pH was proportional to the amount of caseins present (Figure 4.4). However, at pH 4.6 only about 88% of the total calcium was solubilized in heated 4× UF milk, and this amount was both significantly lower than for 1× milk and significantly higher than the amount measured in unheated 4× UF.

In addition to studying the effect of concentration, the present work also determined possible differences in the calcium release during acidification in milk concentrated by DF, and the effect of heating at 80 °C for 15 min. No information is available on the effect of DF and UF on calcium release. Figure 4.5 compares the amount of calcium released in the non-sedimentable fraction as a function of pH for milk concentrated by UF or UF combined with DF. UF and DF samples showed similar trends, both for 2× and 4× milk; however in 2× milk, there was significantly less amount of calcium in DF concentrated milk compared to UF milk (Figure 4.5A). This behaviour was no longer noted for milk concentrated 4× (Figure 4.5B). These observations are in line with the buffering capacity findings (Figures 4.1 and 4.2): at high concentration rates, UF causes some changes to the CCP equilibrium, even without the addition of water during DF.
Figure 4.4 Amount of calcium recovered in the non sedimentable fraction after centrifugation as a function of casein concentration, measured at four pH values (6.0, 5.5, 5.0, and 4.6). Symbols are 1× control (circles), 2× UF (squares), 4× UF (diamonds) milk, unheated (filled symbols) and heated (empty symbols). Results are averages of two separate experiments.
Heating treatment did not seem to have an impact on the release of calcium 2× concentrated milk by UF and DF (Figure 4.5A), but it showed significant differences at 4× concentration, where in both cases, UF and DF samples, there was a greater amount of calcium recovered in the centrifugal supernatants after heating (Figure 4.5B), in agreement with the results for UF retentates (Figure 4.3).

CCP is in dynamic equilibrium with the mineral components in the soluble phase (Holt et al., 1989). It has been previously hypothesized that as milk is already saturated with calcium phosphate, a considerable proportion of soluble calcium and phosphate may be transferred into the colloidal state during heating of concentrated milk (Anema, 2009a). The amount of insoluble calcium (related to the amount of CCP) adjusted for the amount casein in the samples is shown in Figure 4.6, as a function of concentration of protein. Figure 4.6A shows the values for all treatments at pH 5.0, while Figure 4.6B at the natural pH. In Figure 4.6A, the amount of CCP per casein remained constant with protein concentration by UF at pH 5.0, which is about 0.35 ± 0.03, but these amounts were significantly greater than those of DF samples. However, the values of CCP present per casein decreased significantly with membrane filtration and was also significantly lower for DF samples compared to UF samples in Figure 4.6B. The amount seemed to level off to about 0.72 ± 0.03 for UF and 0.61 ± 0.02 for DF milk at 2× SM, which are significantly lower for UF and DF than at 4× SM. It can be concluded that UF and DF have a significant effect on the structure of casein micelles, causing losses of colloidal calcium that may be reflected in a higher release of soluble caseins at natural pH.
Figure 4.5 Amount of calcium recovered in the centrifugal supernatant of 2× (A) and 4× (B) concentrated milk as a function of pH. Milk was concentrated by UF (squares) and DF (triangles) milk. Results show unheated (filled symbols) and heated (empty symbols). Values are the average of two independent experiments, and bars represent standard deviation.
**Figure 4.6** Amount of insoluble calcium divided by the amount of casein present in the milk as a function of protein concentration in the samples: 1× control (circles), 2× UF (squares), 4× UF (diamonds) and 2× DF (triangles), 4× DF (down triangles), unheated (filled symbols) and heated (empty symbols), at pH 5.0 (A) and natural pH (B).
4.5. Conclusions

The buffering capacity and calcium release during acidification depend on the protein concentration, but not proportionally, particularly at volume fractions > 2×. It may be possible to hypothesize that the shear and mixing occurring during membrane filtration cause modifications to the casein micelles and serum environment. There are clear differences in the dynamics of the casein micelles concentrated by DF compared to UF, in spite of the similarities in protein concentration. However, this differentiation depends on the protein concentration. At relatively low concentration, there was a lower buffering capacity and amount of solubilized calcium in the DF milk than UF milk; however, there was no significant difference in the buffering capacity and calcium release in the DF and UF milk at 4× volume fractions. During DF, dilution of the serum phase with water further affects the integrity of casein micelles, resulting in losses of colloidal calcium and compositional changes in the serum. Furthermore, heating milk at 80 ºC for 15 min did not show a significant effect on the distribution of calcium and integrity of casein micelles; however, at high protein concentrations (4× volume fraction by UF and DF), more calcium was solubilized, possibly because of differences in the whey protein aggregates present. These results have important implications in the utilization of retentates and milk concentrates for processing in yogurt and cheese making operations.
CHAPTER 5

INFLUENCE OF HEATING TREATMENT AND MEMBRANE CONCENTRATION ON THE FORMATION OF SOLUBLE AGGREGATES AND THE ACID INDUCED GELATION OF CONCENTRATED SKIM MILK

5.1. Abstract

The objective of this work was to investigate the effects of ultrafiltration (UF) and diafiltration (DF) on the colloidal properties and the acid-induced gelation behavior of concentrated (2× and 4×) heated milk, before and after heating at 80 °C for 15 min. The composition of the soluble fraction, analyzed using a combination of size exclusion chromatography and SDS-PAGE, varied greatly depending on the processing history of the milk retentates (UF or DF, before or after heating). Milk subjected to UF and DF showed a significantly greater amount of non-sedimentable protein (both caseins and whey proteins) compared to UF milk, both in unheated and heated samples. Gelation occurred at a significantly higher pH value for DF than UF milk. At 2× concentration the gel firmness at pH 4.6 was higher for DF milk than for UF milk; on the other hand, this was not the case for 4× concentrated milk, possibly because of limited rearrangements of the casein micelles and heat induced complexes at this volume fraction. This work brings new evidence of the differences in processing functionality between DF and UF retentates, and is of great importance to improve processing parameters.
5.2. Introduction

Membrane processes are widely employed in the dairy industry to standardize or concentrate milk protein content. In ultrafiltration (UF), lactose and small molecular weight components of milk are transmitted through a membrane, retaining proteins and colloidal minerals (Waungana et al., 1999; Karlsson et al., 2005). In spite of the widespread use of this process, the details of the changes in physico-chemical properties of milk protein during membrane filtration and their consequences to the processing functionality of casein micelles are not fully known. Recently, it was shown that the renneting functionality of casein micelles is modified with UF, especially at a concentration ratio > 3× (Ferrer et al., 2011). Little has been reported on the impact of UF or UF in combination with diafiltration (DF) on the properties of casein micelles as they related to their acid gelation.

This study will focus on the effect of the concentration by UF and DF on the physico–chemical properties of heated and unheated retentates and their acid-induced gelation behavior.

Casein micelles are the main building blocks of structure in acid milk gels. The internal structure of the casein micelles is organized in clusters of protein molecules linked by calcium phosphate via their phosphoserine residues. In addition to the electrostatic interactions, these protein particles are held together by hydrophobic, electrostatic and van der Waals forces. Since κ-casein is not involved in the formation of protein/calcium phosphate nanoclusters, it has been described as a chain terminator in the assembly of the caseins (Horne, 2002). Moreover, κ-casein is known to play a major role in the colloidal stabilization of casein micelles by extending its caseinomacropeptide
(CMP) moiety (residues 106-169) into the surrounding serum, providing steric and electrostatic repulsion to the protein particles in milk (DeKruif, 1998).

Casein micelles can be readily destabilized by acid, using lactic acid bacteria or acidulants such as glucono-δ-lactone (GDL). During acidification, as the pH decreases from the natural pH of 6.7 the surface charges of the casein micelles decrease and the calcium phosphate nanoclusters, associated with the phosphoserine residues in the caseins, are progressively diffused into the serum phase (Le Graët and Gaucheron, 1999). The change in pH causes the collapse of the κ-casein hairy layer so that steric and electrostatic stabilizations are diminished. The micelles then aggregate as the proteins approach their isoelectric point (Dalgleish et al., 2004; Donato et al., 2007a). The acidification of milk results in several structural and compositional changes in casein micelles, which lead to their aggregation and gelation at pH ≈ 4.9 (Horne, 1998; Lucey and Singh, 1998).

Although the details of the acid gelation behaviour of casein micelles are well understood in both unheated and heated milk (Alexander et al., 2006; Donato et al., 2007a), much less has been reported on the effect of concentration on the aggregation, in particular, concentration by membrane filtration.

It was previously reported that when water is removed and the volume fraction of casein micelles is increased, the distance between the casein micelles may result in the partial collapse of the κ-casein layer (DeKruif, 1998). As a consequence, the rate of aggregation of casein micelles in a concentrated system would increase, because of an increase in the probability of effective collisions (DeKruif, 1998). In addition, Famelart and others (2004) studied the effect of dilution with water on acid induced gelation of
casein micelles, showing that gelation occurs earlier in diluted milk. The difference was attributed to a reduction of negative charges on casein micelles. The present work studied for the first time on the differences in acidification behavior of casein micelles as they related to their UF and DF processing history.

Heat treatment is an essential process to improve the textural properties of acid milk gels (Lucey et al., 1997a; Lucey et al., 1997b). Heat treatment of milk prior to acidification results in an increasing pH of gelation, reduction in gelation time and a significant increase in the firmness of the gels. Heat treatment at temperatures above 70 °C causes irreversible denaturation of whey proteins (Anema et al., 2003). The denatured whey proteins (β-lactoglobulin and α-lactalbumin) bind covalently to κ-casein or αs-casein and are present either on the micelle surface or in the serum phase (Guyomarc'h et al., 2003; Donato et al., 2007a). The presence of these aggregates has a strong influence on the mechanisms of structure formation and impact the structure and mechanical properties of the gels by bridging between the acidified micelles in skim milk (Singh et al., 1996; Lucey et al., 2000; Donato et al., 2007a).

Much less information is available on the effect of heating on concentrated milk after UF and DF processes despite some work reported on reconstituted milk (Anema, 2000; Anema, 2008b; Anema, 2009b). Previous work (Chapter 3) demonstrated that the composition of the soluble aggregates depends on the concentration factor of milk, and α-lactalbumin and other caseins are found in greater amounts in the soluble aggregates of concentrated milk. It is possible then to hypothesize that UF and DF milk retentates will show modified acidification behavior compared to unconcentrated milk.
The objective of this work was to further extend the knowledge of the gelation behaviour of casein micelles in milk concentrated by membrane filtration. In the present work, the acid gelation of concentrates was followed using rheology and diffusing wave spectroscopy. The major novelty of this work is the focus not only on the effect of the concentration by UF, but also DF on the heating and gelation properties of the retentates.

5.3. Materials and Methods

5.3.1. Sample preparation

The details of sample preparation were described in 4.3.1. Milk samples, pre-incubated at 40 °C were acidified using glucono-δ-lactone and vigorously mixed for 30 s. The amount of GDL added was 1.3% w/w for 1× control; 1.8% w/w for 2× UF and DF; 3.0% w/w for 4× UF and DF, to account for differences in the buffering capacity of the protein. Samples were then incubated at 40 °C, and structure formation was followed using DWS and rheology. The pH was also continuously monitored by an AR 15 pH meter (Fisher Scientific, Mississauga, Ontario) at 40 °C for each sample.

5.3.2. Separation of non-sedimentable fraction

The non-sedimentable fraction was separated from the precipitated casein micelles by centrifugation at 25,000× g for 1 h at 20 °C using a Beckman Coulter Optima LE-80K ultracentrifuge (rotor type 70.1 Ti, Beckman Coulter Canada Inc., Mississauga, ON, Canada) as previously described in 3.3.3.

5.3.3. Size exclusion chromatography (SEC)
The centrifugal supernatant (1 mL) was separated by size exclusion chromatography using a Pharmacia XK 16/70 column with a packed bed height of 67 cm and S-500 Sephacryl high-resolution gel (GE Healthcare) with a nominal fractionation range of $40 - 2 \times 10^4$ kDa (Amersham Biosciences Inc., Baie d’Urfé, Quebec, Canada). For details see 3.3.4. The peak areas were calculated by OriginPro 8 software (OriginLab Corp., Northampton, MA, USA).

To determine differences in the type and composition of proteins eluted by chromatography, 10 mL fractions were collected and then freeze-dried (VirTis, Gardiner, NY, USA) for further analysis.

5.3.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

The freeze-dried samples were then analyzed to determine the polypeptide composition using SDS-PAGE electrophoresis as described in 3.3.6. Samples were analyzed under non-reducing (without the $\beta$-mercaptoethanol) or reducing conditions.

While 20 µL of the reconstituted fractions were loaded for control samples (1× milk), 5 µL of 4× UF and DF were loaded onto the gel for comparison.

5.3.5. Diffusing wave spectroscopy (DWS)

The preliminary stages of structure formation during acidification were followed using DWS, in transmission mode using a 532 nm laser operating at 350 mW (Coherent, Santa Clara, CA, USA). The optical glass cuvette (5 mm, Hellma Canada Limited, Concord, ON, Canada) was immersed in a tank maintained at 40 ºC by a circulating water bath. For gelation measurements, correlation functions and the intensity of transmitted
scattered light were measured for 2 min until gelation. Experimental data were collected and analyzed as previously reported (Gaygadzhiev et al., 2008).

The static and dynamic behaviour of the caseins can be investigated by DWS without dilution of the sample as it is based on the multiple scattering of photons, and this is of particular importance to this work, where the effect of protein concentration is probed. The changes in the physical properties of the sample as gelation progresses can be determined by analyzing the change of the turbidity parameter \((1/l^*)\) or the radius. \(l^*\), the photon transport mean free path (the length scale over with the direction of the scattered light has been completely randomized), is a function of the scattering form factor and the structure factor (Weitz et al., 1993), and any change in either of these two functions will produce a change in the measured \(l^*\). The apparent particle radius can be derived from the Stokes–Einstein equation from the value of diffusivity, measured from the correlation function. The changes in radius could be considered as an indication of changes in sizes of the particles, which may be related to the collapse of the layer during acidification (Vasbinder et al., 2003; Dalgleish et al., 2004). But it can be taken only as an index of aggregation once gelation has started, because the approximation that aggregates are free diffusing no longer applied.

5.3.6. Calcium determination

Calcium determination by ion chromatography and the details of calcium release during acidification were illustrated in 4.3.4 and 4.3.5.

5.3.7. Rheology
Rheological measurements were performed with a controlled stress rheometer, Paar Physica MC 301 (Anton Paar, Graz, Austria) using a constant strain of 0.01 and a frequency of 1 Hz. The temperature of the plate was controlled at 40 °C by circulating water from a water bath (Julabo Labortechnik, GbmH, Germany).

The main parameters determined were the elastic or storage modulus ($G'$), which is a measure of the energy stored per oscillation cycle, the viscous or loss modulus ($G''$) which is a measure of the energy dissipated as heat per cycle, and $\tan \delta$, which is the ratio of the viscous to elastic properties and related to the relaxation of bonds in the gel during deformation. When the elastic behavior of the systems starts to become dominant, rheological measurements can define the gelation point, at which $\tan \delta = 1$ (Lucey et al., 1997a; Lucey, 2002).

To extrapolate the relation between $\tan \delta$ and the amount of serum Ca, the polynomial regression ($R^2 > 0.85$) of Ca release as a function of pH during acidification was initiated (4.3.5); the amount of serum Ca during gelation was calculated by plotting the rheological ($\tan \delta$) and pH as the independent variables into the polynomial regression; then the correlation of $\tan \delta$ as a function of calculated amount of serum Ca was plotted by Sigmaplot (SPSS Inc., Chicago, IL, USA) (see Figure 5.7).

5.3.8. Confocal scanning laser microscopy (CSLM)

Confocal microscopy was employed to characterize the final microstructures formed after acidification. Rhodamine B was added as a preferential stain for protein. After addition of GDL, the sample was placed on a cavity slide, covered with a cover slip, and sealed in a Petri dish. The samples were incubated in a temperature controlled water
bath at 40 °C for approximately 4 h after gelation. The samples were then observed in the CSLM with a 63× oil immersion objective. The upright Leica DM RE microscope (Leica, Wetzler, Germany) was connected to a Leica TCS SP2 system with 3 different visible light lasers, covering 6 excitation wavelengths. The laser was adjusted in the green fluorescence mode, which yielded an excitation wavelength at 543 nm.

5.3.9. Statistical analysis

All experiments are carried out in triplicate (i.e. three separate milk batches and concentrations by filtration), and the average and standard deviations are reported, as described in 3.3.7.

5.4. Results

5.4.1. Analysis of the non-sedimentable fraction

It is known that the properties of acid-induced milk gels are affected by the presence of non-sedimentable aggregates (Donato and Dalgleish, 2006). In this work, the non-sedimentable phase was separated using SEC, and the eluted fractions were analyzed by electrophoresis. Figure 5.1 illustrates the elution patterns of the centrifugal serum fractions from 1× (control), UF concentrated (2× and 4× UF) and UF and DF concentrated milk (2× and 4× DF) before (A) or after (B) heating at 80 °C for 15 min.

The elution behaviour of centrifugal supernatant separated from control (1×) milk was in full agreement with previous literature for both heated and unheated milk (Donato and Dalgleish, 2006). The profiles showed four regions identified in the figure as P1-P4. Peak 1 did not contain protein, while Peak 4 contained residual unaggregated whey
proteins. Peak 4 was the prominent elution peak in unheated samples (Figure 5.1). All samples showed an intermediate peak eluting earlier than the unaggregated whey proteins, but this intermediate peak was different between heated and unheated samples (Peak 2 and 3, respectively). A peak eluting at about 70 min (Peak 2) was observed for supernatants from heated milk samples (Figure 5.1B), while in the case of unheated milk, the peak eluted later, at about 80 min (Peak 3).
Figure 5.1 SEC elution profiles of the centrifugal supernatant for milk concentrated by UF (filled symbols) and DF (empty symbols), unheated (A) and heated (B): 1× control (circles), 2× (squares), 4× (triangles). The chromatograms are representative of three replicate runs.
It has been previously demonstrated that for 1× milk, Peak 2 is composed of soluble complexes of β-lactoglobulin and κ-casein (Guyomarc'h et al., 2003; Donato et al., 2007b). This peak showed a later elution time compared to that of concentrated milk samples. In addition, in concentrated samples, this peak was much larger, and as shown in Chapter 3, the complex contained other caseins and α-lactabumin. Peak 3 was distinct from that of the native whey protein in Peak 4 (Figure 5.1A), but could not be distinguished from Peak 2 in heated samples (Figure 5.1B).

Peak 3 consisted mostly of non-sedimentable caseins, while peak 4 contained unresolved native whey proteins, as previously reported (Donato et al., 2007b). The changes in the peak area as a function of volume fraction of the concentrates, as well as the differences between UF and DF milk retentates, were quantified as shown in Figure 5.2 for unheated and heated milk.

As expected, the amount of soluble material eluted in the peaks increased with protein concentration in the retentates; however, there were also significant differences between UF and DF milk samples, within the same volume fraction. There were differences in the soluble oligomers and caseins eluting in Peak 3 (Figure 5.2A) for 4× concentrated unheated milk, suggesting that there was a greater amount of soluble complexes in 4× DF retentates compared to 4× UF milk. Furthermore, in heated milk, there was a significant difference in the amount of soluble aggregates eluting in Peak 2, with a greater amount for 2× DF and 4× DF compared to the respective UF concentrates (Figure 5.2C).
Figure 5.2 Changes in the area of elution peaks (see Figure 5.1) as a function of the total protein concentration in the original retentate. Unheated milk (A and C), heated milk (B and D). Peak 3 (A), Peak 2 (B) and Peak 4 (C and D, unheated and heated, respectively). Values are the average of three independent experiments, error bars represent standard deviations. Symbols are as for Figure 5.1: 1× control (circles), 2× UF (filled squares), 2× DF (empty squares), 4× UF (filled triangles), 4× DF (empty triangles). Note the difference in the y axis scales.
Figure 5.2 (C and D) illustrates the average changes in the peak area for Peak 4. In unheated milk samples (Figure 5.2C) the amount of unaggregated whey protein increased proportionally with concentration, as expected, with no difference between DF and UF milk. When compared with the results for the area of Peak 3 (Figure 5.2A), which contained non-sedimentable proteins, the data would suggest a greater extent of disruption of casein micelles with DF than with UF for high volume fractions (4×).

With heating (Figure 5.2D), there was a significantly lower amount of residual whey proteins (Peak 4) in DF concentrates compared to UF concentrates, both at 2× and 4× concentrations. This was also reflected in the increase in the area of peak 2, which has been previously shown to contain soluble complexes of whey proteins and κ-casein in skim milk (Donato and Dalgleish, 2006; Donato et al., 2007b; Donato and Guyomarc'h, 2009).

In addition to a greater peak area, the 4× UF and DF retentates also showed an earlier elution for Peak 2 (Figure 5.1B) compared to the 2× concentrated samples, suggesting the presence of larger aggregates or aggregates with different composition. The various fractions eluted by SEC were further analyzed by SDS-PAGE, as shown in Figure 5.3. It is important to note that to be able to compare the samples to the control or to the previous literature the loading volume of control milk (1×) was 4× that of the concentrated milk. Figure 5.3 shows the composition for the fractions eluted 60 – 70, 70 – 80 and 80 – 90 min.

The results were in full agreement with those reported in Chapter 3. The unheated supernatant fractions contained β-1g and some caseins, mostly αs-cas and κ-cas.
Figure 5.3 (A) SEC elution profiles of the centrifugal supernatants for control (filled circles) and 4× milk prepared by UF (filled triangles) or DF (empty triangles), unheated (left) or heated (right). (B) SDS-PAGE patterns (under reducing conditions) of the serum in unheated and heated samples collected from the SEC column at a 10 min interval of elution time ranging from 60 to 90 min, where the maximum values are for the presence of heated-induced complexes. Sample preparation and conditions are described in Materials and Methods. For better comparison, loads of 20 µL for control samples and 5µL for 4× UF and 4× DF were used. The bands in the gels are identified as (i) minor WP; (ii) α₅-cas; (iii) β-cas; (iv) κ-cas; (v) β-lg; (vi) α₅-la.
The band intensities in the supernatant of unheated 4× UF and 4× DF were much higher than those of control samples. The aggregates also eluted earlier (see Figure 5.3A). Hence, it was concluded that unheated 4× UF and 4× DF had more non-sedimentable aggregates and these aggregates were larger in size, compared to control unheated milk.

In unheated skim milk, most of the protein eluted at 70 – 80 min was casein; however, β–lg was also recovered in the fraction eluted between 80 and 90 min in unheated concentrates. No α–la was recovered in unheated milk. It is possible that the presence of β–lg was due to the co-elution of oligomers of the whey protein. In heated samples (Figure 5.3, right hand side), in addition to κ–cas and β–lg, α–la and other caseins were also present in the fractions, indicating the formation of whey protein–casein aggregates.

To better quantify the changes in composition, the ratio between the protein bands was measured by densitometry, and the results are summarized in Figure 5.4, for the fraction eluting between 80 and 90 min (see Figure 5.3). As compared to the fractions collected from unheated milk, there was a dramatic increase in the ratio of whey proteins recovered in the heat-induced complexes. Among all the samples, both fractions (eluted at 70– 80 and 80 –90) showed an increase in the total casein from control to 4× UF, to 4× DF. This would be consistent with the presence of additional non-sedimentable caseins dissociating from casein micelles during extensive filtration and involved in the formation of heat-induced complexes.

Figure 5.4 summarizes the ratio of whey proteins to caseins as well as to κ–cas for fractions eluted between 80 and 90 min. Due to the presence of heat denatured whey proteins interacting with caseins, the ratio of whey proteins and caseins in supernatant
fractions of heated milk was significantly greater than those from unheated milk. The ratio of $\alpha$–la increased with concentration in heated milk in relation to both $\beta$–lg and $\kappa$–cas. In particular, the ratio of $\alpha$–la to $\beta$–lg increased in the heated samples, from 0 to 0.5 and 1 in skim milk, 4× UF and 4× DF (Figure 5.4B, open squares). These results demonstrate that the processing history affects not only the extent of aggregate formation, but also changes in the composition.

The ratio of $\beta$–lg to $\kappa$–cas decreased significantly from 1× to 4× milk and the amount of caseins present in the aggregates increased in heated concentrates as shown in Figure 5.4B (open diamonds). Similar results were also obtained for the fractions isolated between 70 and 80 min. These results are in contrast with what has been previously published in the literature from studies on reconstituted milk powder (Anema, 2000). The discrepancy in the results is probably due to the composition of the serum phase, as concentrated milk from reconstituted skim milk powder contains a greater amount of solids. Indeed, as shown in Figure 5.4, there was a decrease in relative area of $\beta$–lg in the complexes, in the order: 4× DF < 4× UF < control, consistent with an increase in $\alpha$–la in the aggregates. These changes in composition and size of the soluble aggregates may have profound consequences on the gelation behavior of concentrated milk prepared by UF or UF and DF.
Figure 5.4 Changes in the band intensity ratio for α-la/κ-cas (circles), β-lg/κ-cas (triangles), α-la/β-lg (squares) and whey/CN (diamonds) for proteins eluted between 80-90 min (Figure 5.3) from the serum phase of heated (empty symbols) and unheated samples (filled symbols). Results are the average of 3 independent experiments.
5.4.2. Acid induced gelation

5.4.2.1. Unheated retentates: Effect of concentration.

The gelation behavior of retentates prepared by UF or DF was compared to that of 1× skim milk using DWS and rheology. The parameters measured in situ, during acidification as a function of pH, are shown in Figure 5.5 for unheated samples. The size of the casein micelles did not change with concentration, but the values of $1/l^*$ increased with concentration as already reported in the literature (Sandra et al., 2011). In this work, values of $1/l^*$ and the radius were normalized to better compare the changes among treatments.

As previously reported in the literature, gelation of unheated 1× milk occurred at about pH 4.8 (Horne, 2003). The aggregation point occurred at an earlier pH for 2× and 4× milk (Figure 5.5A). Furthermore, there was a significant effect of DF compared to UF at both 2× and 4× concentration. Table 5.1 summarizes the values of the gelation parameters for the unheated retentates. The pH of gelation was similar to that of control milk only for 2× UF, while 4× UF retentates showed aggregation at a higher pH. 2× DF retentates gelled at pH 5.1 and 4× DF milk at pH 5.2 (Table 5.1).

Structure formation can also be followed by measuring the turbidity parameter ($1/l^*$) as a function of pH, as shown in Figure 5.5C. The $1/l^*$ value remained unchanged until a critical pH, after which there was an increase. The critical pH value (Table 5.1) was always higher than the pH of aggregation measured by light scattering. The onset of change in the turbidity parameter was clearly affected by concentration, but, unlike the aggregation point, there were no statistical differences between DF and UF treatments.
Figure 5.5 Changes in DWS and rheological parameters during acidification of unheated control milk (circles) or milk concentrated by UF (filled symbols) and DF (empty symbols): 1× control (circles), 2× (squares), 4× (triangles). Normalized apparent particle radius (A), storage modulus (B), normalized turbidity $1/l^v$ (C), and tan δ (D) are shown as a function of pH. The data are representative of at least three replicates. For statistical analysis of the parameters, see Table 5.1.
Table 5.1 Effect of concentration on the acid induced gelation of unheated control and ultrafiltered and diafiltered milk concentrates. Values are the means of at least three separate experiments. The same superscripts in the same column indicate no significant difference (p < 0.05).

<table>
<thead>
<tr>
<th>Unheated milk</th>
<th>1× control</th>
<th>2× UF</th>
<th>2× DF</th>
<th>4× UF</th>
<th>4× DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1/l change</td>
<td>5.55±0.02c</td>
<td>5.62±0.02b</td>
<td>5.67±0.03b</td>
<td>5.87±0.03a</td>
<td>5.89±0.02a</td>
</tr>
<tr>
<td>aggregation pH (DWS)</td>
<td>4.97±0.03d</td>
<td>4.97±0.02d</td>
<td>5.24±0.05b</td>
<td>5.09±0.02c</td>
<td>5.55±0.03a</td>
</tr>
<tr>
<td>gelation pH (rheology)</td>
<td>4.83±0.03d</td>
<td>4.86±0.02d</td>
<td>5.10±0.05b</td>
<td>4.94±0.03c</td>
<td>5.24±0.09a</td>
</tr>
<tr>
<td>G' value at pH=4.60</td>
<td>2.8±0.1e</td>
<td>56±5d</td>
<td>274±11c</td>
<td>1900±34b</td>
<td>2008±42a</td>
</tr>
<tr>
<td>tan δ value at pH=4.60</td>
<td>0.41±0.01b</td>
<td>0.43±0.01b</td>
<td>0.52±0.01a</td>
<td>0.36±0.00c</td>
<td>0.38±0.00c</td>
</tr>
<tr>
<td>tan δ value at maximum</td>
<td>N/A</td>
<td>N/A</td>
<td>0.69±0.03a</td>
<td>0.56±0.02b</td>
<td>0.60±0.02b</td>
</tr>
<tr>
<td>pH value at Tan δ maximum after gelation</td>
<td>N/A</td>
<td>N/A</td>
<td>4.78±0.02a</td>
<td>4.83±0.03a</td>
<td>4.82±0.02a</td>
</tr>
</tbody>
</table>
Figure 5.5 also summarizes the development of the elastic modulus and loss tangent (tan δ) as a function of pH. The gelation point, defined as tan δ = 1 was in general agreement with that measured by DWS (Table 5.1). At this critical pH value, there was a steep increase in the elastic modulus (G′), signifying the development of network structure. In addition to the changes in the gelation point with increasing concentration of retentates, there was also a statistically significant increase of the G′. The value measured at pH 4.6 for all the gels is indicated in Table 5.1. As expected, due to the greater number of linkages formed, greater concentrations corresponded with a larger value of G′. However, it is important to note that, albeit having similar casein micelle volume fractions, the values of G′ were significantly greater in DF retentates compared to UF retentates.

The changes occurring in the value of tan δ as a function of pH are also worth noting in unheated, concentrated milk. The tan δ behaviour of control milk was well in line with previous literature (Horne and Davidson, 1993; Lucey et al., 1996; Lucey et al., 1998; Lucey et al., 1999; Lucey, 2002; Anema et al., 2003; Donato et al., 2007a). The changes in the value of tan δ for 2× UF milk were very similar to those of control milk, with a plateau value of 0.4 at pH 4.6 (Figure 5.5D). However, all the other samples, 4× UF as well as 2× DF and 4× DF, showed a decrease in the tan δ value and a peak at pH 4.8, reaching a value of about 0.6 before decreasing again. The presence of a peak in the value of tan δ signifies the presence of structural rearrangements in the protein network, and this type of behaviour has been reported in the literature for heated acidified skim milk. The peak in tan δ usually occurs whereby milk coagulation occurs at a higher pH than the isoelectric point of the protein. While 2× DF milk showed a larger value of tan δ
at pH 4.6 compared to control milk, 4× milk samples (both UF and DF) showed a significantly lower value of tan δ, suggesting a higher level rearrangement in the gel structure (Table 5.1).

### 5.4.2.2. Heated retentates: Effect of concentration

The change of light scattering and rheological parameters as a function of pH for heated skim milk and heated 2× and 4× UF retentates are shown in Figure 5.6.

As previously discussed for unheated milk, gelation pH was measured by observing the radius of the protein particles as a function of pH (Figure 5.6A), as well as the growth of the elastic modulus by rheology (Figure 5.6B). As for unheated milk, there was a significant effect of concentration, as well as UF and DF treatments, on the pH of gelation measured with the two techniques. The pH of gelation significantly increased with protein concentration, and as previously shown with unheated milk, there were similarities between control and 2× UF milk. On the other hand, a higher pH of aggregation (as measured by light scattering) and pH of gelation (as measured by rheology) were observed for 4× UF, 2× DF and 4× DF at pH 5.37, 5.45 and 5.57, respectively (Table 5.2).

In the case of skim milk, the sharp increase in the development of the radius occurred at pH 5.26 ± 0.03, a pH significantly higher than that for unheated control (pH 4.86 ± 0.03). These observations confirm the well-known shift toward a higher pH of gelation when milk is heated (Alexander and Dalgleish, 2004; 2005; Donato and Dalgleish, 2006; Donato et al., 2007a).
Figure 5.6 Changes in DWS and rheological parameters during acidification of heated skim milk (circles) and milk concentrated by UF (filled symbols) and DF (empty symbols): 1× control (circles), 2× (squares), 4× (triangles). Normalized apparent particle radius (A), storage modulus (G′) (B), normalized 1/l* (C), tan δ (D) are shown as a function of pH. The data are representative of at least three replicates.
Table 5.2 Acid induced gelation of the heated control and ultrafiltered and diafiltered milk concentrates. Values are means of two separate experiments. The same superscripts in the same column indicate no significant difference (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Heated</th>
<th>1× Control</th>
<th>2× UF</th>
<th>2× DF</th>
<th>4× UF</th>
<th>4× DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH for 1/l(^{+}) change</td>
<td>5.60±0.01(^{c})</td>
<td>5.63±0.02(^{c})</td>
<td>5.90±0.05(^{b})</td>
<td>6.0±0.02(^{b})</td>
<td>6.17±0.03(^{a})</td>
<td></td>
</tr>
<tr>
<td>aggregation pH (DWS)</td>
<td>5.26±0.03(^{d})</td>
<td>5.28±0.03(^{d})</td>
<td>5.45±0.05(^{b})</td>
<td>5.37±0.05(^{c})</td>
<td>5.57±0.04(^{a})</td>
<td></td>
</tr>
<tr>
<td>gelation pH (rheology)</td>
<td>5.31±0.05(^{d})</td>
<td>5.38±0.03(^{d})</td>
<td>5.57±0.04(^{c})</td>
<td>5.62±0.04(^{b})</td>
<td>5.71±0.05(^{a})</td>
<td></td>
</tr>
<tr>
<td>G' value at pH=4.60</td>
<td>184±6(^{d})</td>
<td>679±38(^{c})</td>
<td>831±59(^{b})</td>
<td>2281±64(^{a})</td>
<td>2312±92(^{a})</td>
<td></td>
</tr>
<tr>
<td>tan δ value at pH=4.60</td>
<td>0.34±0.01(^{b})</td>
<td>0.35±0.01(^{b})</td>
<td>0.38±0.01(^{a})</td>
<td>0.31±0.00(^{c})</td>
<td>0.31±0.00(^{c})</td>
<td></td>
</tr>
<tr>
<td>tan δ value at maximum</td>
<td>0.54±0.02(^{a})</td>
<td>0.47±0.01(^{b})</td>
<td>0.48±0.01(^{b})</td>
<td>0.42±0.01(^{c})</td>
<td>0.44±0.01(^{c})</td>
<td></td>
</tr>
<tr>
<td>pH value of tan δ at maximum</td>
<td>4.96±0.03(^{a})</td>
<td>4.84±0.02(^{b})</td>
<td>4.83±0.01(^{b})</td>
<td>4.81±0.01(^{b})</td>
<td>4.80±0.01(^{b})</td>
<td></td>
</tr>
</tbody>
</table>
In concentrated milk, the rapid increase of the apparent radius occurred at the same pH, 5.28 ± 0.03 for 2× UF, but at a significantly higher value (pH 5.37 ± 0.05) for 4× UF. Heated DF retentates showed gelation pH at 5.6 and 5.7 for 2× and 4×, respectively.

The earlier aggregation for heated UF and DF retentates compared to control milk was confirmed by the development of the turbidity parameter. The onset of change for 1/l* occurred at an earlier pH for milk with a larger volume fraction. However, unlike for unheated milk, there were statistically significant differences between DF and UF milk concentrated to the same volume fraction (Figure 5.5B, Table 5.2).

The development of storage modulus and loss tangent in heated milk is shown in Figures 5.6 C and D. For all samples, the development of the gel network was very rapid, reaching the high values of G’, as shown in Table 5.2. Although there were statistically significant differences between DF and UF at the same volume fraction, the values of G’ at pH 4.6 were much closer between the treatments compared to the same treatments before heating (see Table 5.1). The development of tan δ as a function of pH was similar among treatments, with a rapid decrease around the gelation pH and a subsequent increase reaching a maximum at pH 4.8 regardless of the concentration factor or mode of concentration. The maximum value of tan δ reached at pH 4.8 for all samples was about 0.5, with lower values associated with increasing volume fraction. The tan δ value at plateau, measured at pH 4.6 (see Table 5.2), was greater for 2× DF milk and lower for 4× UF and DF.
5.4.2.3. Structure rearrangement and calcium release.

The development of tan $\delta$ and its peak at about pH 4.8 has been previously reported for acid-induced gelation of heated milk (Lucey et al., 2001). This maximum peak has been related to the rearrangements of whey protein aggregates as well as the incomplete release of calcium phosphate from casein micelles. The tan $\delta$ behaviour of concentrated retentates was plotted as a function of calcium release during acidification, as shown in Figure 5.7. For better comparison, the same data is plotted as a function of calcium present in the supernatant as well as % of soluble calcium per total calcium present. In all treatments, regardless of concentration, the maximum value of tan $\delta$ after gelation occurred at pH $\sim$4.8 ± 0.03 (see Table 5.1 and 5.2). Table 5.3 summarizes the amount of initial total calcium and calcium present in the supernatant in all the samples during acid-induced gelation.

Unheated 1× and 2× UF show a smooth decrease in the value of tan $\delta$, as previously reported for unheated skim milk. In these samples, gelation occurred after about 80% of the original Ca was present in the soluble phase (Figure 5.7). In 1× milk after heating (Figure 5.7A), gelation occurred at about 60% of calcium release ($697 \pm 52$ mg/L of soluble calcium), and the maximum in tan $\delta$ corresponded to about 70% of calcium released in solution. At the point of maximum tan $\delta$, the amount of colloidal calcium still present in the micelles was about 4.8 mg/g casein. Figures 5.7B and C illustrate the differences in the tan $\delta$ behavior for 2× and 4× concentrated samples. Only unheated concentrated 2× UF milk showed a Tan $\delta$ behaviour similar to that of unheated skim milk (control). All the other unheated concentrated milk samples (2× DF and 4× UF and DF) showed a Tan $\delta$ signature quite similar to that of heated 1× control.
Table 5.3 Calcium concentration of the control and ultrafiltered and diafiltered milk concentrates. Values are means of two separate experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial concentration of calcium, mg/L</th>
<th>Initial serum calcium concentration, mg/L</th>
<th>Serum calcium concentration at gelation pH, mg/L</th>
<th>Serum calcium concentration at maximum of tan δ, mg/L</th>
<th>Serum calcium concentration at pH = 4.6, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× SM UH</td>
<td>1220 ± 25</td>
<td>294 ± 13</td>
<td>900 ± 43</td>
<td>NA</td>
<td>1021 ± 53</td>
</tr>
<tr>
<td>1× SM H</td>
<td>1205 ± 38</td>
<td>306 ± 15</td>
<td>697 ± 52</td>
<td>877 ± 33</td>
<td>1050 ± 46</td>
</tr>
<tr>
<td>2× UF UH</td>
<td>2163 ± 54</td>
<td>416 ± 27</td>
<td>1650 ± 92</td>
<td>NA</td>
<td>2080 ± 26</td>
</tr>
<tr>
<td>2× UF H</td>
<td>2236 ± 62</td>
<td>436 ± 32</td>
<td>1000 ± 49</td>
<td>1909 ± 53</td>
<td>2131 ± 39</td>
</tr>
<tr>
<td>2× DF UH</td>
<td>1652 ± 41</td>
<td>252 ± 13</td>
<td>816 ± 32</td>
<td>1128 ± 69</td>
<td>1526 ± 63</td>
</tr>
<tr>
<td>2× DF H</td>
<td>1592 ± 39</td>
<td>272 ± 18</td>
<td>535 ± 35</td>
<td>1263 ± 67</td>
<td>1527 ± 29</td>
</tr>
<tr>
<td>4× UF UH</td>
<td>3093 ± 89</td>
<td>526 ± 41</td>
<td>1850 ± 73</td>
<td>2080 ± 78</td>
<td>2592 ± 82</td>
</tr>
<tr>
<td>4× UF H</td>
<td>3176 ± 83</td>
<td>589 ± 44</td>
<td>861 ± 64</td>
<td>2495 ± 111</td>
<td>3071 ± 78</td>
</tr>
<tr>
<td>4× DF UH</td>
<td>2448 ± 64</td>
<td>473 ± 36</td>
<td>1273 ± 57</td>
<td>1904 ± 68</td>
<td>2240 ± 67</td>
</tr>
<tr>
<td>4× DF H</td>
<td>2476 ± 79</td>
<td>538 ± 44</td>
<td>823 ± 67</td>
<td>2133 ± 79</td>
<td>2421 ± 31</td>
</tr>
</tbody>
</table>
**Figure 5.7** Differences in the relation of \(\tan \delta\) and amount of serum Ca, mg/L (Left), and serum Ca of total Ca, % (right) in the samples of 1× SM (circles), 2× UF (squares), 2× DF (diamonds), 4× UF (triangles) and 4× DF (down triangles), unheated (filled symbols) and heated (empty symbols).
In the case of 2× unheated DF retentates, gelation occurred at about 50% calcium release, with a peak in the tan δ at almost 70% calcium release (Figure 5.7B) at a concentration of calcium of 1128 mg/L in the serum phase, corresponding to about 10.1 mg of colloidal calcium per g of casein. 2× UF and DF heated retentates showed a similar tan δ behaviour as a function of % calcium released, both showing a maximum peak at about 80% calcium release. In all cases, the maximum tan δ corresponded to pH 4.8 and similar amounts of soluble calcium present in unheated and heated concentrates, with less soluble calcium for DF than UF samples. In the 2× DF and UF retentates after heating, the amount of residual colloidal calcium was similar (6.34 and 6.27 mg/g of casein, for DF and UF respectively).

In the 4× retentates, both unheated and heated showed a maximum in the peak of tan δ at pH 4.8 (Tables 5.1 and 5.2). 4× UF showed a maximum of tan δ at 70 and 90% total calcium released, respectively, while 4× DF samples showed the maximum peak at 80% and 90%, respectively. The colloidal calcium phosphate residuals in these samples, at the maximum tan δ peak were 9.7 and 6.5 mg/g of caseins for 4× UF unheated and heated, respectively. These values were lower in the DF retentates, 5.2 and 2.45 mg/g of colloidal calcium for unheated and heated samples, respectively. These data clearly suggest that the residual presence of colloidal calcium phosphate does not play a major role in the tan δ behaviour after gelation, as the extent of the maximum peak as well as the position occurs at pH 4.8 with different extents of calcium release, ranging between 10 and 5.2 mg/g for unheated samples and 6.5 to 2.45 mg/g for heated samples.
5.4.2.4. Microstructure of gels

CSLM was employed to probe the structure of the gels approximately 4 hr after the onset of acid-induced gelation at 40 °C. The microstructure of protein gels obtained from retentates concentrated 4× were compared with those of control 1× milk. In the case of the ultrafiltered concentrates, in unheated 4× UF (Figure 5.8C), the gel structure showed a finer structure with high connectivity in the high volume fraction of proteins. Moreover, the micrographs of 4× DF unheated milk that have been acidified (Figure 5.8E) show increased clustering of casein particles in the sponge-like network with greater gel stiffness than ultrafiltered concentrate.

5.5. Discussion

The changes occurring during heating of skim milk and the effect of the whey protein aggregates on the acid-induced behavior of casein micelles are well known (Dalgleish, 1990; Horne and Davidson, 1993; Lucey et al., 1997a; Lucey et al., 1998; Lucey et al., 1999; Vasbinder et al., 2001; Donato et al., 2007a; Donato and Guyomarc'h, 2009). However, less well-known is the influence of concentration by membrane filtration on the properties of retentates. In this work, we studied the effect of concentration using UF or UF combined with DF as membrane filtration selectively concentrates proteins, causing changes to the mineral balance, and this can have a profound influence on the processing behaviour of casein micelles. This work not only focused on understanding the effect of concentration ratios, but also distinguished between UF and DF as these two processes cause major differences to the composition of the serum phase.
Figure 5.8 Confocal scanning laser micrographs of gels made from unheated 1× control (A), heated 1× control (B), unheated 4× UF (C), heated 4× UF (D), unheated 4× DF (E), and heated 4× DF (F). The protein matrix appears red while pores appear dark color. Image size: 50 × 50 µm.
There were significant differences in the composition of the non-sedimentable serum fraction between DF and UF unheated 4× concentrated milk. These differences also became significant at lower concentration ratio (2×) in heated milk. A greater amount of soluble aggregates were present in DF compared to UF milk at the same concentration, and that was reflected in a greater extent of aggregation of whey proteins in heated DF retentates compared to unheated (Figures 5.1 – 5.3).

Previous work reported a decrease in the rate of denaturation of whey proteins as a function of concentration (Anema, 2000). However, the results derived from these studies were on reconstituted skim milk, and the discrepancy may be due to differences in the serum composition (mineral and lactose) (Anema, 2000). Results shown in Figure 5.1 and 5.2 demonstrated that whey protein denaturation occurred to a greater extent in DF concentrates than UF concentration. DF retentates had lower ionic strength and greater amounts of soluble caseins.

Non-sedimentable aggregates eluted earlier in retentates containing greater protein concentrations and contained more caseins (αs, β and κ–cas). There were no differences in the protein composition of supernatant from UF and DF unheated retentates at the same concentration. In heated skim milk, the main proteins present in the collected fractions were κ–cas and β–lg, with a β–lg to κ–cas ratio of 2 (Figure 5.4). The presence of β–lg in the soluble aggregates of skim milk has been previously reported in the literature (Donato and Dalgleish, 2006; Donato et al., 2007a; Anema, 2008b; Donato and Guyomarc’h, 2009). κ–cas was dominant among the caseins, although some αs–cas was also present, and in much greater amounts in the 4× retentates samples. Compared to the control, 4× retentates showed a larger amount of protein in the eluted fraction, in spite of being
diluted back to the original volume ratio. In addition to $\kappa$-cas, $\beta$-lg and $\alpha$-la were recovered in the aggregates of heated retentates. These differences in composition of the serum phase played a major role in the gelation behaviour of milk concentrated by UF and DF. Two levels of concentration were tested in this work, $2\times$ and $4\times$, and it was clear that although an increase in volume fraction of the milk proteins caused an increase in pH of gelation, there was a significant effect of processing history as DF retentates showed coagulation at a much earlier pH than UF retentates with the same concentration ratio, as shown in Table 5.1 for unheated milk. With heating, the composition of the retentates was further modified, causing a significantly earlier gelation pH compared to the unheated retentates, as shown in Table 5.2.

In unheated milk, the aggregation pH of 1x control was $\approx \text{pH 4.9}$, consistent with previous studies (Lucey et al., 1996; Donato and Dalgleish, 2006; Alexander and Dalgleish, 2007; Donato et al., 2007a; Donato and Guyomarc'h, 2009). The pH of gelation measured with rheology was in general agreement with DWS measurements, indicating that in all cases the aggregation of the casein micelles was the major event causing the change in the viscoelastic properties of the gels.

The difference in the aggregation behavior as a function of concentration has been previously discussed in the literature (de Kruif, 1998). An increase in the volume fraction of the casein micelles causes a decrease in the average distance between them, increasing the extent of the attractive forces. In the experiments shown in Figure 5.5, there were no significant differences in the pH of gelation between control and $2\times$ UF, suggesting that this concentration factor is not sufficient to affect gelation in spite of the already very
small average distance between the micelles. It has been shown that at this volume fraction the casein micelles still show free diffusive motion (Krishnankutty Nair et al., 2013), and this may be the reason for the similarity in the gelation behaviour of the casein micelles compared to the control.

On the other hand, this was not the case for unheated 4× UF (Table 5.1). The closer proximity of casein micelles to one another was also reflected in their turbidity measurements as a function of pH for unheated milk (Figure 5.5B). The turbidity parameter (1/l*) measured by light scattering remained constant until a critical pH. This pH value was higher than the pH of gelation: the 1/l* value showed an increase around pH 5.5 for control unheated milk and at earlier pH values depending on the volume fraction, with no statistical differences between UF and DF milk (Table 5.1). This was the case for unheated milk where whey proteins do not play a major role in inducing acid destabilization. In the case of heated milk, the pH of the onset of change in the turbidity was higher than in unheated milk, and DF retentates showed an even earlier onset than UF retentates at the same concentration, indicating the major role played by the changes in composition of the serum fraction. The increase in the 1/l* during acidification is the result of a combination of changes in the refractive index contrast as well as changes in the spatial distribution of the casein micelles, which are no longer hard spheres but adhesive hard spheres (de Kruif, 1998; Alexander and Dalgleish, 2005). High concentration of protein caused an increase in scattering events occurring in a given scattering volume as the spatial distance between the micelles decreased, causing a reduction of the transport mean free path, i.e. an increase in the initial turbidity parameter, 1/l*. 
With the increasing volume fraction of caseins, it is expected that the interactions and gelation point would occur earlier due to the reduction in micelle-micelle average distance (de Kruif and Zhulina, 1996). The increased frequency of collisions, and the increase in the number of bonds and contact area in the gel network resulted in increased gel-firming rates, as previously demonstrated (Anema, 2008a), and resulted in an increase in the $G'$ for concentrated samples. Although the gelation pH of 2× UF was not significantly different compared to that of control skim milk, these samples demonstrated a greater $G'$ at pH 4.6.

It is well understood that the formation of heat-induced whey protein aggregates has important consequences to the acid-induced gelation of milk. The aggregates are present either in the non-sedimentable fraction or they are associated with the casein micelles, and their distribution affects the structure and texture of the final acid gel (Corredig and Dalgleish, 1996; Singh et al., 1996; Dalgleish et al., 1997; Guyomarc'h et al., 2003; Alexander and Dalgleish, 2005; Donato et al., 2007a; Donato et al., 2007b; Anema, 2008b; Donato and Guyomarc'h, 2009).

There was an increase in the aggregation pH and gelation pH (measured by light scattering and rheology, respectively) after heating (compare Table 5.1 with 5.2). In heated skim milk, the gelation point was about 5.2, a significantly higher pH than that for unheated control milk (pH 4.86 ± 0.03). These observations confirmed the well-known shift toward higher gelation pH when skim milk is heated (Alexander and Dalgleish, 2004; 2005; Donato and Dalgleish, 2006; Donato et al., 2007a). In the case of concentrated milk, as for unheated milk, the higher pH of aggregation was attributed in part to the changes in the volume fraction of casein micelles and also to the presence of a
different population of soluble aggregates. As in the case of the skim milk control, it is possible to conclude that the complexes formed bridges between the casein particles and provided increased linkages in the acid gel network (Davies et al., 1978; Mottar et al., 1989).

It is also important to point out that UF and DF retentates contained different concentrations of calcium in the serum phase and showed a larger release of calcium as acidification progressed (Table 5.3). The increase in the amount of $\text{Ca}^{2+}$ in solution may have also caused an earlier destabilization for the micelles and the aggregates. It has been previously reported that free calcium and overall ionic balance may also play a role in acid induced gelation (Famelart et al., 2004; Anema, 2009a; Anema, 2009b). It is important to note that the total amount of calcium in the DF retentates was lower than in the UF retentates, and therefore although the calcium released may play a role in the early destabilization of the casein micelles, the higher $G'$ value at pH 4.6 can be attributed mostly to the presence of more and larger soluble complexes in DF milk than in UF milk.

Unheated control and 2× UF milk (see Figure 5.7) showed a typical tan δ behavior with a sharp decrease with gelation and a continuous decrease, reaching values around 0.42 (for 1× and 2× UF). However, this was not the case for the unheated 2× DF and 4× DF and UF. In these samples, there was a sharp decrease at the gelation point with a subsequent increase and up to a maximum at pH 4.8, regardless of treatment. The extent of the change in tan δ after gelation (Figure 5.7) was much higher for unheated than heated samples, once again suggesting that heated samples showed less structural rearrangements than unheated samples with a smaller value of Tan δ at maximum, but a wider range of change. This transition in the tan δ has been attributed to changes in
protein-protein interaction, due to the solubilization of colloidal calcium phosphate from the micelles and rearrangements in the soluble aggregates (Lucey et al., 1998). These results clearly demonstrate that the solubilization of colloidal calcium phosphate plays a minor role in the rearrangements. At the gelation point, the amount of serum calcium present in 1x control milk was 34.6 and 26.8 mg/g of caseins for unheated and heated milk. In 2x UF milk, the serum calcium present was 32 and 19.2 mg/g of casein for unheated and heated milk. In the 4x UF milk, this concentration was 17 and 8 mg/g of casein, for unheated and heated samples. In the DF retentates, the concentrations were much lower than for UF retentates. Although the amount of calcium solubilized does not seem to be directly related to the early gelation pH of the milk samples, the amount of residual colloidal calcium phosphate may play a role in the values of tan δ and the tan δ changes during acidification, ultimately affecting the rearrangements of the gel network. The amount of colloidal calcium phosphate remaining in the micelles at the maximum value of tan δ was consistently lower in the heated milk compared to unheated milk, and also similar between UF and DF milk samples. However, the peak was much greater for samples containing higher amounts of undissociated colloidal calcium phosphate, suggesting that the release of calcium phosphate is less important than the reorganization of the aggregates present in the continuous phase in the structural rearrangements. Previous authors attributed the maximum in tan δ to the partial loosening of the initial weak gel networks due to the solubilization of CCP or some other physicochemical change in the nature of the casein particles. In particular, the rearrangements were proposed to be a consequence of the early destabilization of whey proteins associated to the casein micelles (Lucey et al., 1998). The transition of tan δ between pH 5.3 and 4.8
also occurred in unheated UF and DF milk. These samples also contained non-sedimentable complexes, composed of whey proteins and caseins. Early destabilization occurred because of the increased presence of soluble aggregates.

As a general summary, a model of the acid gelation of the different systems studied is proposed in Figure 5.9. The nature of the interactions is based on knowledge of the structure of the different protein materials as pH is decreased and the interactions discussed in this thesis, although further work needs to be done to better clarify these reactions.
Figure 5.9 Schematic diagram of the different possible interactions occurring as systems are acidified. The different possible mechanisms are as follows: 11, interactions between casein micelles and casein micelles; 12, interactions between insoluble complexes and insoluble complexes; 13, interactions soluble complexes and insoluble complexes; 14, interactions between soluble complexes and casein micelles; 15, interactions between soluble complexes and soluble complexes; 16, interactions between soluble complexes and casein micelles. (Note: the number of the particles for concentrated milks produced by UF and DF does not exactly represent the extent of concentration).
5.6. Conclusions

This work clearly demonstrated that UF and DF caused changes in the composition of the serum phase in milk and modified the processing functionality of the retentates. There were clear differences in the dynamics of the casein micelles concentrated by DF compared to UF, in spite of the similarities in protein concentration. These modifications caused by UF and DF are caused by differences in the ionic strength and the extent of disruption of the casein micelles. Although very little is known about the effect of DF and heating on the concentrated systems, this work has provided more insights into the effects of concentration processes and heat treatment on the composition of the soluble material responsible for improving acid gel texture will be related to the overall changes occurring during casein micelle aggregation, allowing improvements of processing efficiencies.
CHAPTER 6
OVERALL CONCLUSIONS

Although membrane filtration has been applied in dairy industry for decades, very little was understood on the effect of concentration by ultrafiltration and diafiltration on the physico-chemical properties and processing functionality of the casein micelles. A better understanding of these changes can improve processing efficiencies and obtain novel ingredients. This research presents new findings on the effect of concentration on acid induced gelation. For the first time, it was demonstrated that there is an increased pH of gelation in milk samples concentrated by DF compared to those concentrated by UF.

This study focused on the processing functionality of casein micelles and evaluated the amount of calcium released during acidification, the buffering capacity, and the presence of non-sedimentable material, with and without thermal treatments.

The research did not only further our knowledge on the colloidal properties and behaviour of casein micelles in milk concentrated by UF and DF during acidification, but resulted in a comprehensive understanding of the effect of heating on physico-chemical functionality and properties of casein micelles in concentrated milk.

It was observed that the buffering capacity and calcium release during acidification depend on the protein concentration, but not proportionally, particularly at relatively high concentration. Moreover, the processing history (UF or UF in combination with DF) strongly affected the concentration of colloidal calcium phosphate and increased the level of soluble protein. There were clear differences in the dynamics of the casein micelles concentrated by DF compared to UF, in spite of the similarities in protein concentration.
Concentrating by UF, the volume fraction of protein increased while maintaining the serum composition similar to that of the original milk. In contrast, during DF the dilution of the serum phase with water further affected the integrity of casein micelles and the change in the composition of the soluble phase, resulting in the extensive loss of colloidal calcium along with caseins dissociating into soluble phase. These modifications caused by UF and DF cause variations in the formation, composition and properties of serum materials.

The effect of heat treatment on whey protein denaturation and soluble complex formation depended on a combination of heating temperature, length of heating time and the concentration of milk proteins. With an increase of the heating temperature and heating time, soluble complexes with larger size and greater amounts of whey protein denaturation were observed in the heated samples. The changes in calcium equilibrium and composition and size of the soluble aggregates had profound consequences on the gelation behaviour of concentrated milk prepared by UF or UF and DF. The gelation pH, measured by rheology and diffusing wave spectroscopy, significantly increased with the extent of concentration (p<0.05, as measured by ANOVA), due to a reduction in the interparticle distance and because of the changes occurring to the soluble fraction. In addition, the presence of the non-sedimentable aggregates and dissociated caseins in DF milk caused gelation at a higher pH value than UF milk. At a 2× concentration, the gel firmness at pH 4.6 was greater for DF milk than for UF milk; on the other hand, this was not the case for 4× concentrated milk, possibly because of limited rearrangements of the casein micelles and heat induced complexes at this high concentration.
Overall, the results from this research brought a new understanding of the effects of concentration processing functionality, processing history on the structure-function of casein micelles, and thermal treatment on the composition of the soluble material responsible for improving acid gel texture, which was related to the overall changes occurring during casein micelle aggregation, allowing improvements of processing efficiencies.
CHAPTER 7

REFERENCES


