PROPERTIES OF MILK SYSTEM DURING CONCENTRATION AND SUBSEQUENT HEATING

A thesis submitted in fulfilment of the requirements for the degree of Master of Science (Research)

By

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Dedicated to my children Borjan and Martin

Abstract

Production of concentrated milk encounters several instabilities in regards to the quality of the final product. Main problems that concern industry are aggregation and gelation of milk during heat treatment, age-gelation and thickening during storage. Some shelf life improvements have been achieved by pre-warming/pre-heating of milk prior concentration process intended to denature whey proteins and prevent their further involvement in aggregations with casein micelle. Nevertheless, precise knowledge describing behaviour of these proteins at the molecular level is lacking. Moreover, multitude of factors needs to be considered as they all have an impact on concentrated systems during water removal and heat treatment, including mineral content, protein rearrangements and pH. Hence, the present study aimed at evaluating observations of physiochemical changes in skim milk in regards to reduce water content, sequential heat treatment and the effect of altered pH during heating, all observations performed under laboratory conditions intended to imitate common industrial applications.

During the first study, physiochemical changes of proteins in skim milk were investigated after evaporation process at 55ºC starting from 9% up to two solids concentration, 17 and 25%. Moreover, after reaching desirable concentration level, samples were analysed for protein portioning between the serum and colloidal phases, and how this affected electric dabble layer and particle size distribution, then variations of minerals (calcium, phosphate, magnesium and sodium) and changes in the secondary structure of proteins. Results shown that concentration indeed affected the overall stability of the system, which was dependent of total solids (TS) content. Thus, reaching 17% TS αs- and β- casein dissociated from the micelle and became more prominent in the serum. However, greater total solid concentration (25%) affected the level of κ-casein in the serum by slight increase only, on the other hand, β-LG concentration in the serum

declined as it apparently interacted with the casein micelle. These modifications were confirmed with rearrangement of the secondary structure of the proteins advocated by slight shifting of minerals in the micellar state.

In the second study, it was established how the proteins were affected during heating by determining changes at 75, 85, 95, 100, or 110ºC and prolonged heating of 2.6 minutes at 121ºC. All three concentration level (9, 17 and 25% TS) were examined. Treated samples were analysed for changes in the particle diameter, zeta potential, changes in protein level between the serum and colloidal phase, protein rearrangement in the secondary structure and variations in the mineral content (calcium, magnesium, phosphate and sodium) between the serum and micellar state. According to the results applied temperature induced formation of aggregates, which intensity was dependant of concentration level. Moreover, whey proteins denaturation was delayed in increased solids contented, which moved to higher temperatures as TS increased. However, after reaching specific temperature they were involved in intense aggregation among themselves through thiol-sulfhydryl interchange reactions and with caseins by disulfide interactions forming particles with larger diameter. In skim milk with 9% TS this delay of denaturation of milk proteins was not present and aggregation resulted only in more soluble particles which did not affect the average size distribution. Other important detail was large variations in the casein level in the serum, thus involving intense dissociation of specific caseins at certain temperatures. Observed changes were supported with rearrangement of the secondary structure of the micelle that was variable in regards to concentration level and applied temperature.

The final study investigated the effect of pH adjustment on protein stability during heat treatment of skim milk with different solids content. Concentrated samples with 17 and 25% TS were

altered to pH 6.7, which was native pH of skim milk prior to concentration intending to return them to their native environment in the system. Other set of experiment was performed with all samples including unconcentrated milk which included alkali addition to pH 7.5. Properties of the samples were studied in regards to concentration levels and heat treatment under this condition. SDS Polyacrylamide gel electrophoresis and FTIR were used for protein portioning between the serum and micellar phase and modifications in proteins conformational rearrangement, respectively. For each observed concentration intensive aggregation occurred with formation of small and large aggregates composed of whey proteins, dissociated caseins and casein micelle. The intensity of aggregation increased as pH and TS were moved to higher level. The results in this study resulted in many variations in the casein levels between the serum and micellar phase promoting intense dissociation when concentration and temperature increased. However, conditions created by adjusting pH to 6.7 promoted transfer of dissociated αs- and β-caseins from the serum into the micelle. This transfer occurred at lower heating point for 25% TS in comparison to 17%TS, although this occurrence seized for both at 110ºC, indicating stabilization of the casein micelle to a certain extent or almost restoration of its native state. Adjusting pH to 7.5 had destabilizing effect on all observed proteins during heat treatment resulting in intense dissociation of caseins, covalent bonding and aggregations. In regards to whey protein changes it was observed that heating samples with pH > 6.5 and temperatures up to 110ºC, whey proteins formed soluble aggregated complexes among themselves or with the serum caseins. On the other hand, when temperature was raised above 110ºC in addition to formation of soluble aggregates these proteins were involved in aggregation with the casein micelle.

The present study revealed the findings that any alteration of solids content from the natural value in the skim milk would promote rearrangement of all proteins in the system indicating their

destabilization due to increased particles number. Further heat treatment would induce more intense destabilization in the system by disintegration of the casein micelle, denaturation of whey proteins and formation of aggregates, which size increased when temperature of heating was higher. Moreover, these observations concluded the need of pre-treatment or stabilization of proteins up to point before concentration step that would produce samples with higher heat stability. However, when it comes to the effect of pH it is interesting to point out that slight alteration of pH up to 6.7 in concentrated samples may have a positive effect on stabilization of the micelle up to certain temperature that can be taken in consideration when it comes to heat stability of concentrated milk.

Declaration

"I, Tatijana Markoska, declare that the Master by Research thesis entitled "Properties of milk system during concentration and subsequent heating" is no more than 60,000 words in length including quotes, and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature: 

Date: 09.03.2018

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Chapter 1: Introduction to thesis

1.1 Background

Prolonging shelf life of milk by concentrating using evaporation is an important step in dairy industry. Considering its high nutritional value, the final product is used as a food ingredient in different food preparations and as an essential processing step in further shelf life extension such as in case of production of milk powders. During thermal processing of concentrated milk various important compositional and structural changes of milk proteins take place that affect stability of the final product. These changes often include increased viscosity, aggregation of milk whey proteins and caseins, gel formation and changes in mineral balance as a result of increased concentration and thermal process (Walstra et al., 2005).

Extensive knowledge exists in regards to physicochemical properties of raw milk and how these properties are affected during heating process (Fox and Morrissey, 1977, Fox, 2009, Huppertz, 2016, McCrae and Muir, 1995, Singh, 2004). On the other hand, from a historical point of view there is a knowledge gap when it comes to properties of milk during concentration by evaporation and consequently heat treatment. During evaporation free water is removed more extensively than intramicellar water (Liu et al., 2012), thus that would initiate molecular changes in the mineral levels leading to a reduction in the amount of soluble calcium and increase in the levels of calcium incorporated into colloidal calcium phosphate located within the casein micelle (Bienvenue et al., 2003a, Hardy et al., 1984, Holt et al., 1986, Nieuwenhuijse et al., 1988). Water removal lowers distances between the micelles, which results in compaction of the particles (Cao et al., 2015). It is also expected that during the thermal processing whey proteins including β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and bovine serum albumin (BSA) would interact through thiol-disulphide interactions or disulphide interchange reactions among themselves or

with κ-casein (κ-CN) on the surface of the casein micelle (Sava et al., 2005). However, by increasing total solids and impacting molecular mobility of the particles, the reaction kinetics and final products of various reactions during concentration and heating may be affected, all of which is largely unknown.

Sterilization of concentrated milk creates several problems related to heat stability of proteins leading to coagulation, gel formation and excessive thickening during storage. By heating concentrated milk whey proteins start to denature and form aggregates with casein micelles of different sizes and structures, a process which is highly dependent of protein concentration, temperature, holding time and pH range (Zuniga et al., 2010). From the beginning of production of concentrated milk, manufacturers have tried to prevent these undesirable changes by preheating the milk, followed by evaporation and finally sterilization (Sommer and Hart, 1919). However, the thickening and coagulation still occur to a certain extent even today. Sommer and Hart (1919) proposed a relationship between heat instability of concentrated milk and the composition of milk salts and the difference in concentration. This was followed by Benton and Albery (1926), who used the alcohol test to control heat stability of evaporated milk. They found that adjustment of pH prior to sterilization had an effect on coagulation to a certain extent and addition of citrate or borate to the concentrated system resulted in an enhanced stability to a particular optimum, after which it declined. Therefore, the industry found a provisional solution to the problem by preheating the milk and adding stabilizers prior to evaporation even after some sediment was found in the product.

The problem of age gelation and thickening of the final product still remains as the main issue in production of evaporated milk. Many authors observed preheating of the milk before evaporation as a possible alternative to prevent gelation with control over the pH range and its dependence

over the degree of association between whey proteins and κ casein in the serum or in the micellar phase (Anema and Klostermeyer, 1997b, Anema and Li, 2003b, Anema et al., 2014, Sutariya et al., 2017, Tarassuk and Tamsma, 1956). For example, at pH above pH of raw milk (~6.7) denatured whey proteins and κ-casein are found in the serum phase; on the other hand at a pH below that of raw milk, whey proteins were associated with the casein micelle. Singh and Creamer (1991a) provided more detailed analysis on changes of milk proteins upon heat treatment of concentrated milk with different pH values and heating rate. They found that with more intense heat treatment and higher pH κ-, β- and αs-casein denatured more extensively. Whey proteins associated with κ casein via disulphide interactions create a complex, which remain attached to the micelle as enlarged aggregates. Hence, as a result of the changes in mineral content in the serum and the micellar phase, the amount of the soluble casein in the serum phase decreases. In respect to stabilizers, more reference is paid to trisodium citrate (TSC) and less to disodium hydrogen phosphate (DSHP) as alternatives to control heat stability of concentrated milk (Deeth and Lewis, 2016). Even though, by forewarming and use of stabilizers some of the problems that occur in sterilization of concentrated milk are still present, simply due to many changes in the serum and colloidal phase that are difficult to observe, control and optimize. By heat treatment of milk many changes are observed that promote instability including decrease in pH, saturation of micelle with calcium phosphate, dephosphorylation, bonding between the caseins, association of whey proteins with caseins, although, it is unclear what is directly engaged in the coagulation and in which stage of heat treatment it occurs (Singh, 2004).

1.2 Research aims and objectives

The main aim of the project was to provide a scientific foundation underpinning physical and chemical changes that take place in raw skim milk with different solids concentration as a function of process parameters such as concentration step, pH readjustment and heat treatment.

The proposed project addressed the following questions:

1. What were the structural changes of the whey proteins and casein micelles when they were subjected to concentration?
2. What chemical and physical changes have taken place in the concentrated systems with different pH values during sterilization and how proteins are affected?
3. What would be the most suitable process techniques that could be proposed to prevent aggregation and gelation of concentrated milk?

1.3 Structure of thesis

Structural and compositional properties of concentrated skim milk were studied as a function of concentration, thermal process and pH level. Firstly, the effect of evaporation was studied as a function of different total solids concentrations. Secondly, the further focus was to establish temperature dependant changes in concentrated skim milk systems during heating process as a function of the total solids concentration. Lastly, the temperature dependant changes in proteins were observed as a function of re-established “native pH” environment in concentrated milk by adjusting the pH to 6.7 (original pH of unconcentrated milk) and high alkaline conditions to 7.5. The obtained information would assist researchers and the industry to gain a greater understanding of the chemical and physical changes that take place during heating of

concentrated skim milk systems and thus enable a better process control and more sustainable milk processing. The thesis contains following chapters:

Chapter 1 – Presents the background and general information of the study including aim, objectives and the structure of the thesis

Chapter 2 – Depicts a literature review with detailed information of so far known concentration, heat and pH induced changes on milk.

Chapter 3 – Focuses on physiochemical changes in milk that takes place during concentration process.

Chapter 4 – Focuses on heat induced changes of milk proteins in raw skim milk with different solids concentrations.

Chapter 5 – Focuses on pH adjustment on protein modifications in raw skim milk with different solids concentrations after heating.

Chapter 6 – Provides overall conclusion of the chapters and future directions of optimization of processing conditions in production of concentrated milk with less undesirable consequences.

Chapter 2: Literature review

2.1 Properties of Milk

According to the Code (2016a), milk can be defined as mammary secretion of milking animals excluding colostrum and products with phytosterols, phytostanols and their added esters which is obtained from one or more milkings as a liquid for further processing. The requirements for bovine milk to be placed in the markets are no less than 32 g/kg milk fat and 30 g/kg proteins; however skim milk is limited to 1.5 g/kg of milk fat and no less than 30 g/kg of proteins (Code, 2016a). The main milk product of concern to this thesis is concentrated or evaporated milk, which is defined as a product produced from milk or adjusted milk by partial removal of water by heat or other processes. Evaporated milk distributed on the market should not have less than 7.5% milk fat and no less than 25% total milk solids, on the other hand if the product is sold as skimmed evaporated milk should not contain more than 1% milk fat and no less than 20% total milk solids (Code, 2016b).

Milk is secreted in secretory cells of mammary gland where most of the components are synthesized in the cells and others are taken up from blood. From the cells, secreted fresh milk is transferred into alveoli and through small and large ducts into the cistern and finally released via teat (Walstra et al., 2005). Milk is very complex fluid containing water as a main constituent (approximately 87%), followed by lactose (4.6%), fat (4%), proteins (3.3%) and minerals (0.7%) (Walstra et al., 2005) (Table 2.1). Composition, structure and properties of milk components vary as a result of genetic variations, the stage of lactation, illness of the cow and feed (Fox, 2009).

Table 2.1: Composition of milk (Walstra et al., 2005)

Component	Average content in Milk (% w/w)	Range (% w/w)
Water	87.1	85.3-88.7
Solids-not-fat	8.9	7.9-10.0
Lactose	4.6	3.8-5.3
Fat	4.0	2.2-5.5
Protein	3.3	2.3-4.4
Casein	2.6	1.7-3.5
Minerals	0.7	0.57-0.83
Organic acids	0.17	0.12-0.21

2.1.1 Lactose

Lactose is the main disaccharide in the milk composed of two monosaccharides, glucose and galactose, linked by β1-4 glycosidic bond, present as a α- and β- anomer individualised by orientation of hydroxyl group (Fox et al., 2015a). This reducing sugar is a unique milk component synthetised in the mammary gland from glucose. In milk and milk products, lactose has an important role as an essential and nutritive constituent in production of fermented products, affecting the texture of concentrated and frozen products and inducing changes related to stability in dehydrated milk products (Fox et al., 2015a). Main problem during manufacturing of concentrated milk products related to lactose is its insolubility and crystalline formation that is temperature dependant in supersaturated solutions. Concentrated milk is already saturated with lactose due to evaporation process and when the product is cooled, crystals with different sizes

can be formed that can affect density, viscosity, water activity, freezing and melting points and browning of the product (Fox et al., 2015a). Therefore, in regards to achieve smooth texture in concentrated milk products, a production process needs to be optimised by optimum preheating conditions for desirable effect on viscosity and vacuum cooling combined with seeding for best quality. Lactose also participate in non-enzymatic browning or Maillard reaction that involves interactions between the carbonyl group of lactose and amino groups of proteins mainly lysine (O'Brien, 2009). In this process newly formed products including Amadori compound (C=O), Shiff base (C=N) and pyrazines (C-N) are responsible for undesired browning, off-flavours and loss of nutritive value (lysine) of milk products (Fox et al., 2015a).

2.1.2 Lipids

Lipids in milk are present as fat globules or triglyceride molecules with randomly distributed fatty acids. Main function of milk fat is to serve as an energy source for neonates. Fat enriched products like cream and butter have specific texture and flavour provided by fat globules (Walstra et al., 2005). During production of concentrated milk products, thermal evaporation damages fat membrane mainly as a result of agitation in high velocity heating systems. However, the damage to the fat globule membrane is usually not detrimental since milk is further processed by homogenization as a frequent practice to reduce the diameter of fat globules for better quality of the product. Finally, certain defects of fat globules may result physical defects in milk products especially in cream including oiling off, cream plug and age thickening (Fox et al., 2015a).

2.1.3 Minerals

Minerals in milk are present as organic and inorganic salts. Hence, calcium, magnesium, sodium and potassium are main cations and inorganic phosphate, citrate and chloride are main anions in the milk (Gaucheron, 2005). Some of the mineral ions can be found as purely soluble such as sodium, potassium and chloride; however, some ions appear in a molecular form associated with the milk proteins (casein micelle) like in the case of calcium, magnesium, phosphate and citrate (Table 2.2). These ions interact to create a colloidal calcium phosphate (CCP) which includes other ions such as potassium, sodium, magnesium and citrate with a total amount of 7g/100g dry protein (Walstra et al., 2005).

Salts in milk are distributed in their soluble form or bound to other constituents. Calcium concentration in milk is around 30 mM/L depending of the season and lactation state. Out of this amount more than 60% is bound to the micelle with the remaining portion present in its soluble form in the serum (Gaucheron, 2005). Micellar calcium is further classified in two forms as calcium phosphate salts (CaP) and calcium bound to proteins. Phosphorus in milk is distributed in micellar and soluble form. Micellar phosphorus is also present in two forms as organic phosphate bound with the casein and inorganic phosphate mixed with calcium (calcium phosphate) (Gaucheron, 2005). Dynamic equilibrium exists among salts in solution, among solution and colloidal phosphate and between solution and proteins, therefore every external change in the condition of milk may affect this equilibrium (Huppertz, 2016, Walstra et al., 2005). Finally, changes in mineral composition that take place during processing of milk and evaporated milk effect physicochemical properties of proteins in milk in relation to heat stability.

Table 2.2: Mineral composition in milk (Fox et al., 2015c)

	Range in milk (mM/kg)	Diffusate (%)	Colloidal (%)
Calcium	26-32	33.5	66.3
Magnesium	4-6	67	33
Inorganic Phosphate	19-23		
Sodium	17-28	92	8
Potassium	31-43	92	8
Citrate	7-11	94	6
Chloride	22-34	100	

2.1.4 Proteins

Proteins in general are composed of a different number of amino acids forming a short chain (peptide) or a long chain (polypeptide). This chain structure where amino acids are held through disulphide bridges and covalent bonding is also known as *primary structure* of proteins and is specific for every individual protein (Walstra et al., 2005). Identification of proteins is made based on the side chains (R) of the amino acids (Figure 2.1).

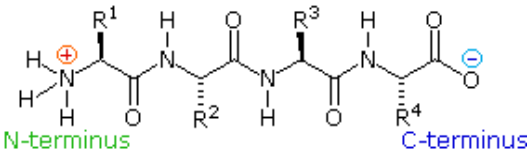

Figure 2.1: Peptide chain

Composition of the side chains in the polypeptides determinates the type of reaction that protein may be involved in (Walstra et al., 2005). For instance, proteins with free amino groups in side

chains as in the case of lysine may react with lactose and contribute to the Maillard reaction. Cysteine in the side chain of peptide chain may react with other cysteine from different peptide and generate cystine by formation of S-S (disulphide) linkage (covalent bonding). When polypeptide chains are linked with steric relationship they develop more complex structures (α helix, β sheets) also known as *secondary structure* of proteins. Further association through steric relationship among protein residues is called *tertiary structure* of proteins (Walstra and Jenness, 1984).

All milk proteins are categorized as whey proteins that are distributed in serum and caseins that are mainly present as large colloidal particles (Table 2.3).

Table 2.3: Average concentration and molecular weight of major bovine milk proteins (Walstra and Jenness, 1984)

Protein	Average concentration in milk (g/L)	Molecular weight (Da)
αs_1-casein	10.0	23,614
αs_2-casein	2.6	25,230
β-casein	9.3	23,983
κ-casein	3.3	19,023
α-lactalbumin	1.2	14,176
β-lactoglobulin	3.2	18,363
Bovine Serum Albumin (BSA)	0.4	66,267
Immunoglobulin	0.8	150,000-1,000,000
Lactoferrin	0.1	80,000
Lactoperoxidase	0.01-0.03	78 000

2.1.4.1 Whey proteins

Whey proteins are found in an aqueous solution of milk termed as a serum or whey. This group of proteins is highly heterogeneous and include a large number of different proteins such as β-lactoglobulin (β-LG) (represents 50% of total whey proteins), α-lactalbumin (α-LA) (25%), Bovine Serum Albumin (BSA), lactoferrin and immunoglobulins (Farrell et al., 2004). Whey proteins are highly hydrophobic with folded peptide chains into a globular and defined secondary structure with a large proportion of α-helix and β-sheets (Walstra et al., 2005). These globular proteins are also known as serum proteins as they exist in milk as monomers or small quaternary structures, which are heat labile and completely denature at 90°C for 10 minutes (O'mahony and Fox, 2013).

β-Lactoglobulin (β-LG) is the main serum protein with a highly defined secondary structure, in milk with native pH (6.7) is present as a dimer containing two -S-S- bonds and one hidden cysteine residue per monomer (Walstra et al., 2005). The presence of free cysteine has a significant role during thermal denaturation of β-LG and disulphide linking with other whey proteins or κ-casein (κ-CN) that affects the heat stability of milk. This protein has very compact globular structure with identified α-helix (10-15%), β-sheets (45%) and unordered structures including turns (47%) (Fox, 2003). Heat denaturation of β-LG was studied extensively (Anema, 2000, McKenna and O'sullivan, 1971, Oldfield et al., 2005, Oldfield et al., 1998b, Sawyer, 2003). When milk is heated at 70°C, native monomers start to unfold and transform into active state with exposed thiol groups and some hydrophobic residues. Following this, activated β-LG interacts via disulphide bonds with other β-LG molecules or caseins and forms irreversibly aggregated complexes. Many processing conditions that involve production of concentrated milk products affect heat stability of β-LG by retardation of heat denaturation as a result of increased

concentration of solids in the system (Anema, 2000). Thus, a number of available active thiol groups of β-LG are reduced due to covalent bonding with lactose molecules that impede denaturation or slow down the overall reaction.

α-Lactalbumin (α-LA) is a spherical molecule synthetised from lactose which contains a strongly bound Ca^{2+} exerting a profound effect on protein molecular stability (Brew, 2003, Walstra, 1999, Walstra et al., 2005). This protein has a regulatory function in lactose synthesis in the Golgi apparatus in the mammary gland. The effect of thermal processing on α-LA is less intense compared to β-LG and at high temperature it forms covalent bonds with β-LG or BSA and is found as aggregated complexes in the system (Havea et al., 2001). In concentrated milk products the percentage of heat denaturation of α-LA is hardly affected by concentration (Anema, 2001).

Immunoglobulins are heterogeneous large glycoprotein molecules present in milk as five classes including IgG, IgA, IgM, IgE and IgD (Hurley, 2003, Walstra et al., 2005). **Bovine serum albumin** (BSA) is present in milk at a low level and contains 17 -S-S- bonds and one –SH group (Walstra et al., 2005). **Protease peptone** is a heat resistant protein fraction to denaturation which does not precipitate at pH 4.6 (Walstra et al., 2005). **Lactoferrin** is a large protein present in the milk as a single chain and is important as an inhibitor of some bacteria by removing Fe^{3+} ions from the serum (Lönnerdal, 2003).

2.1.4.2 Casein

Caseins are the main protein fraction in milk, which represents approximately 80% of the total milk proteins. The role of these proteins in milk is to transfer calcium and phosphorus to the mammary gland without precipitation, which is essential for neonates (Walstra et al., 2005). The

caseins appear as a complex micellar structure in milk composed of four proteins of major importance in dairy industry including αs_1–casein (αs_1-CN), αs_2-casein (αs_2-CN), β–casein (β-CN) and κ-casein (κ-CN) with total concentration in bovine milk of 12-15, 3-4, 9-11 and 2-4 g/L respectively (Huppertz, 2013). Caseins are divided into calcium sensitive caseins (α_{s1}-CN, α_{s2}-CN and β-CN) and calcium insensitive (κ-CN), moreover all caseins are phosphorylated containing a different number of phosphate groups which is partly accountable for their high surface charge (Walstra et al., 2005). All caseins are also highly hydrophobic with exposed hydrophobic groups which are the main attractive force among the caseins (Walstra et al., 2005). Owing to high proline levels, they have less defined secondary structure compared to whey proteins.

αs_1-CN takes 40% of the total casein content in the milk (Huppertz, 2013) with eight phosphorylated groups important for stabilization of calcium phosphate nanoclusters in the micelle (De Kruif and Holt, 2003). αs_1-CN lacks cysteine and cystine, thus is not involved in the disulphide bridging in the micelle (Fox, 2009). This casein has hydrophobic side chains that undergo limited hydrogen bonding which are important for formation of dimers from peptide chains which association is reversible and strongly dependant of concentration (Farrell Jr et al., 2003).

αs_2–CN is an casein with ten phosphorylated groups and is least hydrophobic compared to other caseins (De Kruif and Holt, 2003). In milk is found as a monomer and less as a dimer linked by disulphide linkage or polymerized with κ-CN. It has a fairly defined secondary structure with analysed presence of α-helical structure (30-40%), turns and β sheets (~20%) (Farrell et al., 2009, Farrell Jr et al., 2003).

β-CN is a highly hydrophobic casein present in milk as a polymer with five phosphorylated groups (De Kruif and Holt, 2003, Huppertz, 2013). This casein has a less defined secondary structure with a low level of helical structure (~15%) and more present turns and β sheets (~30%) (Farrell Jr et al., 2003). This deficiency of a defined secondary structure is due to high proline concentration. Moreover, the shortage of cysteine and cystine in the molecule leads to inability for disulphide bridging (Fox, 2009).

Last casein is **κ-CN** which represents 10-12% of all caseins in the milk. It is present in milk as disulphide bonded aggregates with one phosphorylated group (De Kruif and Holt, 2003, Huppertz, 2013). This casein appears on the surface of the micelle with positive and hydrophobic N-terminal end oriented towards the micelle and negatively charged C-terminal oriented towards the serum, which is important for the micelle stability (Farrell Jr et al., 2003, Horne, 2006, McMahon and Oommen, 2013). κ-CN has a less defined thermostable secondary structure with low levels of α-helix (15%), turns structure (~25%) and β-sheets (~30%) (Farrell Jr et al., 2003).

Casein micelle

Casein micelle is a complex of caseins as a spherical aggregate with average size in a range from 20 to 600 nm containing 2-4g water/g protein, out of which 15% is bound to the caseins and the rest is free in the micelle channels (McMahon and Oommen, 2013). Therefore, it has been reported that the micelle has a highly hydrated and sponge-like structure responsible for light scattering of white colour in the milk (De Kruif and Holt, 2003, Horne, 2006, McMahon and Oommen, 2013, O'mahony and Fox, 2013).

In the last few years, the model of the casein micelle was studied extensively (De Kruif and Holt, 2003, Horne, 2006, McMahon and Oommen, 2013). The latest model of casein micelle presented

by Dalgleish (2011) illustrate “core polymers” containing hydrophobicaly bonded mixture of αs_1-, αs_2- and β- casein that further form super polymers or casein micelles that are held with calcium phosphate surrounded by surface κ- casein which is negatively charged and provides overall molecular stability through electrostatic and steric stabilisation. This stabilization is regulated by κ- casein by maintaining the integrity of the micelle and protecting calcium sensitive caseins from dissociation. For instance, during temperature changes some caseins may transfer between the micellar and serum phase and affect the existing equilibrium in the system (Huppertz, 2013). CaP nanoclusters also play an important role in integrity of the micelle and its removal by urea, SDS, ethanol or alkaline pH leads to disintegration of the micelle into small primary particles (McMahon and Oommen, 2013). The primary structure of the casein micelle is maintained by calcium phosphate which stabilises linkages with Ca sensitive caseins through their phosphate centres (De Kruif and Holt, 2003). Ca insensitive casein, κ- casein, located on the surface of the micelle, controls the micelle size by protecting internal caseins of forming new interactions (Figure 2.2).

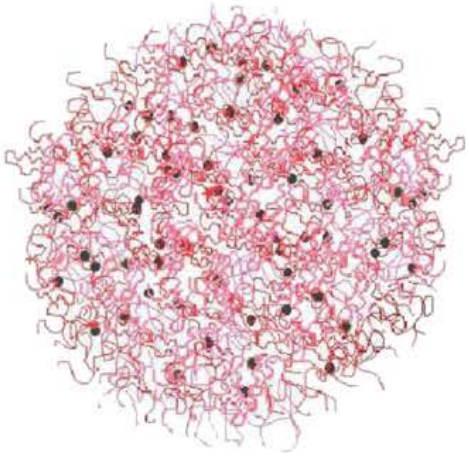

Figure 2.2: Illustrative model of the casein micelle presented as a homogenous protein matrix with CaP nanoclusters distributed in diameter of 18nm. κ-casein is present as rhemorphic polypeptide chains providing steric stabilization the particle (De Kruif and Holt, 2003)

Some properties of casein micelle related to its stability (Fox et al., 2015b, De Kruif and Holt, 2003)

- Casein is a heat stable protein that can maintain its micellar integrity up to 24 hours at 100°C or resist to coagulation when heated at 140°C up to 20 minutes. Heating milk at high temperatures result in covalent interactions between κ- casein and β-LG affecting the stability of the micelle. According to De Kruif and Holt (2003), this stability is a result of three reasons related to biological function of caseins. Firstly, caseins are reomorphic and stable to heat denaturation; next, calcium phosphate holds the Ca-sensitive caseins through strong linkages and stabilizes the internal structure of the micelle; and finally, the surface of the micelle provides a steric stabilization resulted from hydrophilic hairy brush of κ-casein.

- Refrigeration of milk at temperatures from 0-5°C produce dissociation of β-CN and less extensively liberalization of other caseins from the micelle due to weakening of hydrophobic interactions among the casein.
- Commercial homogenization barely affects the casein micelle, on the other hand treatments with high pressure results in various changes of the micelle.
- Micelles show high stability when are subjected to ultracentrifugation followed by sedimentation. Thus the micelle can be re-dispersed to almost native state by crushing and milk agitation.
- When milk is subjected to concentration process, casein micelles become more crowded and destabilize intensively as the concentration factor increases. This effect of concentration on stability of the micelle is due to certain chemical changes that take place during concentration process such as decrease in pH, increase in Ca^{2+} activity and changes in the state of colloidal calcium phosphate.
- pH has a large effect on the casein micelle stability. Therefore, any alteration of native pH to more acidic or alkaline values would affect the integral stability of the micelle. Moreover, the micelle has an isoelectric point at pH 4.6 when individual caseins start to aggregate and precipitate. This mainly results from increased solubility of CCP that at certain point would disintegrate the micelle into small aggregated primary particles. On the other hand, increase in pH to more alkaline values would lead to super saturation of the micelle with additional CCP that again would destabilize the micelle.
- The micelle stability can be interfered with in presence of alcohols, acetones and other solvents by affecting the electrostatic interactions in the micelle. Thus, addition of ethanol or

methanol affects the micelle by dissociation of caseins. This dissociation is pH dependent by increased destabilization of the micelle when pH is increased or decreased.

- Addition of urea and sodium dodecyl sulphate (SDS) also results in dissociation of the caseins from the micelle by cleavage of molecular bonds in the micelle.

2.2 Milk processing

In the dairy industry, raw milk is processed into different fluid products and range of dairy products such as fermented milks, cheese, concentrated milk, dry milk powders, frozen and refrigerated deserts and many more. Milk processing includes some principles necessary for effective management of dairy plant including chemical, microbiological, physical and engineering principles (Chandan et al., 2009). Main aims of dairy industry are achievement of optimum conditions in processing, packing, storage and shipment of a final product where all sensory, chemical, physical and microbiological aspects are met for successful management of the plant. Main processing steps involve separation of raw milk into a cream and skim milk then standardization finalizing it with heat treatment, cooling, packing and storage of the final product. However, every product has defined processing steps that follow standard analytical procedures which are approved by regulatory authorities for optimum processing conditions.

2.3 Concentration of milk by evaporation

2.3.1 Historical development and utilization of concentrated milk

Production of concentrated milk dates from ninety century before the advent of refrigeration when it was used as a beverage. Gail Borden, a young dairy farmer during his trip on boat from London to United States, noticed that due to long travelling time raw milk was easily spoiled. When he went back home he started with experiments and finally developed a product -

condensed milk - produced from skim milk which had a long shelf life and was resistant to spoilage. This product was main dairy product consumed during the First and the Second World War due to its long shelf life and easy transportation. However, this product showed several severe problems during the heat treatment such as coagulation, gelation and thickening during storage. These problems were controlled by preheating the milk before concentration and then sterilization (Sommer and Hart, 1919). It was also observed that addition of stabilizers improved heat stability (Benton and Albery, 1926). Nowadays, preheating and addition of stabilizers are part of standard commercial practice during manufacture of condensed or evaporated milk. The shelf life of final product is dependent on fat content and it can last up to 15 months.

Concentrated milk is used in its reduced form as reconstituted evaporated milk, which is equivalent to normal milk, also used as a mid-product in baking, ice cream processing and candy manufacturing and is the main ingredient or mid-step in production of milk powder. Evaporated milk after re-dilution with water is again returned to its native condition in regards to flavour and nutritive value.

Concentrated milk is produced in two forms including evaporated milk and sweetened condensed milk. The difference between these two types of milk is only in the sugar content, hence, in sweetened condensed milk there is a certain amount of sucrose added during manufacturing process in order to saturate the solution with sugar, which is important for preserving the quality and long shelf life of the product (Walstra et al., 2005). While these products have been developed long time ago, most of processing is based on the empirical knowledge thus due to lack of fundamental understanding, instability of the concentrated products during manufacturing and storage is still present.

2.3.2 Manufacturing process of evaporated milk

The process for production of evaporated milk is straightforward engaging several steps from raw milk until the final product (Figure 2.3). The manufacture begins when raw milk immediately upon arrival is standardised by tests for odour, taste, bacteria, sediment, protein and fat composition. Then milk is pasteurized with purpose to enhance milk stability and prevent protein coagulation and bacterial growth. Preheating conditions that are used frequently as an industrial practice involve 80-95°C for 10-30 min or over 100°C for a short period of time (Deeth and Lewis, 2016). Subsequently, heat treated milk is piped to an evaporator for water removal at temperatures that may start at 90°C while the concentrated product would exit the unit at around 40-45°C. The evaporation of bulk water is usually performed in a falling-film evaporator and the rest of the water is separated from the milk with a circulation evaporator (Walstra et al., 2005). Determination of the total solids concentration is mostly performed by the means of refractive index measurements or means of density. Once a required total solid content is achieved as predetermined by Food Standards, the evaporated milk is homogenised and further stabilized by addition of additives (Deeth and Lewis, 2016). In regards to stabilisers addition the amount to be added is determined experimentally and most attention is paid to disodium citrate (TSC) and disodium hydrogen phosphate (DSHP). The milk is finally vacuum sealed in pre-sterilized cans and sterilised at 121^{o}C for 15 minutes, cooled and shaken to break any formation of a curd or lumps.

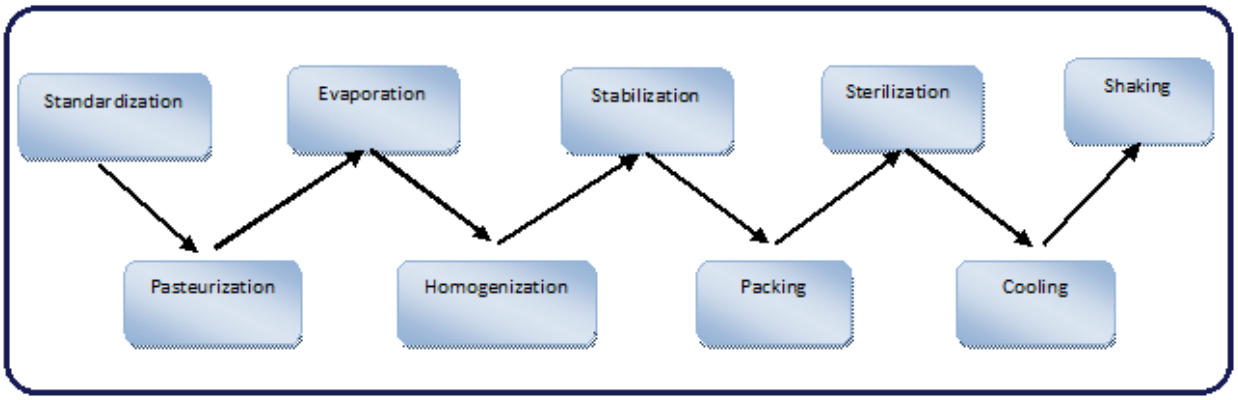

Figure 2.3: Manufacturing process of concentrated milk (based on Deeth and Lewis, 2016)

However, industry endeavours to find appropriate approaches and solutions of many problems that appear in the process of production of evaporated milk. Some of the problems include fouling of evaporators and heat exchangers, which maintenance imposes a high cost; next, concentrated milk shows instability during sterilization, which may result in formation of large aggregates and finally main concern for industry is age gelation. However, finding optimum conditions in the process of manufacture of concentrated milk vary seasonally and with milk from different cows.

2.4 Changes in milk during concentration process

Removing water from the milk leads to increased solids content that would change the total equilibrium in the system. Particles become present in a higher concentration and the distance between them is minimized that would promote modifications in the existing interactions and formation of new chemical bonding. Moreover, these modifications can be classified into chemical and physical changes of concentrated milk in regards to increased concentration levels.

2.4.1 Chemical changes

2.4.1.1 Effect of evaporation on pH

Concentration process lowers the pH of milk which is less pronounced in comparison to preheating treatment or sterilization (Nieuwenhuijse et al., 1988). This difference in pH profile is a result of the temperature used for the treatment; hence, evaporation is performed at low heating temperatures compared to other more intense heat treatments. This acidification of concentrated milk appears as a result of increased concentration of organic acids resulting from lactose degradation, shifting of minerals from soluble to micellar state and release of H^+ (Huppertz, 2013). The effect of lactose degradation during concentration process is less pronounced due to low heating temperatures used in the process, however increased solids concentration also means increase of lactose concentration in the system that may enhance the probability for interactions among crowded molecules, especially between lactose and lysine from proteins, but this process is mainly reversible under current conditions (Nieuwenhuijse et al., 1988). The effect of minerals is more pronounced in this process since the additional colloidal calcium phosphate in the micelle induce liberalization of H^+ which reduces the pH and the milk becomes more acidic (Walstra et al., 2005).

$$Ca^{2+} + H_2PO_4^- \longrightarrow CaHPO_4 + H^+$$

Decrease of pH is usually by 0.3 and 0.5 units for concentration factor 2 and 3 respectively which would decrease isoelectric pH of the milk (Walstra et al., 2005).

2.4.1.2 Changes in proteins

When water is removed from the system, the distance between the particles is minimized and they become more prone to interactions. Preheating the milk prior to concentration induces denaturation of whey proteins to denature. During heating at a low temperature for evaporation purpose, their concentration in native form is low which prevents further aggregations with the casein micelle. From the beginning of its production, it was established that preheating had a positive effect on heat stability of whey proteins in concentrated milk during further processing (Sommer and Hart, 1919, Tarassuk and Tamsma, 1956), however there is lack of public knowledge in supporting literature reporting as to why this pre-treatment has been required and how concentration process in raw milk affects the stability of whey proteins. Moreover, during evaporation there are only slight changes noticed on the levels of native whey proteins in the milk in comparison to the effect of preheating (Oldfield et al., 2005). This resistance to denaturation during evaporation process is due to low temperature used for water removal and high concentration of total solids in the milk. The effect of evaporation process on two major whey proteins in the milk is different. In addition, it is reported from Anema (2001) that α-LA is more resistant to denaturation in comparison to β-LG during evaporating conditions or is almost unaffected in concentration range of 9.6-38.4% TS in skim milk. However, this resistance is present for both whey proteins at different levels. In concentrated systems lactose concentration is high and have protective function on denaturation of whey proteins by enhancing interactions between this sugar and proteins and increased hydration of proteins (Arakawa and Timasheff, 1982). Lactose level in the milk also affects the particle growth, moreover at high lactose concentration, denaturation reaction of whey proteins is retarded and the aggregate growth is high (Spiegel, 1999). However, the level of aggregation of whey proteins was observed to be

lower than the level of denaturation, because the temperature of evaporation has a minor effect on this modification (Brodkorb et al., 2016).

Increased solids concentration induces a collapse of the structure of the casein micelle by formation of swollen and diffuse micelles that at high concentration are fragmented into smaller structures (Singh, 2007). Thus, this modification of the micelle structure prompts more intense reformation and new interactions among proteins when milk is further processed by sterilization. The presence of aggregates during evaporation was confirmed in the study of Cao et al. (2015) in which they observed reduced –SH content in evaporated milk in comparison to unconcentrated milk, which was related to aggregation of β-LG through interchange disulphide reaction with α-LA or creation of intermolecular disulphide bridges with κ- casein.

In regards to the effect of evaporation on the casein micelle structure it was reported by Anema and Li (2003b) that a slight dissociation of the total casein was observed from the micelle during mild heating which was pH dependant. In concentrated milk with pH 6.3 and 6.5 (which is native pH of concentrated milk), they observed that β- and αs- casein slightly dissociated from the micelle. As previously described by Aoki et al. (1990) and Bienvenue et al. (2003a), β-casein is a predominant casein involved in solubilisation at low temperatures. This pH dependence of solubility of β-casein and αs-casein at low temperatures was explained by destabilization of the micelle resulting from changes in colloidal CaP. Hence, the micelle loses its integrity because of saturation with colloidal CaP, which may affect its stability and result in release of some individual caseins. κ-casein is not affected by pH at the same extent as β- and αs-casein at room temperature but does become soluble as temperature increases. On the other hand, whey proteins do not show pH dependence on their structure at temperature below 60°C (Anema and

Klostermeyer, 1997b). This correlation of colloidal CaP with dissociation of caseins depends on temperature and pH was previously observed by Dalgleish and Law (1988).

2.4.1.3 Changes in particle size

Reduction of water content in the system leads to increased voluminosity of the micelle, which occurs due to aggregation of some micelles and interactions of whey proteins on the micelle surface (Singh, 2007). It was observed by Liu et al. (2012) and Bienvenue et al. (2003b) that when total solids concentration is increased the diameter of the particles may be reduced due to shrinking of the micelle resulting from saturation with colloidal calcium phosphate (CCP) and dehydration of the micelles. However, this decrease in size at even higher concentration is reversed, and an average diameter of particles increases. Hence, increased concentration promotes formation of large particles due to non-covalent interactions among particles promoted by reduced net negative charge of the micelle and decrease in electrostatic repulsion between the particles (Bienvenue et al., 2003a, Nieuwenhuijse et al., 1991b). It was reported that the increase in the micelle voluminosity is mainly do shifting of soluble calcium and caseins into the micelle (Liu et al., 2012, Singh, 2007) or attachment of the whey proteins onto the micelle surface (Cao et al., 2015). If milk is preheated before concentration, the final product have particles with increased diameter compared to samples that were not heat treated before evaporation (Bienvenue et al., 2003b). This observation is due to attachment of denatured whey proteins to the κ-casein of the micelle which may affect the micelle stability by reducing electrostatic repulsion.

2.4.1.4 Changes in mineral balance

Mineral composition is affected when water content in milk is reduced. During evaporation process, certain amount of soluble calcium and phosphate is transferred from the serum into the micellar state due minimised capacity of the mineral system to maintain soluble calcium and phosphate in their soluble form as a result of water removal and increased temperature (Liu et al., 2012). However, this shifting of minerals into the micelle is less pronounced during evaporation and more definite when the concentrate undergoes sterilisation (Hardy et al., 1984). Ionic strength of milk during concentration increases (Lewis, 2011). However, it was reported by Nieuwenhuijse et al. (1988) and Hardy et al. (1984) that concentration of ionic Ca^{2+} after evaporation process slightly decreased and after sterilization it experienced even greater reduction. This decline of ionic Ca^{2+} resulting from water removal is related to pH reduction in concentrated milk (Nieuwenhuijse et al., 1988). As previously described, pH drop is a consequence of shifting of minerals to the colloidal state and liberation of H^{+}. In regards to other minerals it was reported by Nieuwenhuijse et al. (1988) that the concentration of soluble Mg decreases and the amount of soluble Na increases proportionally with concentration step.

2.4.2 Physical changes

2.4.2.1 Rheology

When total solids in milk are increased, the balance in the system is disrupted by lowering distances among the particles which promotes various interactions and cross linking among molecules resulting in a transition from Newtonian to non-Newtonian flow behaviour (Velez-Ruiz and Barbosa-Canovas, 2000, Fernandez-Martin, 1972). This change in milk is reflected in an increase in viscosity. Hence, the viscosity decrease in order skim milk > milk > whey > whey

permeate which is related to the volume of the particle (Walstra et al., 2005). However, the increase in particle size is regulated by preheating, moreover in preheated milk the apparent viscosity is high due to large molecules formed from denatured whey proteins and their linking to the casein micelle (Bienvenue et al., 2003b). This changes in voluminosity of the particles in regards to preheat treatment was observed by Bienvenue et al. (2003a) who reported particles with increased diameter in samples that were subjected to preheating and only small variation in particle size in non-treated concentrated milk. Moreover, increased solid concentration leads to nonlinear increase in viscosity which is due rearrangement of caseins and formation of new bonds which are converted from reversible to irreversible linkages during prolonged storage of the concentrate (Bienvenue et al., 2003a). This modification in viscosity and changes in voluminosity are due to formation of new molecular structures governed by week interactions easily disrupted by high shear.

Liu et al. (2012) in their research showed that during the entire process of milk concentration, water was preferably removed from the serum and less from the micelle. This behaviour was due to greater mobility and availability of intramicellar water, however, water bound to the micelle started to be removed only after a considerable amount of bulk water was evaporated. When large proportion of water is removed from the system, water activity (a_w) decrease, which can be measured by determining the relative humidity, at which a product does not absorb or realise water (Walstra et al., 2005). Water activity measures the available water for microbial growth in the food, moreover, as higher the water activity the more perishable the product can become. This decrease in water activity also increases hydrophobicity of the particles. Turbidity of concentrated milk is expected to increase due to enhanced concentration of lactose and minerals

in the system associated with the micelle which becomes denser with high refractive index (Liu, 2014).

When milk is evaporated, an undesirable deposit is created on the surface of the evaporator wall termed fouling. This deposit formation is more pronounced in multiple-effect evaporators where the cost for sanitation and milk losses can be more than a total running cost (Walstra et al., 2005). Main reasons for this problem in industry are deposit formation from denaturation and aggregation of whey proteins and low solubility of calcium phosphate during heating that precipitates in the system (Jeurnink et al., 1996). The caseins assembled in the micelle with their repulsion force resulting from presence of κ-casein on the surface do not participate in the sediment formation as such, however they can become incorporated into the precipitate due to attachment of aggregated whey proteins onto their surface (Walstra et al., 2005, Jeurnink et al., 1996). Industry has observed several factors that are important for avoiding fouling in milk manufacture including temperature level, preheating, difference in temperature between medium and liquid, formation of gas bubbles, pH of milk, concentration level and many more (Walstra et al., 2005). When the product is evaporated due to increased solids concentration, the flow rate is low and the temperature distribution becomes non-linear, which would result in even more severe fouling. Fouling of heat exchangers is greater when temperature above 70ºC is used for the process and less prominent at lower temperatures (Walstra et al., 2005). Presence of air bubbles in the milk serve as a nucleus that incorporates precipitants and induces fouling that is more intense if the milk is destabilised mainly by acidification (Jeurnink et al., 1996). Therefore, finding optimal conditions for evaporation and heat treatment are main concern in concentrated milk manufacturing.

2.5 Packing and sterilization of concentrated milk

Stabilized evaporated milk is filled into cans by leaving some head space of around 10 mm needed for appropriate mixing during sterilization. After filling, cans are vacuum sealed and sterilized with continual agitation. In practice, it is common to perform in cans sterilization at high temperatures to prevent bacterial growth, inactivate enzymes, improve and preserve the quality of the product and to ascertain specific properties of the products (Walstra et al., 2005). Sterilization of concentrated milk is carried out at 120°C for 15 minutes; since it is basically a static process, the cans are usually agitated to reduce coagulation and gel formation (Deeth and Lewis, 2016). Mixing is also important for prevention of attachment of aggregated proteins on the internal can surface that may reduce the heat transfer coefficient of the wall and affect the quality of the sterilization process (Early, 1998). Shelf life of caned concentrated milk products is usually one year at room temperature, however in practice longer storage times are commonly observed.

2.6 Heat treatment of milk and concentrated milk

The main aim of applying heat treatment on milk and milk products is to produce commercially sterile products, which addresses full safety to costumers by inactivation of enzymes and killing of pathogens including *Mycobacterium tuberculosis, Coxiella burnetii*, *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes, Campylobacter jejuni* and potential external bacteria (Walstra et al., 2005). This practice also ensures a product with specific properties, high quality and long shelf life. The main heat treatments that are part of industrial practice for milk and milk products to be placed and sold in the market are pasteurization and sterilization. Temperature range used for pasteurization is 60-80°C and 100 to 150°C for sterilization. However, the

reaction to take a place for both heat treatments are needed three different period including heating period, holding period and cooling period where evaluation of all period is essential for determination of overall effect of temperature on quality of the final product (Lewis and Deeth, 2009).

Pasteurization process is used with an aim to prevent public health hazards that arise from present pathogenic microorganisms and to induce minimal physical, chemical and organoleptic changes in the final product (Lewis and Deeth, 2009). Pasteurized products induce minimal changes of flavour, colour and appearance of the milk and can be stored in a refrigerator for few days.

In regards to sterilization, the main objective of the process is production of a commercially sterile product with a long shelf life with the main attention focused on deactivation of heat resistant spores of *Clostridium botulinum* (Lewis and Deeth, 2009). The sterilization is mostly conducted in bottles or sealed containers or by Ultra High Temperature (UHT) processing followed by aseptic packing. The principal advantage of UHT processing is minimized changes of physical, chemical and organoleptic characteristics of a final product in comparison to in-container sterilization.

Ability of milk to withstand high temperatures under severe processing conditions without appearance of visual coagulation or gelation of a final product is known as heat stability (Singh, 2004). The method that is frequently applied for determination of heat stability of milk and milk products is commonly termed Heat Coagulation Time (HCT) or the time between the starting point of placing the milk into a heated oil bath up to appearance of visual coagulation (Deeth and Lewis, 2016). HCT depends on many factors including milk composition, preheating conditions,

concentration, pH and other parameters. Unconcentrated milk during heat treatment show high stability at natural pH (6.6-6.7), while at high pH the stability decreases with the minimum stability around pH 6.9-7.0 (Huppertz, 2016, Darling, 1980). Controversially, concentrated milk is less heat stable than unconcentrated with maximum stability at pH 6.5-6.6 , any changes in pH makes the product even less stable to heat treatment (Huppertz, 2016, O'connell and Fox, 2003). The heat instability is referred to colloidal instability which results in coagulation mainly governed by changes and collapse of the micelle brush (κ-CN) important for steric and electrostatic stabilization of the micelle (Huppertz, 2016).

2.7 Changes in concentrated milk during heat treatment

Heating conditions promote several changes in the product mainly occurring during in-container sterilization that continue during storage. These changes can be divided into chemical changes that facilitate protein heat modifications such as denaturation of whey proteins, protein variations among the serum and micelle, disintegration of the casein micelle, Maillard reaction, dephosphorylation and many more reactions, and physical changes including fouling of heat exchangers due to sedimentation and gelation (Deeth and Lewis, 2016).

2.7.1 Chemical changes

2.7.1.1 pH

When milk is heated at high temperatures and native pH (~6.7 for unconcentrated and ~6.5 for concentrated milk) pH decreases. In concentrated milk products, pH is preliminary reduced by evaporation process and in the system with increased solids concentration pH reduction during heat treatment is more intense (Walstra et al., 2005). Main reasons for pH reductions are production of organic acids due to degradation of lactose, precipitation of minerals (mainly

calcium and phosphate) in the colloidal state that results in liberation of H^+ accompanied by dephosphorylation of caseins (Fox et al., 2015b).

The significance of pH on heat stability of milk and milk products was for the first time revealed by Rose (1961). For unconcentrated milks, two HCT-pH profiles were established, A and B, where the former is characterised with maximum stability at ~6.7 and minimum stability at ~6.9 while the latter shows increase in heat stability as a function of pH increase. In regards to concentrated milk the heat stability is lower in comparison to that at the same pH of unconcentrated milk.

Alkalization prior to heat treatment

Alteration of pH to more alkaline values prior heat treatment was observed to affect the proteins by changes in their solubility (Anema and Li, 2000, Anema, 1998, Anema and Klostermeyer, 1997b, Vaia et al., 2006, Vasbinder and de Kruif, 2003). A hypothesis exists that slight increase of pH of milk prior treatment may promote higher stability by compensation of pH decrease that takes place during heat treatment and achieving the "natural equilibrium" of the milk. Addition of alkaline solution in milk affects the structure of the casein micelle. Moreover, consequent increase in pH from 6.0-12.0 leads to deprotonation of carboxyl groups of aspartic acid (Asp) and glutamic acid (Glu) that leads to high electrostatic repulsions, destruction of salt bridges and a loose and expanded structure of the casein micelle (Liu and Guo, 2008). It was reported by Vaia et al. (2006) that alkalization of milk reduces turbidity of milk due to disruption of casein micelles, which are main light-scattering particles in skim milk, and increase solvent quality of the milk. This disruption is more progressive at high pH and elevated temperature. On the other hand, increased solids content results in resistance to given alkaline disruption which was

suspected to be a result of lower solvent quality and less disruption in protein-protein interactions due high solids content. The reason for given alkaline disruption of casein micelle was reported by Vaia et al. (2006) to be increased solvent quality in milk due to decreased level of ionic calcium an phosphate at high pH, which further affects the cohesive interactions among caseins by micellar disruption. This micellar disruption is confirmed by intense dissociation of individual caseins from the micelle which is pH and temperature dependant (Anema and Li, 2000, Singh and Creamer, 1991a). Moreover, heat treatment of concentrated samples prepared from reconstituted milk adjusted to pH>6.7 reflected intensive dissociation of individual caseins from the micelle in the serum phase (Anema, 1998). Thus, liberation of the individual caseins from the micelle was reported to be temperature dependant for specific caseins and more intense in samples with high solids content. Maximum dissociation of αs- and β- casein is observed at a temperature between 60-80^{o}C, followed by re-association at a temperature up to 100^{o}C after what further increase in temperature again results in intense dissociations. Correspondingly, the dissociation behaviour of κ-casein is described by a continuous increase over the entire temperature range. However, in alkalized concentrated milk up to high pH values (8.0-11.0), the casein micelle undergoes extensive alkaline disruption into separated nanoclusters and improvement of solvent quality for the caseins, which is more pronounced when heating temperature is applied (Vaia et al., 2006). In addition, this disruption leads to increase of net negative charge of the caseins and their dissociation from the micelle.

2.7.1.2 Denaturation of whey proteins

When milk is treated at high temperatures, whey proteins demonstrate high sensibility that includes denaturation followed with aggregation and finally with gel formation. However, in unconcentrated milk, gelation is not common due to low levels of whey proteins, while in

concentrated milk this is more expected reaction (Huppertz, 2016). This instability of whey proteins is dependent on the process that is applied and the temperature-time profile, for example denaturation is almost 100% during in container sterilization and less pronounced by UHT treatment (Deeth and Lewis, 2016). This denaturation mainly involves β-LG and α-LA due to low concentration of other whey proteins in the milk and thus do not have a major influence on total heat stability. β-LG and α-LA start to unfold or denature at 40°C, moreover at low temperatures, below 70°C, this reaction is reversible (Deeth and Lewis, 2016). Irreversible denaturation takes place at a temperature above 70°C when native molecules start to open and transfer into active state by exposure of a sulfhydryl group at $Cyst_{121}$ (responsible for covalent interactions) and some hydrophobic residues involved in hydrophobic interactions (Deeth and Lewis, 2016, Oldfield et al., 1998b). After denaturation, whey proteins start to interact with each other or with κ-casein at the micelle surface or form soluble aggregates. This reaction is reliant on pH of heating and solids concentration. Thus, heating at pH>6.6 leads to formation of a network of soluble aggregates containing whey proteins and κ-casein, however heating at pH<6.6 results in attachment of whey proteins on the micelle surface (Vasbinder and de Kruif, 2003) and the casein micelle is more or less stable on coagulation surrounded by network of denatured whey proteins (Huppertz, 2016, Nieuwenhuijse et al., 1991b).

In regards to solids concentration it was reported by Anema (2000) that at high solids concentration during heating at 95°C the denaturation of β-LG is retarded in milk due to increased lactose concentration responsible for interactions with β-LG and decrease of free thiol groups required for aggregation. α-LA is more resistant to heat treatment in comparison to β-LG and its denaturation and aggregation by interactions with κ-casein is present after prolonged heating (Oldfield et al., 1998a). Moreover, this aggregation is pH dependant, and in concentrated

milk heated at low pH whey proteins may form a gel like structure by forming aggregates in the serum; on the other hand, heating at high pH they are found attached to the micelle surface associated with κ-casein. It was observed by Dumpler and Kulozik (2016) that heat induced aggregation of whey proteins in concentrated milk products is depicted by four forms including whey-κ-CN complexes, soluble denatured whey complexes, native whey proteins and co-sediment of whey-casein aggregates. In order to reduce the level of aggregation in the concentrated milk, preheating of milk prior concentration is applied in industrial practice. With this procedure whey proteins are denatured prior to concentration and form gel-like structure in the serum that prevents heat gelation with the casein micelle during sterilization and improve overall heat stability of the concentrate (Huppertz, 2016). Still, this increased heat stability applies only when concentrated milk is preheated in acidic side of the maximum, contrary at alkaline side of the maximum heat stability preheating has little effect (Nieuwenhuijse et al., 1991b).

2.7.1.3 Disintegration of the casein micelle

Casein micelle in unconcentrated milk has high stability when is heated at high temperatures up to certain time. However, in concentrated milk products, heat stability is lower due to changes in the native equilibrium in the system induced by evaporation process. It is known that the stability of the micelle in the system is kept by the hairy brush of κ-casein, which stabilizes the micelle with the C-terminus located on the surface of the micelle carrying net negative charge due to presence of many glutamine residues (Huppertz, 2016, De Kruif and Holt, 2003). The net negative charge of the micelle surface is -13 mV at 20°C resulting from dissociated carboxyl and ester phosphate groups (Singh, 2004). Therefore, this negative potential gives electrostatic and steric stabilization to the micelle, which consistency is strongly related to heat treatment of the

product (Huppertz, 2016). The main factor involved in dissociation of the caseins during heat treatment from the micelle is pH of the heated sample and other factors to a lesser extent such as interactions of whey proteins with the micelle, solids content and mineral composition. When evaporated milk is heated at native pH the hairy brush starts to disintegrate and separate from the micelle becoming more soluble and involved in new interactions with whey proteins (Figure 2.4), which is dependent on temperature and concentration level (Anema and Klostermeyer, 1997a, Anema and Klostermeyer, 1997b, Singh and Creamer, 1991a, Singh and Creamer, 1991b, Dumpler et al., 2017). Hence, if pH is increased more than optimal pH for heat stability in milk (6.5-6.6) dissociation of all caseins from the micelle is extensive and leads to destabilization in the system and coagulation (Anema and Klostermeyer, 1997a, Anema and Klostermeyer, 1997b, Huppertz, 2016, Singh and Creamer, 1991a, Singh and Creamer, 1991b). Inside the micelle β- and αs- caseins are associated through hydrophobic and electrostatic bonds with CCP that plays an important role in their integration in the micelle (De Kruif and Holt, 2003, Singh, 2004). However, in concentrated milk it was reported that CCP is found in new alternative form resulting from precipitation of minerals in the colloidal state during evaporation process (Aoki et al., 1990). Thus, this re-equilibrated CCP show lower ability for cross-linking of caseins in the micelle, which during sterilization results in lower stability compared to the native one in unconcentrated milk. Another important factor that affects the stability of the micelle during sterilization is dephosphorylation or loss of phosphate groups that would decrease the binding capacity of CaP and liberation of β- and αs-caseins (Singh, 2004). Following, β- and αs-caseins dissociate from the micelle during heat treatment and as in the case of κ- casein, the extent of dissociation is pH and temperature dependant (Anema, 1998, Nieuwenhuijse et al., 1991b, Singh and Creamer, 1991a). Therefore, the dissociation of caseins from the micelle during heating

mostly depends of pH prior to heating, while also other factors influence this dissociation such as the presence of whey proteins, solids concentration and composition of the serum and the micelle (Huppertz, 2016).

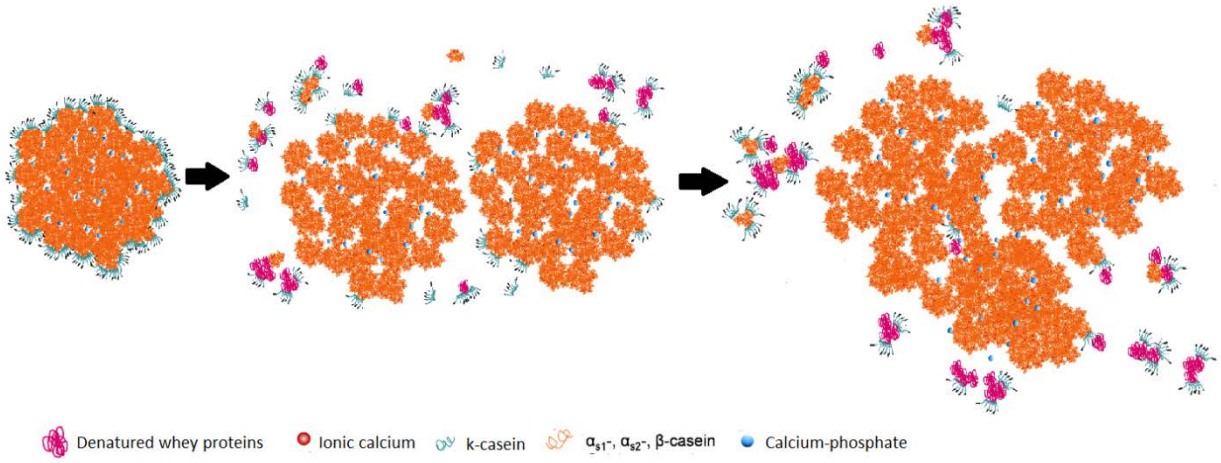

Figure 2.4: Heat induced denaturation and coagulation of casein micelle and whey proteins (adapted from Dumpler et al. (2017)

2.7.1.4 Changes in mineral equilibrium

The mineral equilibrium that exists between the soluble and micellar states depends on solids concentration and temperature changes. Mineral composition in concentrated milk play important role in heat stability. Alternative form of CCP in the micelle found after evaporation process is affected with more intense precipitation of soluble calcium and phosphorus into the micelle during sterilization process (Huppertz, 2016, Nieuwenhuijse et al., 1988, Singh, 2004, Aoki et al., 1990). In addition, soluble calcium and phosphate shift into the micellar state and saturate the micelle with additional CaP that behaves differently than the native CaP and destabilize the micelle (Singh, 2004, Singh et al., 1995). Concentration of Ca^{2+} slightly decreases when milk is evaporated and latter continues to decrease during heat treatment (Chandrapala et

al., 2010, Nieuwenhuijse et al., 1988, Singh, 2004). This reduction may promote association of calcium ions with the carboxyl acid group of the micelle surface and neutralize its charge leading to destabilization (Fox and Morrissey, 1977). However, it is known that heat treatment of milk promote dephosphorylation (Belec and Jenness, 1962), thus, calcium ions may form new association with released phosphate esters that form new balance in the system (Chandrapala et al., 2010). These modifications of Ca^{2+} are reversible to some point after cooling (Deeth and Lewis, 2016). Finally, mineral balance in the concentrated milk product is affected by the process of water removal to a lesser extend and more intensively by sterilization.

2.7.1.5 Millard reaction

Non-enzymatic browning of milk as a result of high temperature treatment is regularly reaction that takes place during sterilization of milk and evaporated milk. The process is carried out by interactions among carbonyl group of lactose with ε-NH_2 group of lysine and formation of lactosamine. This reaction is undesirable for milk products due to unacceptable changes in colour, alteration of milk flavour by formation of strong burned flavour, reducing the nutritional value of the milk by loss of lysine- main protein involved in the reaction (Fox et al., 2015a)

2.7.1.6 Particle Size

In concentrated milk, heat treatment leads to fluctuation of the micelle size which is dependent on applied preheating treatment prior to concentration, pH of heating and solids concentration. Hence, in high concentrated milk products due to low solvent quality the crowded proteins undergo intense conformational changes and associations that lead to increased voluminosity of the particles. In regards to the effect of pH on changes in molecules size during heating, it was reported by Anema and Li (2003b) that when milk is heated at pH 6.5 the diameter of the

particles increased by 25-30 nm and at slightly higher pH (6.7) the increase in size is only 5-10 nm. These changes in the particle diameter are more intense if the milk is not preheated before concentration and less intense in preheated concentrated milk because of less availability of β-LG to be involved in aggregations due to its denaturation during the preheating treatment (Bienvenue et al., 2003a). Changes in particle size in milk affects milk rheology and the quality of the products that may result in age-gelation.

2.7.2 Physical changes

2.7.2.1 Fouling of heat exchangers

Heating concentrated milk leads to forceful deposit formation on the wall of heat exchangers. Formed aggregates during heating process if persist in the product without attaching on the exchanger surface would be preserved as a sediment in the final product (Deeth and Lewis, 2016). In regards to the type of heat treatment applied on milk it was observed that fouling is more prominent during preheating compared to sterilization. Hence, the temperature at which the deposit is formed is 80-105°C with appearance of white voluminous deposits which involve 50-70% protein and 30-40% minerals (Deeth and Lewis, 2016). During sterilization of concentrated milk due to increased solids concentration, high temperature difference across the wall of heat exchangers and slow flow rate promote fouling in the system (Walstra et al., 2005). A possible solution for preventing of deposit formation during sterilization of concentrated milk is preheating the milk prior concentration that may limit participation of whey proteins in aggregation during sterilization.

2.8 Fourier transform infrared (FTIR) spectroscopy

Conformational changes of the proteins in the milk can be easily observed by FTIR spectroscopy. With this technique, infrared spectrum of wide spectral range is obtained from an absorption or emission of a sample. This method is fast alternative without high requirements for sample preparation and application (Grewal et al., 2017a). Changes in proteins that take place during concentration step and heat treatment induce conformational rearrangement of the secondary structure. Moreover, with FTIR the changes in covalent-bonding or other weak interactions including electrostatic and hydrophobic bonding can be observed. The absorption of the bands in the region 1700-1600cm^{-1} relates to Amide I region that assign strength of C=O and N-H groups (Kher et al., 2007). The obtained spectrum in this region gives information of distribution of individual peaks that assign certain structure of polypeptide chain (Figure 2.4).

Table 2.4: Peaks distribution in Amide I region (Grewal et al., 2017a, Lefèvre and Subirade, 1999)

Secondary structure	**Peaks distribution range**
Aggregated β sheets	~1690
Antiparallel β sheets	~1680, 1620-1635
Turns	~1660-1670
α helix / loops	~1650
Unordered structures	~1645
Side chains	~1612

Peaks distribution in the spectrum obtained from milk samples can be depicted by different intensity and appearance which is dependent of the applied treatment. Moreover, in concentrated milk the solvent quality decrease, changes in pH, minerals equilibrium and ionic strength leads to conformational rearrangements of proteins due to high possibility of new interactions (Walstra et al., 2005).

2.9 Summary

Concentration of milk by evaporation is an important processing step in dairy industry that causes numerous changes in properties which are dependent on other conditions. In the current literature little is known about physio chemical changes in milk proteins that take place during concentration step of raw milk. Moreover, the existing knowledge is insufficient in regards for understanding the impact of increased solids content on conformational rearrangement of proteins and formation of new interactions. Moreover, practical application of processing conditions for production of concentrated products are established in early twenty century, however the scientific knowledge of changes in molecular level is lacking. Chapter 3 of this thesis would present the physiochemical behaviour of whey proteins and casein micelle resulted from the effect of different solids content studied in both serum and micellar state, conformational rearrangement of the molecules and how the other milk component influence newly formed condition.

Heat treatment of concentrated products is the main processing operation that affects the stability of concentrated products, which application requires high precision for obtaining heat stable product. Moreover, concentrated milk products are less stable than unconcentrated milk and are prone to aggregation and gelation. These undesirable conditions of concentrated products are

barley understand due to insufficient knowledge of the reasons that induce these behaviours. In Chapter 4 the effect of different heating temperature on proteins rearrangement among serum and micelle and their conformational distribution would be studied. Additionally, the effect of salt equilibrium on protein changes would be taken in account.

The last factor which is of major importance in regards to heat stability of concentrated products is pH. The pH undergoes reduction during evaporation and more intensively during heat treatment of concentrated products. Hence, return of pH to “native state” and to a more alkaline value before heat treatment would be examined and how the new re-equilibration would affect the proteins when they are subjected to different heating temperatures (Chapter 5).

Chapter 3: Physiochemical changes during concentration of raw skim milk

3.1 Introduction

Raw milk is a highly perishable food commodity and is generally processed using heat with or without any additional manipulation of its composition to make it safe for consumption or extend its shelf life. One approach to shelf life extension involves partial or almost complete water removal that would change water activity of the final products resulting in diminishing rates of many detrimental biological, biochemical and chemical processes (Labuza, 1982). Concentration of milk presents an intermediate processing step in production of dairy powders or concentrated or so-called condensed milk products. Production of concentrated products involves removal of water from pasteurized milk followed by an in-can sterilization. A final product if processed properly is rendered commercially sterile, and has a long shelf life at ambient temperature. One of the main problems during production of evaporated/concentrated milk is heat induced instability of milk proteins, which leads to fouling in the evaporators, a major industrial concern, and may also destabilize the final product through formation of aggregates and age-gelation.

Fouling on the stainless steel surface of evaporators results mainly from precipitation of whey proteins, which in practice is minimized by forewarming/preheating of skim milk prior to concentration. Forewarming denatures whey proteins before concentration step preventing their further cross-linking among themselves and with the caseins (Webb and Bell, 1942), and thus reducing the sedimentation of aggregated whey proteins and intense fouling of heat exchangers and evaporators (Jeurnink et al., 1996). Additionally, formation of air bubbles on the equipment wall serves as a nucleus for precipitation of aggregated whey proteins, precipitated minerals or cross-linked caseins which are controlled by degassing the milk and applying high pressure and high shear rate (Jeurnink et al., 1996).

Concentration of milk results in decrease in the water activity, changes in the mineral balance between the soluble and colloidal phases and close packing of colloidal particles, which all leads to establishment of a new equilibrium in the system (Walstra et al., 2005). In this new equilibrium characterized by lowered pH, greater ionic strength and increased total solid concentration, electrostatic repulsions are minimized (Singh, 2004). As a result, the casein micelles are distributed as particles of different sizes held together through relatively weak interactions (Bienvenue et al., 2003b). Lowered pH also partially destabilizes the casein micelle, which, along with other re-equilibrated constituents, leads to compromised heat stability of concentrated milk and its inability to withstand severe heat treatment leading to high fouling in heat exchangers and age-gelation of the final sterilized product.

Increase in pH and reduction in ionic strength before heat treatment may produce greater repulsion and less cross-linking of proteins (Bienvenue et al., 2003a), which may promote greater stability and reduce the age-gelation in concentrated milk. However, it is unclear how this new equilibrium would affect the casein micelle when solids concentration is increased. Only practical and empirical observations in regards to changes observed in concentrated milk exist and have been reported in the early twenty century. The fundamental knowledge pertaining to these changes is still lacking in the public domain. A number of studies have reported on the changes induced by evaporation process of a reconstituted or preheated milk system (Anema and Klostermeyer, 1997b, Bienvenue et al., 2003a, Hardy et al., 1984, Liu et al., 2012, Liu, 2014, Martin et al., 2007, Nieuwenhuijse et al., 1988). However, it would be valuable to take a step back and establish all subtle changes that occur during concentration of raw skim milk system. While these changes during evaporation may be subtle, they may also create a foundation for some major physical and chemical processes that are augmented during sterilisation and

consequent storage. Therefore, the work in this chapter was aimed to establish changes during concentration step in the production of concentrated dairy products. The emphasis was given to understanding the impact of changed environmental conditions on the state of proteins especially their secondary structure.

3.2 Materials and methods

3.2.1 Sample preparation

Raw milk was provided by Murray Goulburn Cooperative Co. Ltd (Laverton North, Victoria, Australia). Experimental skim milk samples were prepared by centrifugation at 40°C at 3225 g for 30 minutes (Avanti J-26XP centrifuge, Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). Skim milk was processed usually immediately; however, to minimize microbial changes it was stabilized by addition of sodium azide (0.01 %). Total solids content (TS) was determined by oven drying over night at 105°C. The raw skim milk was concentrated using a rotary evaporator (Rotavapor® R100, John Morris Scientific, Deepdene, Victoria, Australia) at 55°C until the required TS concentration of 17 or 25% was reached, 2 hours at the most. The proportion of TS in the sample was monitored by recording the amount of water removed from the initial sample. The evaporation temperature was not raised above 55°C in order to minimize the changes of the main components of milk. The pH was measured before and after evaporation using a pH meter (Metrohm AG, Oberdorfatrasse, Herisau, Switzerland).

3.2.2 Particle size and zeta potential (ζ)

Particle size and zeta potential of particles in the samples were measured using a dynamic light scattering (Zetasizer-Nano ZS, Malvern Instruments, Worcestershire. UK) and the data was processed using a Dispersion Technology software (version 5, Malvern Instruments). Preparation

of the samples for the analysis was achieved by diluting them in a 1:100 proportion in simulated milk ultrafiltrate (SMUF). SMUF was prepared as described by (Liyanaarachchi et al., 2015) applying ultrafiltration to raw milk. The permeate was obtained at 15°C using a SEPA CF membrane module and polyethersulfone (PES) membrane (190 x 140 mm) with a molecular cut-off of 10 kDa (Sterlitech Corporation, Kent, WA, USA). Refractive index was set at 1.345, 1.358 and 1.37 for 9, 17 and 25% TS concentration, respectively. One millilitre of diluted sample well mixed was transferred into cuvette and the three measurements for particle diameter and electric charge of the particles were collected for each sample what was followed with repetition from different batches of milk.

3.2.3 Determination of mineral content

Calcium (Ca), magnesium (Mg), phosphate (PO_4) and sodium (Na) were the elements analysed by an Inductively Coupled Plasma (ICP) atomic emission spectrometer (ICP Multitype, Shimadzu Corporation, Kyoto, Japan) following the method previously described by (Grewal et al., 2017c) with minor modifications in the sample preparation. The serum phase and bulk milk samples with different solid concentrations were dried overnight at 105°C and then ashed in a muffle furnace at 550°C for 24 hours. After ashing, the samples were re-suspended in 70 % nitric acid and diluted with Milli-Q water up to 100 ppm of solids. Standards of four minerals (Ca, Na, Mg, and P) were prepared in the concentrations ranging from 0 to 100 ppm. The amount of every mineral was expressed as mM per litter of concentrated sample.

Ionic calcium (Ca^{2+}) was measured in the supernatant of samples with different solids concentration and native pH following the method of (Chandrapala et al., 2010). For this method, a calcium ion selective electrode (Ca^{2+}) was used along with a pH meter (inoLab, WTW GmbH, Ingolstadt, Germany). Calcium chloride was used for preparation of a standard curve of

concentrated solution in range from 0-2 mM with R^2=0.996. The ionic strength was adjusted with KOH to 0.08 M and calculation of Ca^{2+} activity ($a_{Ca^{2+}}$) was performed as follows:

$$a_{Ca^{2+}} = \frac{C_{Ca^{2+}}}{C^0} \gamma_{Ca^{2+}}$$

Where C^0=1 mM/L and γ_{Ca2+} = 0.425 and 0.403 are given as activity coefficient of the Ca^{2+} as given by Debye-Huckel approximated at 25°C and 60°C, respectively.

3.2.4 Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Changes in proteins during concentration step were studied by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions as described by (Grewal et al., 2017b) with minor modifications. Serum phase was separated from the pallet by ultrahigh centrifugation at 21ºC for 1 hour at 100,000 g using a Beckman Ultra L-70 centrifuge (Beckman Coulter, Australia Pty. Ltd, Gladesville, NSW, Australia). After centrifugation, the liquid phase was carefully transferred in a tube and used for the analysis. The samples were prepared by dilution of the supernatant in 1 mL of SDS sample buffer (0.0625 M Tris-HCl buffer (pH 6.8), 10 % (vol/vol) glycerol, 2.5 % (vol/vol) of 0.4 % (wt/vol) bromophenol blue solution, 20% (vol/vol) of 10 % (wt/vol) SDS). A molecular weight marker (SeeBlue Plus2 Pre-Stained Protein Standard, Thermofisher Scientific, Victoria, Australia) was loaded in the middle of the SDS gel to separate reducing and non-reducing samples. In reducing SDS-PAGE samples, the covalent bonds were cleaved by using β-mercaptoethanol in concentration of 26 µL per mL of diluted sample in the SDS buffer, vortexed and boiled in water bath for 4 minutes and cooled before running. Reduced and non-reduced samples (10 µL) were loaded in 12.5 % gels and run in a Protean II xi cell (Bio-Rad Laboratories) in a SDS running buffer (0.1% SDS, 0.025 M Tris and 0.191 M glycine, pH 8.6). The electrophoresis was

performed under specific conditions (210V, 70mA, 6.5W for 1.2 hours) and the gel was then stained using staining solution (0.15% Coomassie Brilliant Blue R250 dye, 72% isopropanol and 3% acetic acid) for one and a half hour with slow shaking and then distained with distaining solution (10% isopropanol, 10% acetic acid) overnight. Gels were imaged using ChemiDoc imager (ChemiDoc MP, Bio-Rad Laboratories, Richmond, CA, USA).

3.2.5 Quantification of portioning between soluble and colloidal states

SDS-PAGE analysis was conducted in duplicates and the absolute quantity of individual proteins in each individual band of non-reduced and reduced proteins gels was averaged. The intensities of different bands, which represented individual whey proteins and caseins, were determined using an Image Lab.2.1 software connected with a densitometer (BioRad Chemidoc MP imaging system, Gladesville, NSW, Australia). Protein quantity of each band was recalculated to the value of unconcentrated milk in order to produce comparable results. The difference in the band intensity among the proteins with different solids concentration was noted to determine the effect of concentration on their partitioning.

3.2.6 Fourier transform infrared (FTIR) spectroscopy

All samples, including un- and concentrated, were analysed using a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, Boston, MA, USA) in the range of 4000-600 cm^{-1} with a resolution of 4 cm^{-1} and averaging 16 scans for each spectrum as described by Grewal et al. (2017a) for evaluating the conformational changes of the proteins. At the start of measurement, the background spectrum was scanned with a blank diamond ATR cell using the same instrumental conditions as for the sample spectra acquisition. The measurements were performed by adding a drop of milk samples on ATR cell. The same procedure was used for each concentration level in

duplicates. The acquired spectra were then exported to optical spectroscopy software (Spectrograph, version 1.2.7, Oberstdorf, Germany). Special attention was paid to the original spectra in a region between 1200 and 800 cm^{-1} and the second derivative of a region between 1700-1600 cm^{-1} for evaluating changes in C-O stretching of carbohydrates and secondary structure of the proteins, respectively. For the amide I region derivatization along with smoothing was performed to enhance peaks resolution and better identification of the protein's structure (Grewal et al., 2017a).

3.2.7 Data analysis and statistics

Principal component analysis (PCA) was employed to identify the regions separated based on concentration factor. This multivariate analysis technique gives an overview of all information in the data by generating a set of principal components (PCs) as coordinated axes with minimum loss of information. For the purpose Unscrambler software (version 9.8;CAMO AS, Trondheim, Norway) was used by exporting FTIR spectra of all samples and the quality along with visual inspection was confirmed (Grewal et al., 2017a). Groupings of the performed PCA were presented in score plots whilst wavenumbers were identified by loading plots indicating high loading that separated the samples into a different groups.

The data was analysed using one-way ANOVA with the concentration as the only factor and Tukey's Studentised Range (HDS) test for comparison of the means of samples analysed in duplicate on two separate occasions using Statistical Analysis System (SAS) software. Level of significance was preset at $P<0.05$.

3.3 Results

3.3.1 Effect of total solids concentration on physiochemical properties of the raw milk system

The current work differs from other reported studies in that the water was removed from raw skim milk and hence all the constituents were in their native state during the concentration process. When skim milk was concentrated up to 17 and 25 % TS, pH gradually declined by 0.2 and 0.25 units reaching final values of 6.5 and 6.45, respectively. As expected, this change in pH of the system, accompanying increase in solids concentration, induced substantial changes in the mineral equilibrium of the system. Significant reduction ($P<0.05$) of ionic calcium (Ca^{2+}) was observed in the samples with high total solids concentration (25%) (Table 3.1). Moreover, concentration of all observed minerals (calcium, phosphorus, magnesium and sodium) decreased significantly (<0.05) when concentration was enhanced by 1.9 or 2.8 times that indicates slight loss of minerals in the process as precipitants (Figure 3.1A). The intensity of this reduction was higher at higher solids content. In regards to concentration of these minerals in the serum, only slight but apparently significant (<0.05) reduction was observed likely due to transfer of the soluble minerals into the micelle (Figure 3.1B).

The concentration process did not have a significant ($P>0.05$) effect on the particle size measured as the diameter of the particles (Table 3.1). Similarly, there was no significant difference between the ζ potential of samples with different concentration of the total solids. Apparently, concentration had minimal effect on particle size and negative charge of the particles. However, changes in pH and salt equilibrium induced changes in the partitioning of milk proteins between the micellar and the serum phase.

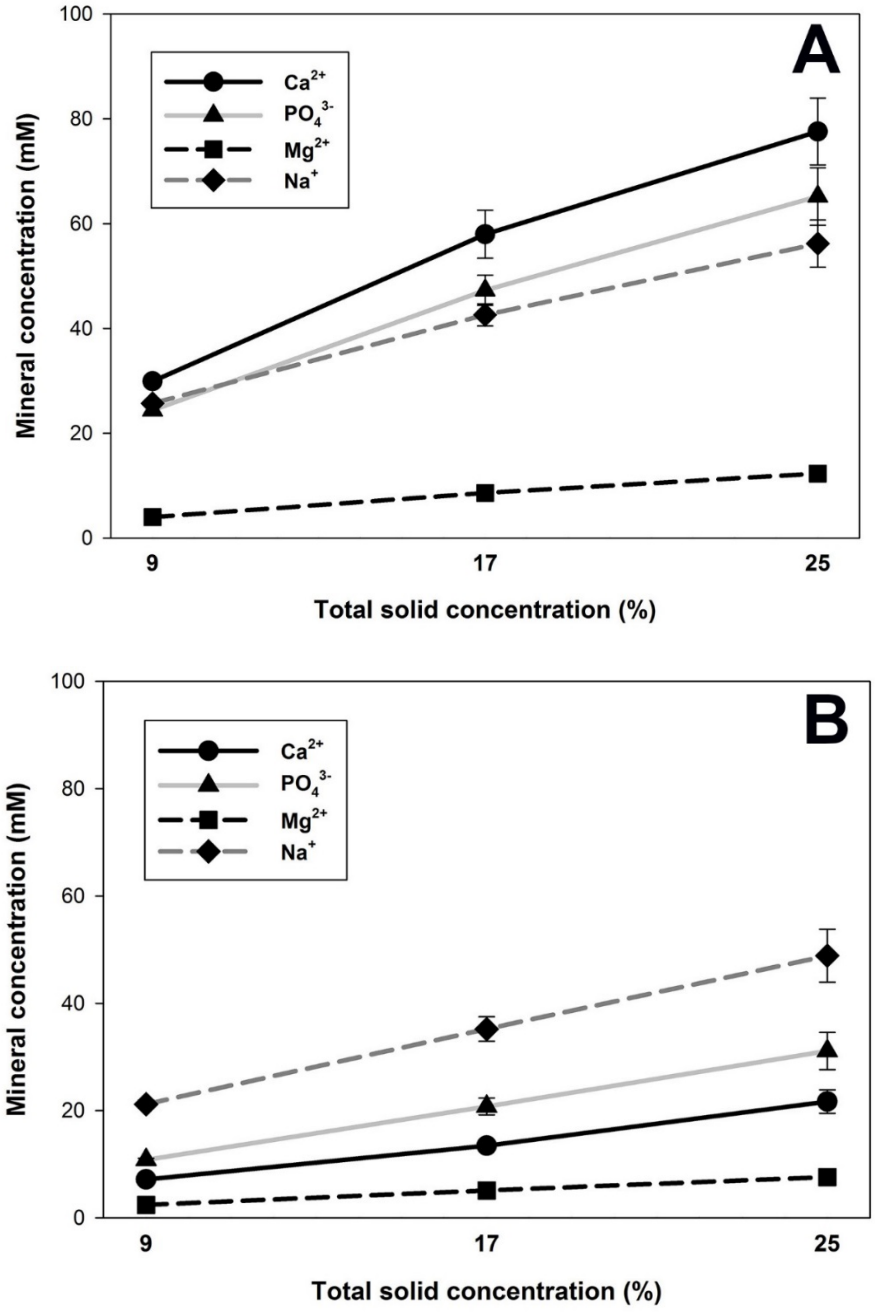

Figure 3.1: Effect of concentration on concentration of selected ions in the bulk (A) and the serum phase of the raw skim milk (B)

Table 3.1: Particle diameter, zeta potential and ionic Ca^{2+} concentration of samples with different solids concentrations (9, 17 and 25%); *SEM – pooled standard error of the mean. [a]Means in rows with different lower case superscripts differ significantly (P<0.05)

	Total solids concentration, %			SEM*
	9	17	25	
Average particle size, nm	149.9[a]	164.6[a]	166.1[a]	21.7
Zeta potential, mV	-15.66[a]	-16.71[a]	-15.56[a]	0.42
Ionic Ca^{2+}, mM	1.57[a]	1.57[a]	1.46[b]	0.006

In the non-reducing SDS-PAGE patterns of skim milk with different solids concentrations, the bands corresponding to aggregates were present on the top of the stacking and resolving gels which intensity increased with the concentration (Figure 3.3). Furthermore, in reducing SDS-PAGE patterns, the intensities of bands corresponding to individual caseins changed with different concentration of total solids implying changes in their presence in the serum (Figure 3.3).

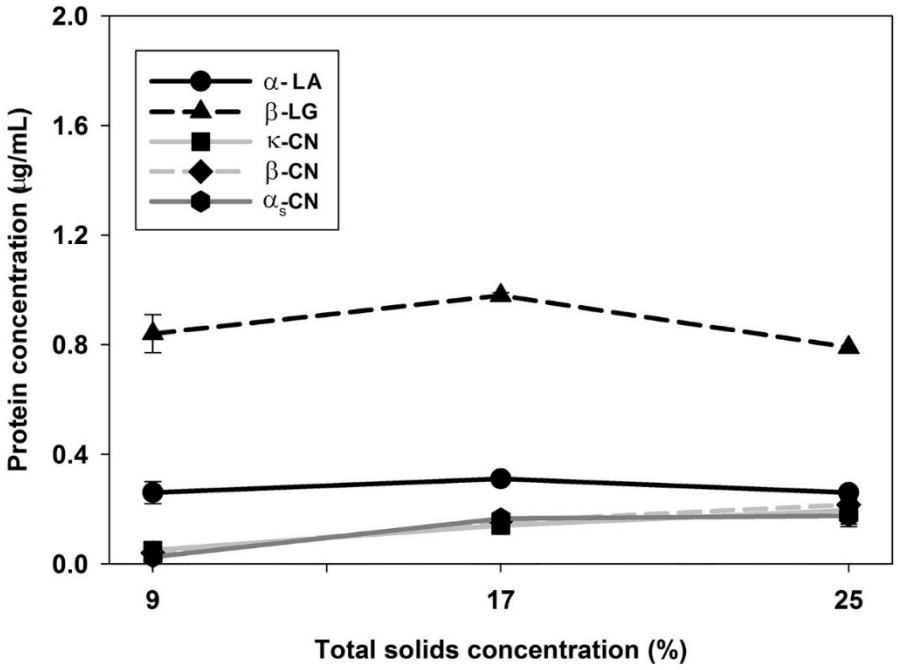

Figure 3.2: Protein concentration in the serum phase of raw milk containing 9, 17 or 25% total solids. The present means are recalculated in µg of protein as in unconcentrated milk.

Concentrations of all proteins in concentrated samples were recalculated to the value of unconcentrated milk in order to enable comparison of the results. It appeared that proportion of κ- casein (κ-CN) increased proportionally with concentration factor. Interestingly, concentration of β-casein (β-CN) and α_s-casein (α_s-CN) in the serum of concentrated samples was greater than expected due to concentration increment. Concentration of all caseins increased in the serum phase probably due to their dissociation from the micelle, suggesting that the casein micelle undergoes a partial disintegration during the concentration process. Amongst whey proteins, α-lactalbumin (α-LA) did not show significant ($P>0.05$) change in its concentration after water removal in skim milk. On the contrary, proportion of β-lactoglobulin (β-LG) started to decrease in the serum significantly ($P<0.05$) when solids content was increased to 17 and 25% reaching values of 5.5 and 7.9 g/L, respectively, from its initial concentration of 3.5 g/L. Concentration of

β-LG in 17% and 25% TS was lower by 11% and 17%, respectively, compared to theoretical values, which may be attributed to the association of β-LG with the casein micelle and subsequent sedimentation after ultracentrifugation.

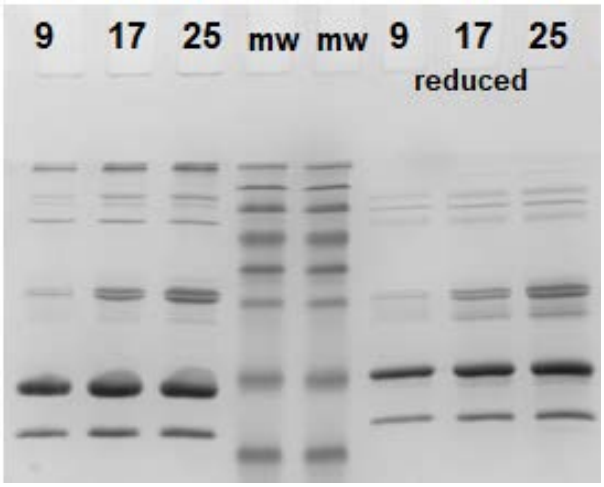

Figure 3.3: SDS PAGE of milk with three different solids concentrations. First three lines on the left are non-reduced and three different lines on the right are reduced by β-mercaptoethanol.

3.3.2 Changes in FTIR spectra due to concentration

In the FTIR spectrograms, the regions between 1200-900 and 1700-1600 cm^{-1} (Amide I) were studied for elucidating conformational changes of lactose and proteins, respectively (Figure 3.4 and 3.6). In the region 1200-900 cm^{-1}, the observed peaks included 1118, 1075, 1041 cm^{-1} that corresponded to C-O stretching of sugars (lactose) in milk (Zhou et al., 2006). The intensity of these peaks increased concomitantly with the rise in total solids concentration. Furthermore, increscent of a peak at 1075 cm^{-1} with concentration factor allocate covalently bonded phosphate groups to the caseins in the milk (Jaiswal et al., 2015).

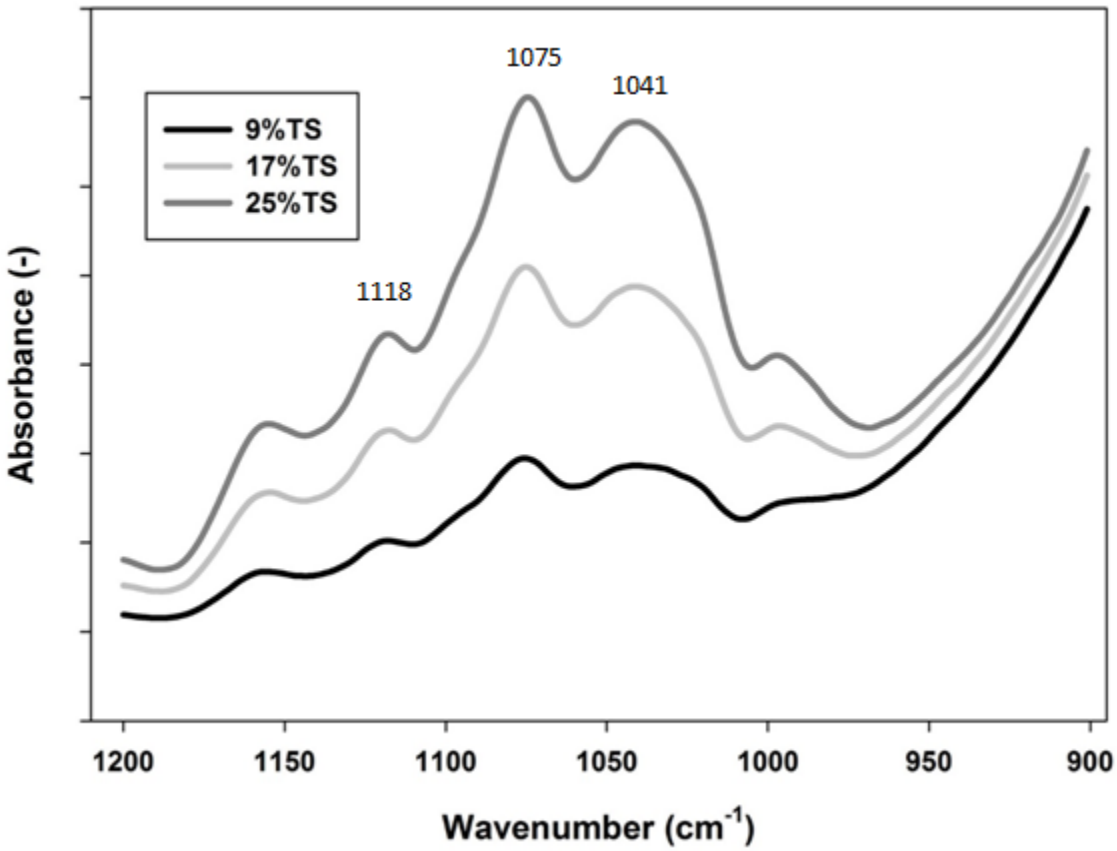

Figure 3.4: FTIR spectra of the raw skim milk samples concentrated to different total solid concentrations (9, 17 or 25%) obtained in a region between 1200-900cm^{-1} depicting absorbance governed by carbohydrates.

PCA was performed in the region from 1200-900cm^{-1}, where PC1 and PC2 explained 91% and 3% of the variance, respectively (Figure 3.5). Samples were classified in three groups according to concentration level and the loading plot (Figure 3.5B). PC2 separated the samples by higher loading for most concentrated samples for 1118, 1075 and 995 cm^{-1}. Moreover, the loadings were less intensive as concentration factor decreased indicating modifications in lactose molecules and changes in vibrations of $–PO_3^{2-}$ when solids content was altered.

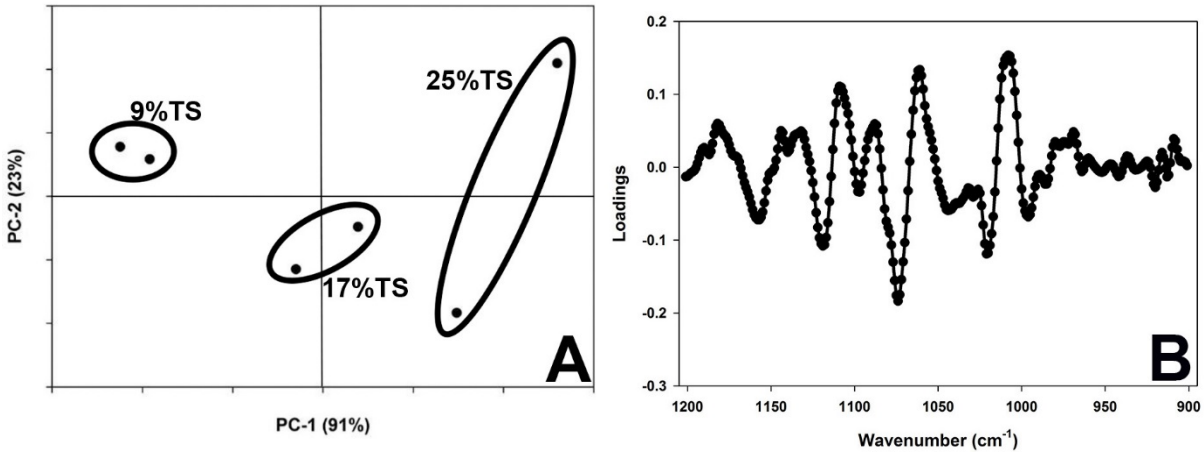

Figure 3.5: Principal component scores (A) and loading plots (B) for region 900-1200cm^{-1} for raw skim milk with different solids content.

In Amide I region (1700-1600 cm^{-1}), used for identification of peaks assigned to C=O stretching of proteins, the second derivative of the FTIR spectra was calculated in order to visualize otherwise hidden peaks (Figure 3.6). The observed peaks include 1695 cm^{-1} (aggregated β sheets), 1670 cm^{-1} and 1663 cm^{-1} (β turns), 1653 cm^{-1} (α helix), 1645 cm^{-1} (unordered structures) and 1632 cm^{-1} and 1622 cm^{-1} (antiparallel β sheets). The variation in the intensity of the peaks was predominantly governed by the increase in the total solids concentration; however, there were some noticeable deviations. For example, the spectrum of the 17% concentrated sample in comparison to the original sample is characterized by enhanced intensity of every peak almost proportionally with the concentration step; however, in the peak intensity depicting the α-helical structure has increased even more. Further sample concentration (25% TS) produced even more intense variations in the peak distribution, especially in a decrease of peak at 1622 cm^{-1} that denote antiparallel β sheets with a slight increase in α- helix (1653 cm^{-1}) and formation of randomly distributed structures (1645 cm^{-1}).

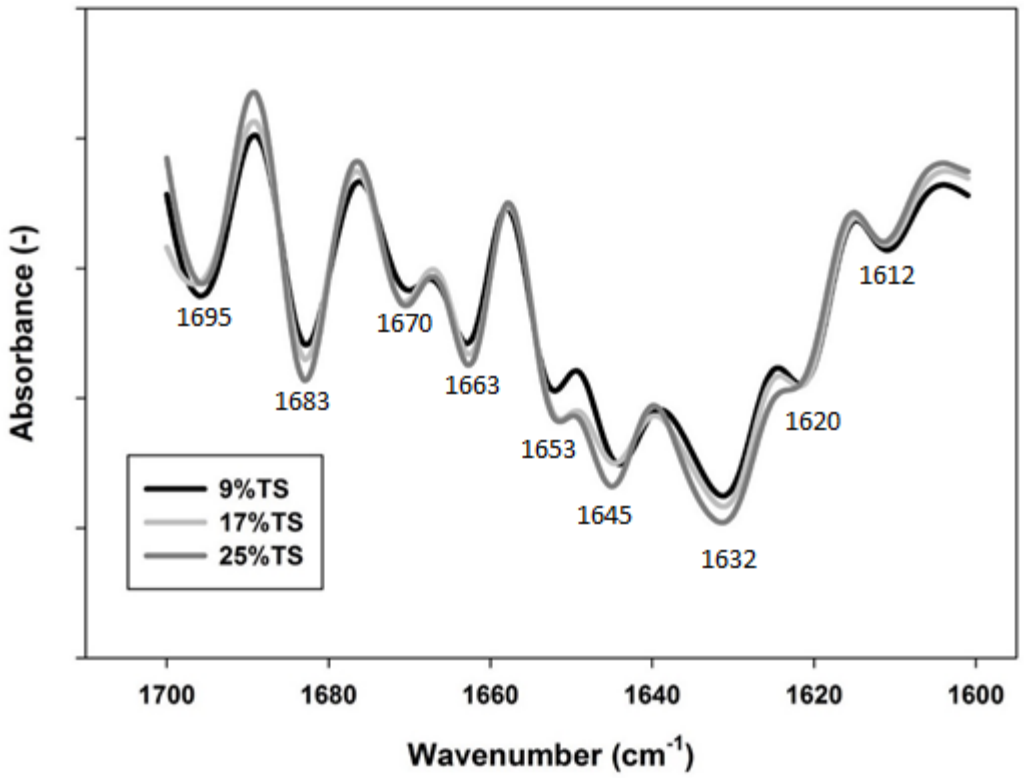

Figure 3.6: Second derivative of Amide I region of samples with different solids concentration (9, 17 and 25 %). Averaged spectra of two measurements.

In the assessed samples, PC1 and PC2 explained 65% and 20% of the variance, respectively (Figure 3.7). As detected by the second derivative spectra, PCA separated samples based on the concentration. When solids concentration increased from 9 to 17 and 25%, the PC2 indicated increscent in wavenumbers loadings. Hence, in 25% concentrated milk high loadings were observed for regions 1645-1652 cm^{-1} that again relates to presence of more random structure and α- helix, on the other hand β-sheets were more frequent in lower concentrations. Moreover, the results confirmed that increase in concentration level changed substantially the secondary structure of proteins, which was more pronounced at high solids content.

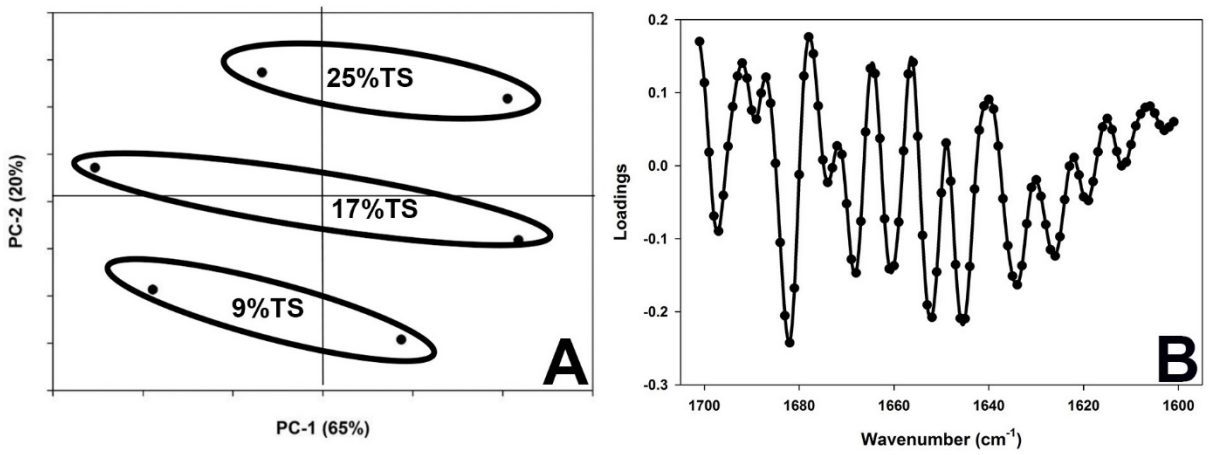

Figure 3.7: Principal component scores (A) and loading plots (B) for Amide I region for raw skim milk containing different solids content.

3.4 Discussion

As indicated in the introduction, this study wanted to bring greater understanding in regards to physical and potentially chemical changes during concentration step in the processing of condensed milk. For decades, it has been a common industrial practice to preheat milk prior to concentration in order to reduce the level of aggregation of whey proteins during evaporation of skim milk. Preheating denatures whey proteins and hence prevents their further cross-linking with the casein micelle during evaporation, which appears to govern a number of instabilities taking place during sterilisation (Webb and Bell, 1942).

In general the observed changes appeared to be subtle and more physical in nature. For example, concentration did not affect ($P>0.05$) average particle size and net negative charge of the milk particles. The results confirmed findings of a previous study (Bienvenue et al., 2003b) that non-preheated milk after evaporation process did not result in particles with enlarged diameter. Slight reduction of β-LG was observed in the serum of samples with 25% TS probably due to its

attachment to the micelle, however it is not clear whether this attachment took place on the surface since only minimal modification in the particle size was noted. Furthermore, it can be argued that unfolding of β-LG to a certain extent has taken place in order for it to associate with the casein micelle, which explains its decrease in the serum phase. The denaturation of whey proteins starts above 60°C (Dannenberg and Kessler, 1988) and temperature of evaporation in the study did not exceed 55°C. Nevertheless, transition and unfolding of β-LG from dimmer to monomer has been reported to start at as low as 50°C (Liu, 2014) under certain conditions, hence exposing its free thiol group, which may later react through thiol-disulphide bonding with other whey proteins or κ-CN (Brodkorb et al., 2016, Walstra, 1999). Additionally, unfolding of β-LG dimmers at lower processing temperatures may also expose some hydrophobic residues, which may engage in weak interactions (Oldfield et al., 1998b). Recently Mediwaththe (2017) showed that even a moderate shear, similar to rotations of the rotary evaporator, may induce subtle changes including change in hydrophobicity and even initiate aggregation at 20°C.

In addition, in this study when sample was concentrated up to 25% TS, β-LG appeared to be weakly attached to the micelle and separated after centrifugation into the pallet. This attachment apparently did not change significantly the micelle size. Modification of β-LG properties was only observed when milk was highly concentrated (25% TS). It appears that such an increased solids concentration may have induced more close packing of the molecules and thus greater probability of new interactions. On the other hand, in samples with lower total solids content this type of behaviour was not the case likely due to greater repulsive forces that prevented these molecules to engage in new interactions and create new particles. However, types of interactions that are involved here could not be clearly identified and require further assessment.

In regards to stability of the casein micelle, partitioning of individual caseins into the serum phase increased with the rise in concentration of total solids. The observation was contrary to a previous study by Liu et al. (2012), who observed that when preheated skim milk was concentrated (using the same method as in the current study), caseins were more confined in the colloidal phase during the process of water removal. However, slight dissociation of β-CN and κ-CN from the micelle at pH around 6.5-6.7 with the mild heating up to 60°C of reconstituted milk has been reported by Anema and Klostermeyer (1997b). They observed that the dissociation of β-CN and κ-CN is strongly dependent on the pH and the heating temperature. For any particular temperature and pH, the dissociation of caseins followed the trend: κ>β>α_s-CN. Although in agreement, the trend of dissociation of individual caseins from the micelle was not linear in our study as β-CN and α_s-CN were more present in the serum in the samples at intermediate total solids concentration (17% TS) whereas the κ-CN became the most prominent casein in the serum at higher solids concentrations (25% TS). Similar behaviour has also been observed by Dumpler et al. (2017). In their report, dissociation followed the trend α_s>β>κ-CN with small aggregates of β-CN and κ-CN observed in the serum depending on the temperature and concentration. However, their study was more about the effect of temperature on already concentrated samples and not the effect of the increased solids concentration alone, which was the highlight of our study.

Changes in mineral composition affect equilibrium of the milk system and hence have a large effect on the protein stability. Similar to previous observations, calcium and phosphate ions shifted from the serum into the micellar state during concentration step and saturated the micelle with additional colloidal calcium phosphate (CCP) (Hardy et al., 1984, Holt et al., 1986, Nieuwenhuijse et al., 1988, Liu et al., 2012). Sodium and magnesium also shifted into the

micellar state, however the observed saturation was more pronounced at higher solids content (25%). On the contrary, concentration of ionic Ca^{2+} was slightly reduced with the increase in solid concentration, which is in agreement with previous report by (Nieuwenhuijse et al., 1988). Newly established equilibrium in more concentrated systems apparently has driven calcium and phosphate to be more involved in associations with the casein micelle by saturating the micelle with additional colloidal calcium phosphate. This saturation may have destabilised the micelle by disruption of hydrophobic bridges between the caseins and their consequent dissociation into the serum (Nieuwenhuijse et al., 1991a, Singh et al., 1995). In addition, this saturation of the micelle with additional mineral content also participates in liberalization of H^+ ions that reduce pH of the system (Nieuwenhuijse et al., 1991a).

The region (1200-900cm^{-1}) corresponds to C-O stretching vibrations of lactose (Zhou et al., 2006). The change in intensity of peaks in this region could be attributed to the interactions between lactose with amino acids of proteins mainly lysine (Maillard reaction). The rate of Maillard reaction is strongly dependent on the concentration of reactive amino groups in the system, which proportion increases with the concentration of TS (Turner et al., 2002). Furthermore, at high total solids concentration, along with increase in NH_2 groups, lactose concentration is also great, which further accelerates the Maillard reaction. Moreover, increase in intensity of peaks at 1118, 1075 and 1041 cm^{-1} when concentration is increased indicates high C-O stretching due more frequent interactions between lactose and lysine. Thus, the availability of NH_2 groups is high in high solids content that stimulate the reaction. Increased intensity of a peaks at 1075 cm^{-1} and 995cm^{-1} correlates to changes in stretching vibration of phosphoserine when solids content is altered indicating high bond energies from bonding of reduced serum PO_3^{2+} to serine residues of caseins (Grewal et al., 2017a).

Peak assignment in the in amide I region has been performed according to the previous work by Grewal et al. (2017a). It is known that β-LG has a more defined secondary structure compared to other dairy proteins (Sawyer, 2003). Therefore, the changes in the secondary structure in concentrated samples may be related to rearrangement of the backbone of β-LG. Furthermore, observed changes in the region between 1620-1650 cm^{-1} has been related previously to the changes in monomer-dimmer equilibrium of β-LG (Lefèvre and Subirade, 1999). This is explained by the appearance of only one component in the region from 1620-1635 cm^{-1} in samples with 25 % TS, which has been attributed to the dissociation of β-LG from its dimeric to monomeric form followed by conformational transition (Lefèvre and Subirade, 1999). Likewise, this suggestion is also supported by decrease of β-LG in the serum phase observed in the samples with high total solids concentration as observed in SDS-PAGE patterns. These patterns depict its denaturation in a more crowded environment accompanied by exposure of its burred hydrophobic zones and free reactive thiol group (Cys_{121}), which would be further involved in interactions with κ-CN on the micelle surface (Sawyer, 2003). Changes in the secondary structure were further confirmed by the observed increase in intensity of a peak at 1645 cm^{-1} in the highly concentrated sample, which clearly indicates presence of random structure (Liyanaarachchi et al., 2015, Grewal et al., 2017c). This can be again correlated to dimmer/monomer transition of β-LG and subsequent formation of intermolecular β sheets leading to aggregation (Liyanaarachchi et al., 2015). However, since the temperature of evaporation did not exceed 55°C, the denaturation of whey proteins under these conditions was less intense but apparently still present. Moreover, increase in the intensity of a peak at 1645 cm^{-1} assigned to the random structure may also be related to greater presence of caseins in soluble aggregates formed in the serum phase (Grewal et al., 2017c, Grewal et al., 2017a).

Furthermore, increase in α-helical structure may also relate to loop structures created by liberated caseins in the serum. Some other explanations for increase in a peak intensity at 1645 cm^{-1} in the samples with 25%TS exist and include lactosylation of milk proteins (Grewal et al., 2017a), which is directly related to the concentration of reactive ε-amino group of lysine (Turner et al., 2002). Since, the concentration of all solids in the system was increased, it could be expected that probability of this chemical reaction would be also great.

3.5 Conclusion

The water removal from the raw milk has a considerable effect on all constituents in the milk system. The concentration of total solids impacts the existing equilibrium of the system leading to new interactions and the dissociation of individual caseins from the micelle. The effect of water removal induce dissociation of αs- and β-casein when solids concentration was almost doubled, on the other hand κ-casein did not dissociate substantially at this level of concentration, however after threefold concentration step it became significantly more present in the serum phase. The effect of concentration on β-LG was more pronounced in 25% TS concentration characterized by its denaturation and reduction in the serum phase, which was again confirmed with rearrangement in the secondary structure at the given concentration level. The findings report the effect of concentration on constituents of a raw/native milk system indicating significant destabilizations in skim milk proteins when water content is reduced signifying the importance of stabilization of milk constituents prior evaporation treatment. In regards to improving the stability of skim milk concentrates, pre-warming as practical application in the industry appears required, although some alternative approaches may also be examined.

Chapter 4 Heat induced changes of milk proteins in concentrated raw skim milk

4.1 Introduction

In the dairy industry, milk is concentrated to a certain level of total solids (TS) with a purpose to obtain a product with high nutritional value and long shelf life. Production of evaporated milk is very straightforward and involves removal of more than a half of the water and creation of a product with long term high stability. Increased solids consequently lower water activity thus creating conditions unfavourable for various biological and chemical processes. However, while this process may appear fairly simple, a number of changes in the system have been noticed in the past including intensive fouling of evaporators and heat exchangers during processing, aggregation and formation of sediments, coagulation and age-gelation of the final product (Adochitei and Drochioiu, 2011, Benton and Albery, 1926, Jeurnink et al., 1996, McKenna and O'sullivan, 1971, Singh et al., 1995, Tarassuk and Tamsma, 1956). Therefore, in order to achieve a heat stable product industrial practice has been to apply forewarming before water removal followed by addition of stabilizers and sterilization.

Milk in its natural concentration can withstand fairly high temperatures applied during a sterilization process. On the other hand, concentrating milk often results in certain system modifications influencing heat stability by partial or complete coagulation during sterilization. Therefore, concentration factor is usually maintained no more than 2.6 times or ~22% TS, which is considered as a limit after which an intense coagulation takes place (Walstra et al., 2005). Moreover, many practical applications are implemented in dairy industry in order to improve the quality of the product and prevent undesirable consequences including addition of stabilizers. However, many other factors have a great influence on stability of processed products such as seasonal variations, lactation period and breeding of dairy cattle, acidity, solids concentration and other process variations.

Evaporation itself is not sufficient to achieve required preservation of the final product, therefore for prevention of biological, including bacterial and enzymatic, contamination sterilization is applied as the final processing step (Van Den Berg, 1962). Certain instabilities of concentrated milk products result mainly from heat induced coagulation, which relates to temperature time combinations required for deactivation of certain microorganisms, which are usually higher than the limit of heat and colloidal stability of the product (Singh et al., 1989). Sterilization of evaporated milk in the industrial practice is performed as in-can sterilization at 120°C for appropriate time required to achieve 12D level of deactivation of spores of *Clostridium botullinum* (Early, 1998). Furthermore, heat stability of concentrated milk products is defined as their ability to withstand high temperatures (Singh, 2004). An extensive body of knowledge exists that covers heat stability of unconcentrated milk (Huppertz, 2016, Singh, 2004, Fox and Morrissey, 1977, McCrae and Muir, 1995); on the other hand, there is not much information about how concentrated systems transition and behave during concentration in regards to their physicochemical changes that may consequently lead to detrimental changes during heating. Main physical issues during manufacturing of evaporated milk appear to result in coagulation during sterilization, sediment formation and age gelation (Huppertz, 2016)

Moreover, above mentioned heat instabilities of concentrated milk products mainly refer to destabilization of milk proteins or whey proteins and casein micelle, which are involved in aggregate formation. The aggregation process involving these two classes of milk proteins depends on many variables created in the milk composition by water removal during evaporation step and further heat treatment and involves parameters such as high ionic strength, reduction of pH, redistribution of minerals among the serum and colloidal state that destabilize total equilibrium in the system (Huppertz, 2016).

It is widely known that whey proteins are very heat sensitive proteins. They start to unfold around 60°C from their native state and expose their reactive groups, which are responsible for further interactions and aggregate formation (Anema and McKenna, 1996, Brodkorb et al., 2016, Oldfield et al., 2005, Oldfield, 1996, Oldfield et al., 2000, Roefs and Kruif, 1994, Singh, 1995, Zuniga et al., 2010). Due to their high reactivity, whey proteins have high destabilizing effect on the overall system during heat treatment of evaporated milk, therefore preheating of milk before concentration step is applied in order to denature whey proteins before concentration and prevent further aggregation (Walstra et al., 2005). Nevertheless, it was reported that in concentrated milk denaturation of whey proteins is retarded at elevated temperatures due to greater solids concentration in the system that hinder mobility of the particles and obstruct the space thus limiting their ability to open their structure and engage in further interactions (Anema, 2000, McKenna and O'sullivan, 1971). However, available information is lacking as for solids levels and temperature limits at which this retardation takes place in non-preheated concentrated skim milk products.

The casein micelle is also destabilised during heat treatment mainly due to dissociation of κ-casein from the surface (Anema, 1998, Anema and Klostermeyer, 1996, Anema and Li, 2003b, Nieuwenhuijse et al., 1991a, Dumpler et al., 2017), which is responsible for steric and electrostatic stabilization of the micelle (Huppertz, 2013). Dissociated κ-casein would be consequently involved in covalent and hydrogen interactions with aggregated whey proteins creating further aggregated gel-like structure. At this stage, the casein micelle may completely disintegrate due to removal of stabilizing hairy brush that would lead to liberation of internally located caseins and their further involvement in cross-linking.

Moreover, all these findings were reported based on observations of heat treated pre-warmed or reconstituted milk, which obscures a thorough understanding due to already applied treatment. On the other hand, the reported observations obtained in experiments using concentrated raw milk date as early as 1930's with knowledge in regards to changes during heat treatment being rather empirical, which therefore imposes a need for gaining fundamental understanding in relation to heat induced unfolding and aggregation of milk proteins, specifically related to subtle changes in the secondary structure of proteins. In Chapter 3, it was observed how concentration step affected individual proteins in the system and how whey proteins and caseins behaved at different concentration levels. Thus, the work in this chapter attempted to provide greater understanding how proteins behaved in raw concentrated skim milk systems upon exposure to commercially applicable heating regime. The main focus would be positioned on protein distribution between the serum and micellar phase of milk and conformational rearrangements of molecules during heating.

4.2 Materials and methods

4.2.1 Sample preparation

Fresh raw milk was collected from a local manufacturer (Murray Goulburn Cooperative Co. Ltd, Laverton North, Victoria, Australia). Milk was prepared for experimental purposes by adding sodium azide (0.01%) for prevention of bacterial growth during manipulation with the samples. Skim raw milk was prepared by centrifugation at 40°C at 3225 g for 30 minutes using an Avanti J-26XP centrifuge (Beckman instrument Australia Pty. Ltd, Gladesville, NSW, Australia). After fat removal, skim milk was pre-planned for evaporation process and for that purpose the total solids (TS) content was measured by oven drying overnight at 105°C. Evaporation process was

performed by a rotary evaporator (Rotavapor® R100, John Morris Scientific, Deepdene, Victoria, Australia) at 55ºC for up to 2 hours. The totals solids concentration was measured and recorded periodically starting from 9% TS at original milk to the final concentration of 17 or 25% TS.

Each sample at 9, 17 or 25% TS was divided into six 10 mL aliquots for heat treatment and seventh aliquot was kept as a control. The prepared samples were placed in an oil bath set at 121°C and removed when the temperature reached a required level including 75, 85, 95, 100, 110 or 121°C. The difference in holding time during heating at 121°C was performed to achieve commercial heating conditions of concentrated milk. One aliquot was removed at each temperature while the final sample was kept at 121°C for 2.6 minutes. Every sample was immediately submerged in an ice bath and prepared for further analysis. Separation of the serum phase in the milk was performed by ultrahigh centrifugation at 21°C for 1 hour at 100,000 g using a Beckman Ultra L-70 centrifuge (Beckman, Coulter, Australia Pty. Ltd, Gladesville, NSW, Australia).

4.2.2 Particle size and zeta potential (ζ)

The size distribution and zeta potential of heat treated samples and controls with different solids concentrations was measured following the same method as described previously in section 3.2.2.

4.2.3 Determination of mineral content

The total and serum concentration of selected minerals was determined in heat treated samples and the control containing different solids concentration. Calcium (Ca), magnesium (Mg), phosphate (PO_4) and sodium (Na) were the elements analysed by an inductively coupled plasma

emission spectrometer (ICP-AES) sequential plasma spectrometer (ICP-9000 system, Shimadzu Corporation, Kyoto, Japan). Preparation of the heat treated samples and supernatants of each samples was conducted as described in section 3.2.3.

The effect of heat treatment of different concentrated samples on ionic calcium (Ca^{2+}) was measured accordingly to the method previously described by (Chandrapala et al., 2010). Moreover, the method was explained in more details in section 3.2.3.

4.2.4 Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used for determination of protein distribution in the serum phase in regards to kinetics of denaturation upon exposure to the heating protocol. Every analysis was carried out in duplicate from randomly selected samples. The electrophoresis was performed following the same steps as explained in details in section 3.2.4.

4.2.5 Fourier transform infrared (FTIR) spectroscopy

Immediately after cooling of heat treated samples, they were analysed for the effect of the treatment in regards to solids concentration on the secondary structure of proteins. Second derivative was applied for greater resolution of the peaks in the region 1700-1600 cm^{-1} (Amide I) related to conformational changes in proteins. Sample preparation was performed as described in section 3.2.6.

4.2.6 Statistics

General linear model (GLM) and Tukey's Studentised Range (HDS) test was used for data analysis. All the results were arranged in a split block design with the concentration as the main plot and the temperature as the subplot. The overall design was replicated on two occasions,

which served as a block, using Statistical Analysis System (SAS). $P<0.05$ was considered as a level of significance.

4.3 Results

4.3.1 Effect of heating and concentration level on physiochemical properties of the raw skim milk systems

4.3.1.1 Particle size

Heating of skim milk with different solids concentration resulted in variation of particle diameter significantly ($P<0.05$) only in samples with alerted solids content and at particular temperatures. For example, particle size distribution and average particle size of unaltered milk (9%TS) did not change significantly ($P>0.05$) during the heat treatment, moreover it maintain the more or less the initial diameter, which was within a previously reported range (McMahon and Oommen, 2013). When the concentration was increased to 17% TS, the particle size remained fairly consistent up to 110°C but it changed ($P<0.05$) during 2.6 min holding at 121°C indicating extensive aggregation of milk proteins (Table 4.1). Further concentration increase, to 25% TS, resulted in slight but not significant ($P>0.05$) changes of the average particle size at 100°C. Indicatively enlargement of the casein micelle was noticeable around 85°C but major increase in the particle size took place at and above 110°C. Extent of these changes is likely related to greater probability of protein/protein interactions in a more crowded environment due to reduced repulsion and closer packing of the molecules that induce prompt response to temperature changes.

Table 4.1 Particle size diameter (nm) of concentrated raw milk samples containing 9, 17 or 25% total solids during heating and after sampling at indicated temperatures. The results are means of six measurements from two different batches of milk. Means in a row with different superscript lower case letters and in a column with different superscript upper case letters differ significantly ($P<0.05$); *SEM – pooled standard error of the mean.

Total solid concentration, %	Average particle size, nm						
	Control	75°C	85°C	95°C	100°C	110°C	121°C
9	150^{aA}	149^{aA}	160^{aA}	158^{aA}	167^{aA}	159^{aA}	146^{aA}
17	164^{aA}	171^{aA}	169^{aA}	175^{aA}	177^{bA}	187^{aA}	414^{bB}
25	166^{aA}	165^{aA}	182^{aA}	217^{aA}	204^{bA}	392^{bB}	358^{bB}
SEM*				21.7			

4.3.1.2 Zeta potential (ζ)

As indicated in Chapter 3, zeta potential of concentrated samples did not differ significantly ($P>0.05$) from the unconcentrated milk. However, this consistency was disturbed during heating as surface potential varied greatly (Figure 4.1). Surface potential of particles in the unconcentrated sample initially significantly ($P<0.05$) declined to -17.4 mV at 95°C followed by a significant ($P<0.05$) rise to -14.4 mv at 110°C remaining relatively constant until the end of the heating protocol.

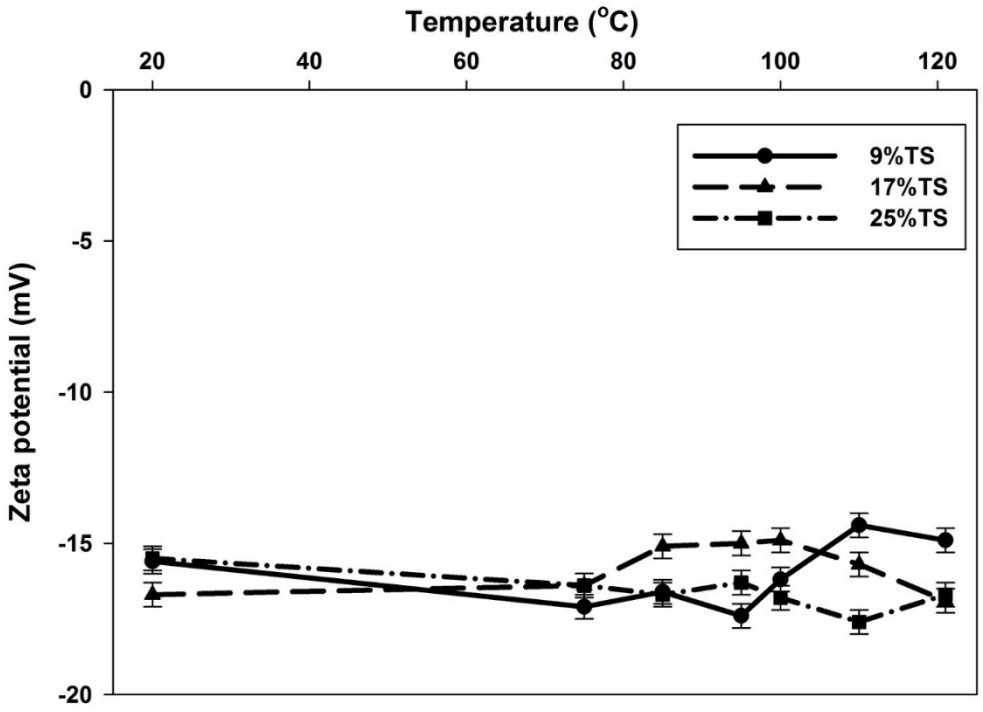

Figure 4.1: Zeta potential (mV) of concentrated raw milk samples containing 9, 17 or 25% total solids during heating and after sampling at indicated temperatures. The results are means of six measurements from two different batches of milk.

Heating concentrated samples has produced different outcomes, which could have been due to induction of different electrostatic interactions leading to modifications of electronic charge on the surface of the particles. For example, zeta potential of the 17% concentrated skim milk during heat treatment initially became significantly less negative increasing from -16.7 mV at 75°C to -14.9 mV at 100°C. Further heating apparently resulted in creation of particles with a significantly ($P<0.05$) more negative ζ potential, almost equal to that at the start of the heating. On the other hand, zeta potential of particles in highly concentrated system (25%TS) experienced a significant drop from its initial value of -15.5 mV down to -17.6 at 110°C. Prolonged heating at

121°C did not result in a substantial change of the surface potential, indicating that most of the changes took place well before this temperature.

4.3.1.3 Minerals

Ionic balance in milk presents equilibrium between minerals in the soluble and colloidal phases. A number of different conditions including heating and total solids concentration may affect this balance. The concentration of minerals (Ca, Mg, Na and Pi) was measured in the supernatant of the treated samples. As indicated by Figure 4.2 divalent cations represented by Ca and Mg appeared affected the most by heating, followed by inorganic phosphate, while Na appeared fairly consistent and not much affected by heating (Figure 4.2D). As an obvious trend, Ca, Mg and phosphate ions experienced very gradual decline in the serum during heating in their natural concentrations. Only above 100°C, this decrease was apparent and significant ($P<0.05$). This was expected as the serum calcium in normal milk during prolonged heating at 121°C declines as a result of transfer of soluble calcium to the colloidal state (Nieuwenhuijse et al., 1988). Similarly, during heat treatment of milk with increased solids concentration (17%) induced a significant ($P<0.05$) decline of the soluble form of calcium, however this change was induced sooner (<100°C). This result is indicative of intense shifting of the serum calcium into the casein micelle. Upon further concentration (25%), concentrated milk system experienced a similar but more pronounced shifting of the soluble Ca than that observed at 17% TS. The maximum reduction of the soluble calcium was observed even before 75°C and a steep decline just before 100°C; however, this was then followed by resolubilisation of Ca from the micelle as its concentration increased from 11.8±0.5 mM to 14.5±0.5 mM.

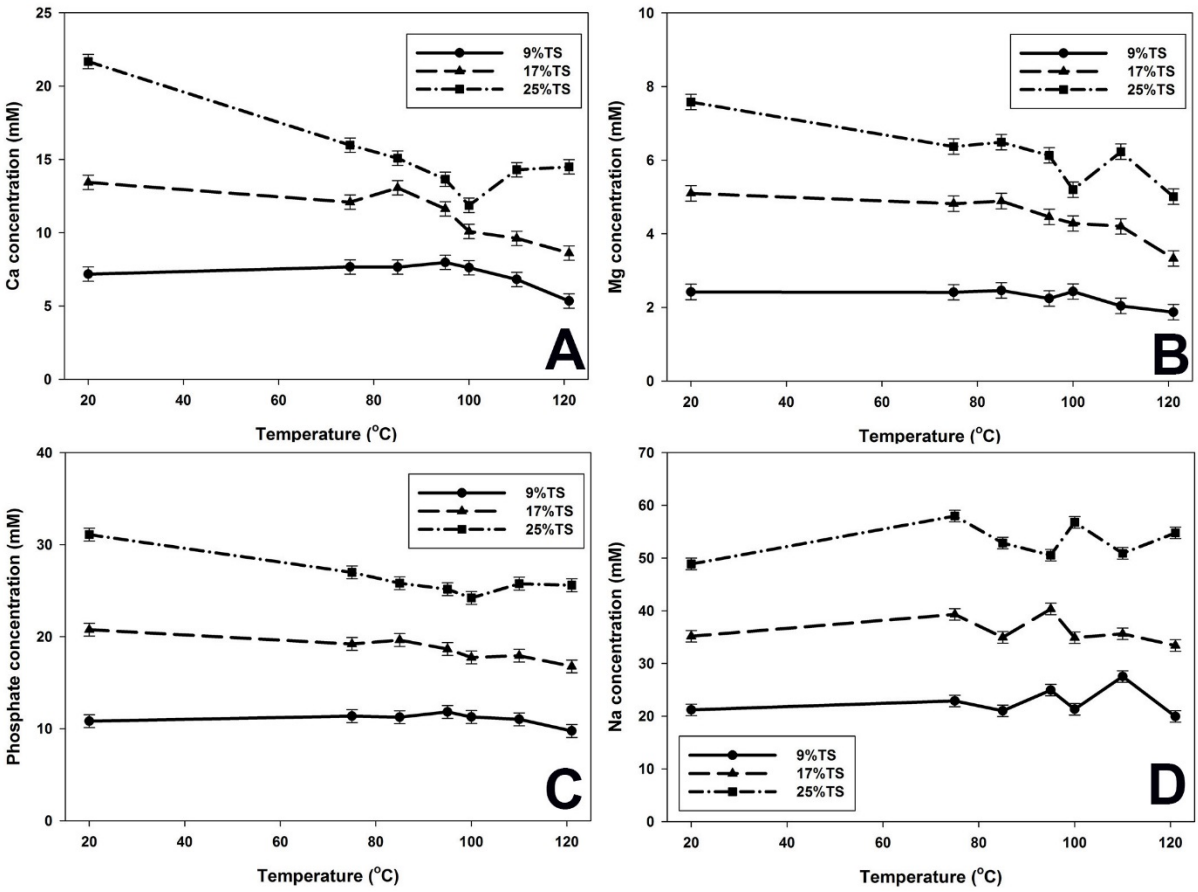

Figure 4.2: Mineral concentration in the serum phase (mM) of three samples containing 9, 17 and 25% TS. The results presenting calcium (A); magnesium (B); phosphate (C); and sodium (D) are means of four measurements from two batches of milk.

Equilibrium between soluble and micellar magnesium followed a similar trend to that of calcium but to a lesser extent slightly declining above 100°C at its natural concentration, slowly or rapidly declining <100°C at 17 or 25% TS (Figure 4.2B). Similarly, to Ca, Mg resolubilised above 100°C in a sample containing 25% TS increasing its concentration in the serum but then declining again. Since no visible salt precipitation was noticed, it may be assumed that Mg re-equilibrated to its colloidal form.

Inorganic phosphate in all heat treated milk samples followed a similar pattern to that of Ca, although changes appeared significant ($P<0.05$) only after heating was completed (Figure 4.2C). Hence, in unconcentrated milk only slight reduction of Pi was observed when milk was treated at 121°C for 2.6 minutes. In samples with 17 and 25% TS heating resulted in a slight shift of Pi into the micelle with maximum transfer at 121°C.

Ionic Ca^{2+}

Concentration of ionic Ca^{2+} in the unconcentrated sample was similar to previous reports (Chandrapala et al., 2010, Nieuwenhuijse et al., 1988). As noted in Chapter 3 and depicted in Table 4.2, the initial concentration of ionic Ca appeared significantly ($P<0.05$) lower at 25% TS. It would be expected to observe a rise in ionic Ca concentration in the serum due to increased solids concentration, however its concentration remained fairly constant then declined upon concentrating milk up to 17 and 25% TS, respectively.

Table 4.2: Ionic Ca^{2+} concentration in skim milk with 9, 17 and 25% TS during heating and after sampling at different temperatures. The results are means of four measurements from two different milk samples. Means in a row with different superscript lower case letters and in a column with different superscript upper case letters differ significantly (P<0.05); *SEM – pooled standard error of the meanesults are means of four measurements from two different milk samples. Means in a row with different superscript lower case letters and in a column with different superscript upper case letters differ significantly (P<0.05); *SEM – pooled standard error of the mean

Total solids concentration ,%	Ionic Ca^{2+} concentration (mM/L)						
	Control	75°C	85°C	95°C	100°C	110°C	121°C
9	1.57^{aA}	1.67^{cA}	1.67^{cA}	1.75^{dA}	1.78^{eA}	1.79^{fA}	1.63^{bA}
17	1.57^{aA}	1.52^{bdB}	1.54^{cB}	1.51^{bdeB}	1.49^{defB}	1.48^{efB}	1.36^{gB}
25	1.46^{aB}	1.38^{bC}	1.41^{cC}	1.41^{cC}	1.36^{dC}	1.32^{eC}	1.19^{fC}
SEM*				0.006			

At 17% TS, consequent heat treatment reduces the level of ionic Ca in the system. Starting concentration of ionic Ca was 1.57 mM and the effect of heating was minor at every 10°C increase up to 121°C when the concentration was reduced to 1.36 mM. Highly concentrated milk

samples (25%) also experienced reduction in ionic Ca^{2+}, which increased as applied temperature rose. Moreover, the maximum reduction ($P<0.05$) was observed at 121°C. Thus, lower heating temperatures from 75 to 95°C affect the ion composition only slightly, however further increase in temperature lead to extensive reduction, which was 1.19 mM at 121°C.

4.3.1.4 Proteins

Heating of unconcentrated milk resulted in changes in the protein composition in the serum phase. Clearly, whey proteins (α-LA and β-LG) were the most affected as their concentration declined substantially with rise in temperature to their maximum reduction at 121°C (Figure 4.3 A,A1). The reduction of whey proteins concentration in the serum stems mainly from interactions via covalent bonding between these proteins and the casein micelle during heat treatment and their separation into the pallet by ultracentrifugation. Furthermore, heating led to appearance of these proteins in the serum as a part of aggregated whey protein-κ-casein.

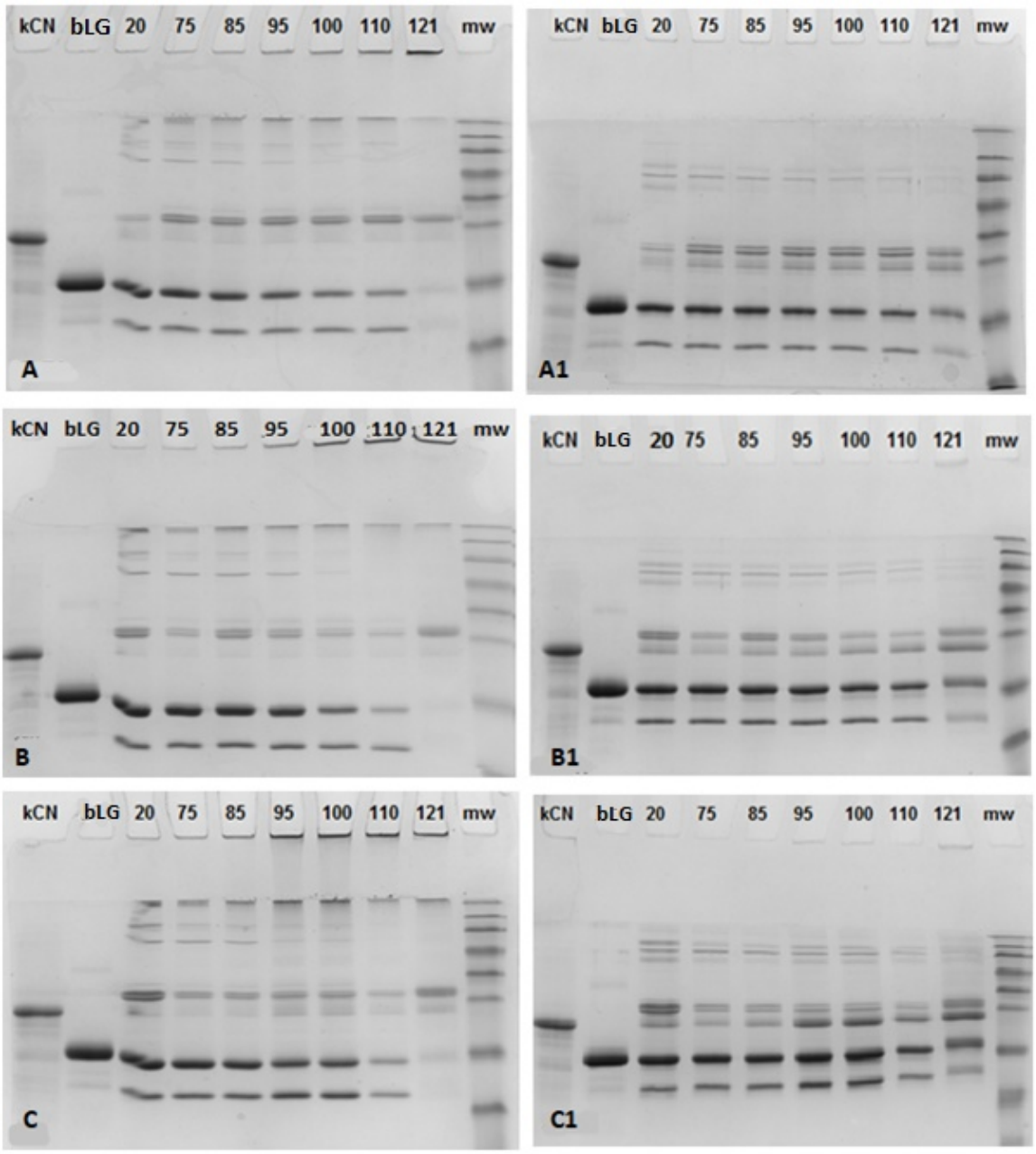

Figure 4.3: SDS PAGE of supernatant obtained by ultracentrifugation of milk samples containing 9% TS (A, A1), 17% TS (B, B1), and 25% TS (C, C1). Where A,B,C are non-reduced gels and A1,B1,C1 are reduced gels.

All caseins including κ-, αs- and β-casein appeared to become more prominent in the serum when the temperature increased from 75°C up to 110°C (Figure 4.3 A1). However, prolonged

heating at 121°C resulted in reduction of the caseins levels in the supernatant likely due to new and reinforced covalently driven interactions among these proteins or with the whey proteins that created new, this time larger aggregates likely with the micelle (Figure 4.3A1). These individual caseins including αs- and β-casein in the serum at all temperatures were present as more or less soluble monomers; on the other hand, κ-casein was involved in formation of aggregated complexes with whey proteins at all heating temperatures. Moreover, upon prolonged heating of skim milk at 121°C, the level of αs-CN (more precisely αs_2-CN) as indicated by the SDS gels has declined indicating involvement of this protein in the covalent interactions and formation of aggregates in the serum phase or association with whey proteins within the micelle (Figure 4.3).

In comparison to unconcentrated samples, the samples concentrated up to 17% TS showed greater variations in the protein composition in the serum phase during heating. While concentration of α-LA and β-LG in the serum phase steadily declined following the rise in temperature, these proteins started to engage with other proteins creating covalent bonds at higher temperatures compared to the unconcentrated milk (Figure 4.3B,B1). The heat stability of whey proteins in this concentrated milk (17%) appeared higher as depicted by slight denaturation as the temperature increased up to 100°C. Above this temperature, these proteins appeared more involved in creation of covalent bonds among themselves or with caseins, which resulted in their complete incorporation into large aggregates when temperature reached 121°C (Figure 4.3B,B1).

Caseins also experienced large variations in regards to their presence in the serum during heating of 17% concentrated sample. When milk is heated up to 75°C, the concentration of all caseins was low in the serum phase (Figure 4.3B,B1). Further temperature rise, to 85°C, resulted in an intense increase of the bands assigned to κ-, αs- and β- caseins in the supernatant, which is related to their heat induced dissociation from the casein micelle (Anema and Klostermeyer,

1997b). However, additional rise in temperature minimized the dissociation of β- and αs-caseins from the micelle as they appeared to reassociate with the micelle up to 121°C, at which temperature their concentration in the serum again increased. In contrast to other caseins, κ-casein concentration continuously increased in the serum following the temperature rise indicating separation of the hairy brush from the micelle. This behaviour of κ-casein appeared somewhat similar but more intense to that in unconcentrated milk; on the other hand, αs- and β-caseins were more affected at this solids concentration and encountered greater variations in solubility at different temperatures compared to unconcentrated milk sample. In regards to caseins presence in the serum during heating, it was obvious that mostly κ-casein was involved in covalent interactions with whey proteins resulting in formation of aggregated structures (Figure 4.3B,B1).

When most concentrated sample (25% TS) was heat treated, whey proteins showed greater stability to heat in comparison to samples with less solids. First notable observation is that whey proteins (α-LA and β-LG) decreased in the serum phase. This was similar to that of previously observed samples (9 and 17% TS) (Figure 4.3A,B,C), however in a more crowded environment denaturation of whey proteins appeared more impeded in comparison to less concentrated milk samples. Slight denaturation of these proteins started at lower temperatures, however it was not as intense up to 110°C, after which temperature they started to engage in covalent interactions and aggregate formation. From the gel obtained under non-reducing conditions (Figure 4.3C) it can be deduced that denaturation of whey proteins started around 110°C and, upon completion of heating at 121°C, all of α-LA and β-LG were incorporated into created aggregates through intermolecular thiol-disulphide interactions and disulphide interchange reactions (Mediwaththe et al., 2018).

Concentration of αs- and β-caseins initially decreased following the temperature increase from 75°C up to 110°C; however, after completion of the treatment at 121°C these proteins appeared to dissociate from the micelle and become more prominent in the supernatant. On the other hand, concentration of κ-casein in the serum remained consistent up to 95°C. After this temperature, further increase induced this protein to separate from the micelle. A slight reduction of the serum κ-casein can be noticed at 110°C. It is not clear whether κ-casein/β-LG complex reacted with αs_2-casein remaining associated with the micelle or this reaction created larger complex involving these three proteins, which consequently precipitated upon centrifugation (Figure 4.3C). Even concentration of β-casein declined at this temperature, which may indicate that it was fairly strongly associated with the micelle at this temperature. Interestingly, band intensities of αs-, β- and κ-caseins under non-reducing conditions increased in the serum after completion of heating (Figure 4.3C).

4.3.2 FTIR

When samples with different solids concentrations were subjected to heating, increase in covalent interactions among proteins has been observed by conformational changes of proteins. For every solids concentration, a spectrogram was generated that indicated the effect of temperature on subtle protein changes and impact on consequent interactions. For Amide I region, the spectra were derivatized and the smoothing was performed to provide for a better separation of the peaks.

The obtained spectra of milk samples at all concentrations clearly indicated the impact of heating on protein conformation resulting in variations of peak intensities observed at 1700-1695 (aggregated β-sheets), 1663 (turns), 1653 (α-helix), 1645 (random structure), 1632 and 1623

(anti-parallel β-sheets) and 1609 cm^{-1} (side chains). The identification of the peaks was according to the observation of Grewal et al. (2017a).

In unconcentrated milk, the effect of applied heating on protein conformation resulted only in slight variations of peaks assigned to β sheets (Figure 4.4 A). Most variations at this concentration started to be noticed at 85°C with increase in β-sheets (peaks 1620 and 1698 cm^{-1}) and decrease of peaks assigned to turns (1663 cm^{-1}). More intense vibrations in the spectra appeared upon conclusion of heating at 121°C with decline in antiparallel β-sheets (1620 cm^{-1} and 1698 cm^{-1}) followed with increase in β-turns (peak 1663 cm^{-1}) and random structure (1645 cm^{-1}).

Heating the sample containing 17% TS to 121°C is characterised by large variations in the peak intensity assigned to α-helical structure (1653 cm^{-1}) (Figure 4.4 B). When temperature increased from 75-100°C, this peak intensity declined persistently. At 100°C, the peak was completely absent; however, it reappeared after conclusion of heating. This observation indicates dynamics of structural behaviour of well-defined structures in milk proteins. In a similar fashion, the intensity of a peak assigned to antiparallel β-sheets (1623 cm^{-1}) gradually decreased as temperature increased up to 100°C and then it was reinstated after 121°C to almost original state. Loss of β sheets and helical structure at 100°C could be assigned to formation of new structures, which was confirmed by greater intensity of turns (1663 cm^{-1}) and unordered structures (1645 cm^{-1}). Moreover, a peak at 1698 cm^{-1} related to aggregated β-sheets was greatly affected by heat treatment especially at 75°C where it was initially reduced with further heating resulting in more intense reduction and shifting to the right.

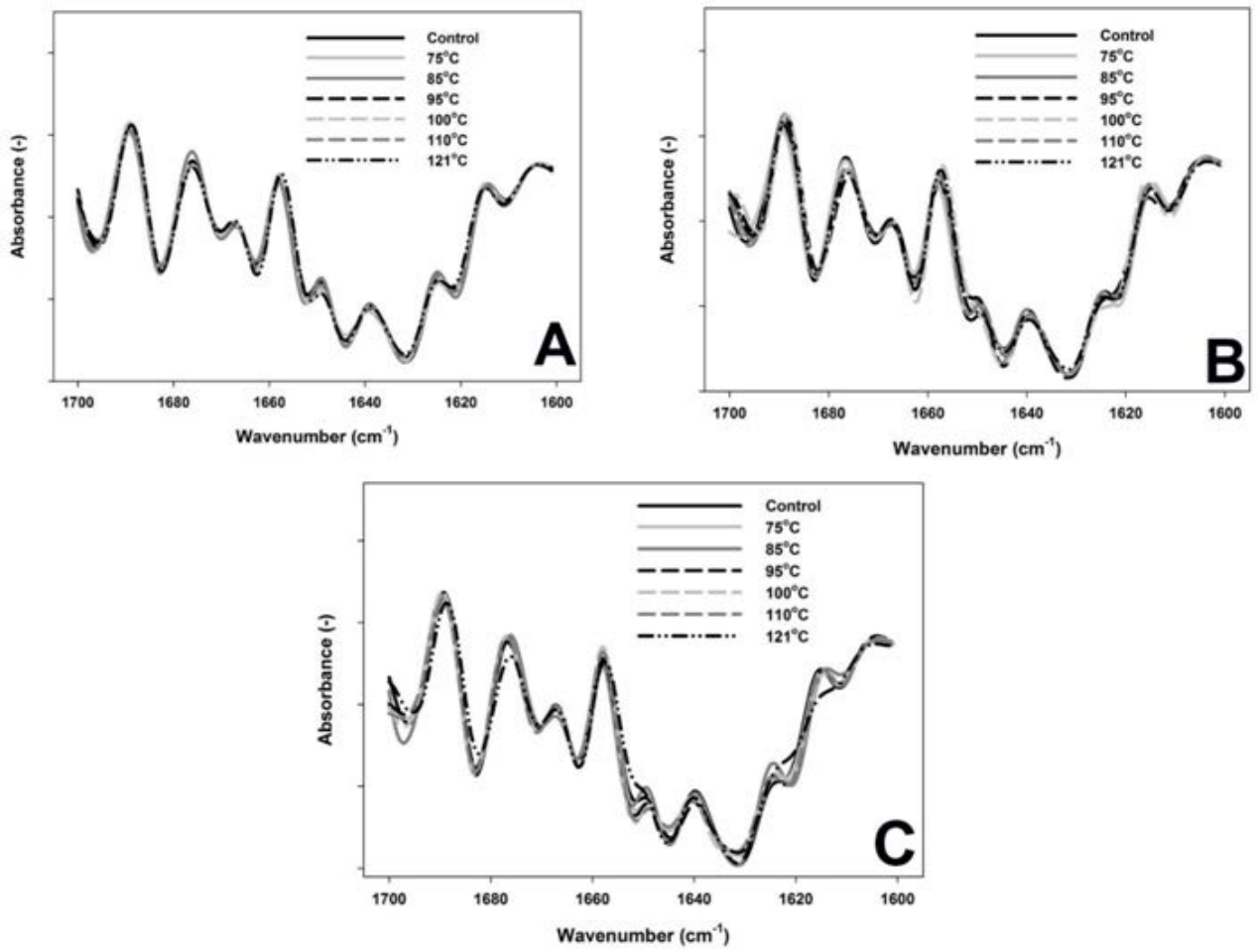

Figure 4.4: Second derivative of the FTIR spectra of samples containing 9% (A), 17% (B) and 25% TS (C) heated to 121°C. The samples were assessed when the temperature reached indicated levels.

The spectra obtained analysing the most concentrated milk samples (25% TS) indicated that temperature treatment affected the secondary structure of proteins more extensively in comparison to the samples with lower solids concentrations (Figure 4.4 C). While the loading at 1653 cm^{-1} assigned to α-helix increased when temperature rose from 75-110°C, further heating to 121°C resulted in a loss of the given peak. Simultaneously, loss of defined structures upon completion of heating was observed by reduction in side chains, anti-parallel β-sheets and

aggregated β-sheets, detected by minimised intensities of peaks at 1610, 1620 and 1695 cm^{-1}, respectively. These losses of defined structures consequently generated formation of new unordered structures as indicated by intensification of a peak at 1645 cm^{-1}.

4.4 Discussion

4.4.1 Effect of different heating temperatures on whey protein aggregation in regards to solids concentration

In industrial practice, milk is preheated prior to concentration process to denature the whey proteins and prevent their further interaction with the caseins during sterilization process. The subtle but important changes observed in Chapter 3 indicated that the whey proteins were primarily affected by concentration step, which likely rendered them more reactive. To improve our understanding of how these changes may govern behaviour of major milk constituents during heating in a concentrated system, a heating protocol, involving sequential sampling at selected temperatures, of skim milk with different solids concentration was applied on samples containing 17 or 25% TS and the observations were made in relation to unconcentrated sample (9% TS).

Thermal processing of milk affects the heat stability of whey proteins via their denaturation followed by aggregation. Heat induced denaturation of β-LG and α-LA was studied extensively (Liyanaarachchi et al., 2015, McKenna and O'sullivan, 1971, Oldfield et al., 2005, Oldfield, 1996, Oldfield et al., 1998a, Oldfield et al., 1998b, Oldfield et al., 2000, Sava et al., 2005, Singh, 1995, Verheul et al., 1998, Zuniga et al., 2010, Roefs and Kruif, 1994). The mechanism of heat induced β-LG denaturation was proposed by the model of Roefs and Kruif (1994) based on three steps of polymerisation reaction. The process starts with activation when heated molecule unfolds and exposes a free sulfhydryl group on the surface available for interaction. The next

step involves propagation when the active βLG reacts with another native β-LG molecule and form activated dimmer that further exposes sulfhydryl group leading to termination step when two activated monomers or aggregated dimmers form new disulfide bridges and formed aggregates have no more exposed sulfhydryl groups for further aggregation. In concentrated skim milk, the whey proteins are found in four different groups of heat induced aggregates including casein micelle dissociated complexes of whey proteins with κ-casein, denatured whey protein complexes in the serum phase, co-sedimented aggregated whey proteins-caseins complexes and native whey proteins (Dumpler et al., 2017).

Progression and extent of the proposed denaturation mechanism appears to be concentration dependant (Anema, 2000, McKenna and O'sullivan, 1971, Anema and McKenna, 1996). Hence, heat treatment of milk containing greater solids concentration would modify and in many cases retard the unfolding and aggregation. Anema (2000) observed retardation of β-LG denaturation in samples with high percentage of total solids when heated at 75ºC. Heating at 100ºC had a similar effect on the protein regardless of the solids concentration. In the current study, this process appears to be retarded in a concentration depended manner. Hence, rise in solids concentration to 17% or 25% resulted in a delay of denaturation up to 100ºC or 110ºC. After these temperatures, extensive denaturation has taken place. At the final temperature of 121ºC, ~100% of the whey proteins were aggregated mainly through covalent interactions. β-LG has one reactive cysteine (Cys_{121}) and two disulfide bonds (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}) (Sawyer, 2003), which become exposed by denaturation. On the other hand, α-LA does not contain any free cysteine residues in its structure but four disulfide bonds (Cys_{6}-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{60}-Cys_{77} and Cys_{73}-Cys_{90}) (Brew, 2003), which engage in aggregate formation through intramolecular disulphide interchange reactions with β-LG (Huppertz, 2016). However, the

intensity of α-LA aggregation is lower and dependant on aggregation of β-LG, hence aggregation of α-LA with the casein happens mainly through βLG-αLA complexes (Oldfield et al., 2000). This reaction of whey proteins at high temperature is extreme and all dimmers are found in aggregated structures that can interact with κ-casein on the micelle surface and form particles with large volume or form soluble aggregates with the serum κ-casein that are not big enough to sediment in the pallet after ultracentrifugation.

Aggregation of whey proteins reflects on the size distribution, which again appeared concentration dependant by increased volume fraction of molecules when samples were treated (Anema and Li, 2003a, Dumpler et al., 2017). Moreover, these interactions in the current study did not affect the particle voluminosity for unconcentrated milk but had a major effect on the concentrated milk samples. Hence, increase in particle size for samples with 17% TS observed at 121ºC is related to increased number of large molecules in the system. Increase in particle size in heated milk was due to attachment of aggregated whey proteins onto the micelle surface (Anema and Li (2003a). Anema and Li (2003a) also observed that the rate of denaturation of the whey proteins was greater than the level of association with the casein micelle. Association of denatured whey proteins with the casein micelle takes place through thiol disulphide interactions with cysteine (Cys_{11} and Cys_{88}) of κ-casein, which are located on the para-κ-casein on the internal side of the micelle (Walstra, 1999). For interaction to take place it is required to achieve correct orientation of active groups of whey proteins to penetrate into the micelle and reach active sides of κ-casein (Anema and Li, 2003a). This finding may be correlated with our results at 100ºC in 17% TS as intense denaturation of β-LG and α-LA took place without impact on the particle size voluminosity. Therefore, observed start of extensive denaturation at this temperature

for the given concentration may relate to formation of small aggregated complexes that were not large enough to separate by ultracentrifugation and remained in the serum.

At 25% TS, the top band of each temperature lane related to serum aggregation increased in intensity starting from 75ºC until maximum aggregation at 121ºC. However, Anema (2000) reported retardation on denaturation of whey proteins in heated reconstituted milk from the increased solids content at 75ºC after what at 100ºC he observed equal denaturation in all concentrations. Contrary in our observations whey proteins were more or less stable on denaturation up to 110ºC when intense denaturation happens. In addition, as a result of more crowded environment molecules do not have enough space to open their structures and expose their reactive groups needed for formation of new interactions. Moreover, in the temperature range from 75-110ºC, the created aggregates were mainly composed of κ-casein molecules with less participation of other soluble caseins or between αs- and β- casein and whey proteins. Likewise, at temperature of 110ºC whey proteins are main participating proteins in the serum aggregates and further heating at 121ºC resulted in intense interaction between whey proteins and κ-casein in the serum.

Main interactions involved in aggregate formation in the serum phase resulted from -S-S- bridging. Moreover, when the whey proteins start to unfold and expose their reactive thiol groups available for further interactions at the same time their hydrophobic residues are also exposed which can be involved in possible interactions (Oldfield et al., 1998b). Therefore it cannot be excluded that hydrophobic interactions also take place when proteins are subjected to close packing and high temperatures. It appears that in a concentrated system initially formed aggregates serve as nuclei for further aggregation. Considering limited mobility due to their size, only a small fraction of nuclei would be formed that would grow rapidly in size. On the other

hand, in less concentrated skim milk due to greater mobility particles aggregate into small structure that can remain soluble up to a certain defined temperature, at which these aggregates would rapidly grow.

4.4.2 Disintegration of the casein micelle

In unconcentrated skim milk, heat treatment affected heat stability of the casein micelle by slightly increasing partitioning of individual caseins in the serum. The partitioning appeared to be constant for all caseins from 75ºC up to 110ºC, after which point prolonged heating at 121ºC lowered the levels of individual caseins in the serum phase. Similarly, Anema and Li (2003b) reported that in reconstituted unconcentrated milk heated at high temperature only minimal dissociation occurred and in particular involved κ-casein. When milk is heated at high temperature, the net negative charge of the micelle is reduced due neutralization of electric double layer to a more positive value (Anema and Klostermeyer, 1996). Reduced ζ potential observed in our study may lead to lower repulsing power of particles and interactions among denatured whey proteins with the casein micelle, which may prevent further dissociation of individual caseins from the micelle structure, which is confirmed by the SDS gel at 121ºC (Figure 4.3 A,A1). Additional colloidal calcium phosphate inside the micelle, resulting from the shift of calcium and phosphate from the serum at observed temperature, also may have had some significance in formation of more stable nanoclusters that prevented dissociation of caseins from the micelle. Other explanation for reduced level of caseins in the serum at high temperature is that soluble caseins form aggregated complexes with whey proteins that associate on the micelle surface and separate in the serum after ultracentrifugation (Anema and Li, 2003a). Thus, β-LG interact through sulfhydryl interactions with the serum κ-casein or with α-LA by disulphide interchange reactions and form aggregates as evidenced in the top band of every line in the non

reducing SDS gels. This reaction increases in intensity up to 121ºC when all whey proteins are involved in new interactions and there are no any free dimmers left in the native state. However, these interactions did not have significant effect on the micellar voluminosity. Moreover, when native skim milk is treated at high temperature the casein micelle may suffer minimal changes that do not reflect on the total voluminosity.

In concentrated skim milk, both increase of solids concentration and applied heat treatment resulted in more intense disintegration of the casein micelle. It is known that concentrated milk have a lower pH value as a result of evaporation (Chapter 3), which undergoes further reduction when concentrated samples are heat treated (Nieuwenhuijse et al., 1991a, Singh, 2004). One reason that induces this pH reduction in heated concentrated milk is shifting of soluble calcium and phosphate into the micelle and saturation of the micelle with additional CCP. Newly formed alternative form of CCP may have different properties than the native CCP and likely lower potential for maintaining micellar integrity (Anema and Klostermeyer, 1997b, Aoki et al., 1990). Moreover, destabilization of the micelle structure leads to liberalization of the caseins in the serum. The effect of CCP applies only for calcium sensitive caseins due to their binding in the micelle through CCP nanoclusters; on the other hand, κ-casein is hydrophobically bound to the micelle surface (Singh, 2004).

Dissociation of caseins from the micelle in 17% concentrated samples was first observed at 85ºC with no previous dissociation of caseins at lower temperature although this was expected as reported by Anema and Klostermeyer (1997b). They observed dissociation of caseins from the micelle at temperatures as low as 60ºC. At 85ºC reduced Ca^{2+} ions in the serum may attach to the negatively charged sides of the micelle surface and alter the net negative charge to a more positive value of the surface hairy layer (Bienvenue et al., 2003a, Fox and Morrissey, 1977). This

destabilizes the micelle surface with reduction of electrostatic and steric repulsion resulting in dissociation of κ-casein from the micelle into the serum. These changes may lead to opening of the micelle structure and liberalization of calcium sensitive caseins into the serum. Moreover, when concentrated milk is heated there is an intense shifting of serum calcium and phosphate into the micelle (Hardy et al., 1984, Nieuwenhuijse et al., 1988), which may behave as alternative CCP (Aoki et al., 1990) that can destabilize the micelle and lead to liberalization of the calcium sensitive caseins (β- and αs-casein) from the micelle. At temperatures from 95ºC to 110ºC, the concentration of αs- and β- caseins in the serum was reduced. In addition, high temperature induces new interactions among whey proteins and caseins that can attach to the micelle surface and prevent dissociation of internal caseins from the micelle. This stabilization may act up to a certain heating point or 121ºC when the micelle was saturated with CCP and caseins were again separated from the micelle into the serum.

Heating the most concentrated samples (25%) resulted in even greater destabilization of the casein micelle. Slight dissociation of β- and αs- casein was observed at temperatures as low as 95ºC and 100ºC with some reduction in solubility at 110ºC. Again our results indicated that heating more concentrated samples increased the micellar stability of caseins to a higher temperature level. κ-Casein followed a similar trend of dissociation as other caseins following the temperature increase. Upon completion of heating at 121ºC, all caseins were present in the serum in a great proportion. In a system containing more solids, particles are closely packed and electrostatic repulsion is lowered (Singh, 2004). It is apparent that the casein micelle passes through various phases during heating, for example at 95°C and 100ºC, the casein micelle underwent substantial disintegration likely due to intense shifting of serum calcium and phosphorus into the colloidal state. This event has likely brought the micelle into a

supersaturated state that could no longer maintain the structural integrity and calcium sensitive caseins started to dissociate. Surface hairy layer is disrupted by reduction of electrostatic stabilization provided by reduction of negatively charged sides of C terminus of κ-casein probably by attachment of ionic Ca^{2+} and its neutralization (Fox and Morrissey, 1977). However, due to restricted mobility space further heating to 110ºC diminished this reaction. Moreover, at this point the intense denaturation of whey proteins was observed that could have further lead to new interactions with dissociated caseins and formation of protein complexes or their attachment to the micelle surface. Attachment of denatured and aggregated proteins to the surface possibly stabilised the surface of the micelle up to a certain point since prolonged heating at 121°C destabilized the micelle again. At this point, the micelle is found in the environment characterized by a slight increase of serum calcium and intense reduction of ionic Ca^{2+} that could lead to liberalization of individual caseins into the serum and thus closing a loop with new interactions and aggregation.

4.4.3 Changes in the secondary structure of proteins

Heating of unconcentrated milk did not result in intense alterations of the intensity of the peaks distributed in the FTIR spectra. At 85°C increase in a peak at 1620 cm^{-1} and 1698 cm^{-1} followed with decrease in a peak at 1663 cm^{-1} assigned to increase of aggregated β sheets on behalf of declining β turns. Moreover, these peaks were allocated to formation of aggregated intramolecular β sheets during β-LG aggregation (Lefèvre and Subirade, 1999). When temperature further increased, observed variations of these peaks could be related to slight moving of the intramolecular backbone as a result of heat treatment. Moreover, at the final heating temperature a decline of a negative peak at 1620 cm^{-1} and 1698 cm^{-1} followed with an increase in a peak at 1663 cm^{-1} and peak at 1645 cm^{-1} is related to decrease of β-sheets and

aggregated β-sheets followed with increase in β-turns, respectively, which characterizes formation of more unordered structures. In the control, the applied heat treatment over the observed temperature range affected the peaks that represented α-helix and β-sheets more or less to remain in similar proportions indicating only slight variations in a conformational rearrangement of the proteins.

Heating concentrated milk containing 17% TS was not only characterized by increased protein concentration in the system but also reflected with greater variations in peaks related to conformational changes. Increase in temperature from 75ºC up to 100ºC resulted in reduction and loss of a peak assigned to α-helix (1653 cm^{-1}). This was followed with a slight increase of a peak at 1622 cm^{-1} and shifting to 1624 cm^{-1} as the temperature rose which is related to conformation of β-sheets. Moreover, a reduction of α-helical structure leads to opening of the structure of the molecule enabling greater exposure of hydrophobic sides and formation of new stronger hydrogen bonds driving molecular associations and aggregation (Ngarize et al., 2004). According to Oldfield et al. (1998b) when hydrophobicaly bonded aggregates are formed at low heating temperature these can easily be transformed into disulfide bonded aggregates. This was confirmed in the SDS patterns, in which intense covalent aggregation was present at 100ºC. This aggregation was confirmed by increase in turns (1663 cm^{-1}) and rise in a peak at 1645 cm^{-1} assigned to unordered structures, which showed maximum intensity at 100ºC. The SDS results also confirmed intense aggregation of β-LG and α-LA at this temperature. The FTIR spectra can also be related to aggregation of these proteins since they are known to have more defined secondary structure compared to caseins (Sawyer, 2003). However, in our results we also observed intense shifting of soluble calcium and ionic Ca^{2+} into the micelle which can be taken

into account for increase of a random structure at the expense of loops or helical structure that arise from caseins and movement to higher bond energies (Curley et al., 1998).

Further increase in temperature (121ºC) leads to reformation of α-helix (1653 cm^{-1}) and β-sheets (1623 cm^{-1}) and loss of random structure (1645 cm^{-1}). Reformation of α-helix may also be related to formation of a random coil structure due to increased solubilisation of caseins from the micelle and their involvement in formation of soluble aggregates (Grewal et al., 2017a). This is indicative from our results since intense solubilisation of the caseins from the micelle was observed in the SDS patterns of heated milk at 121ºC along with their involvement in covalent aggregation.

Heat treatment of 25% concentrated samples resulted in even greater variations in peak distribution in FTIR spectra in comparison to those of 17% concentrate. When β-LG is found at elevated concentrations in the system and is subjected of heat treatment, its secondary structure becomes more disordered in comparison with lower concentration levels (Lefèvre and Subirade, 1999). Intensification of helical structure when temperature was raised from 75 to 110ºC is an indication of reduced unordered structures and antiparallel β sheets. This could also depict behaviour of α-LA as a protein with a more defined helical structure (Brew, 2003) and still present in its native unfolded state. Furthermore, this increase in peak intensity attributed to helical structure can also correlate to formation of new random coil structure due to precipitation of caseins in formation of aggregates (Grewal et al., 2017a). Moreover reduction of native β-sheets with simultaneous increase of aggregated β-sheets (1698 cm^{-1}) suggest intense β-LG aggregation in the system (Liyanaarachchi et al., 2015). Increased particle size at 110ºC can be correlated with these variations in the spectra suggesting that both β-LG and caseins are involved in aggregation process with formation of particles with increased diameter. However, prolonged

heat treatment at 121ºC also resulted in further extensive variations in the conformation of proteins. High shifting of calcium and sodium into the micelle can lead to deformation and disappearance of α-helix (Curley et al., 1998) which is followed with increase of random structures that again involve both caseins and whey proteins. Moreover, α-LA at this temperature is involved in disulphide interchange reaction with β-LG, for which to happen it requires unfolding and exposure of its disulphide groups that can explain great loss of α-helical structure in the system. QI et al. (1997) proposed a model of molten globule of β-LG at temperatures above 70ºC, at which turn fraction remain in almost native state, α-helix is completely lost, and β-sheets are lost for ~ 50% accompanied with increase of random structure. However, in our study this distribution of peaks appears at high temperature of 121ºC, which may likely be related to increased concentration of total solids and β-LG in the samples hindering the overall process at low temperature. Moreover, presence of a component in the range of 1620-1635 cm^{-1} is a characteristic of monomeric form of β-LG or all dimmers are already denatured and unfolded into monomers (Lefèvre and Subirade, 1999). Summarizing, concentrated milk containing 25% TS after heating at 121ºC for 2.6 minutes is characterized by elements characteristic to a molten globule or unfolded structures that are mainly composed of denatured aggregated whey proteins with included aggregated caseins.

4.5 Conclusion

Applied heat treatment on concentrated milk affects total equilibrium in the system by impacting components of the system, their partitioning and aggregations, which were observed to be dependant of concentration level and temperature. Moreover, as concentration of total solids in milk increased whey protein aggregation was hindered and shifted towards higher temperatures. At 17% TS concentration, after induction of aggregation small soluble aggregates were initially

formed which grew rapidly into large particles. However, in the most concentrated system (25% TS), intensive denaturation and aggregation of whey proteins was observed with immediate formation of large aggregates. The casein micelle also experienced substantial changes during heating of the concentrated milk systems. The heating temperature had a pronounced effect on the mineral equilibrium in the system which likely destabilised the micelle and led to liberalization of individual caseins into the serum phase. This casein partitioning was apparently concentration and temperature dependant, hence most frequent casein liberated from the micelle was κ-casein, followed to a lesser extent with αs- and β-casein. The FTIR spectra confirmed that impact of heating on conformational changes of proteins was augmented with the concentration of total solids. From this study it can be concluded that the system needs to stabilize prior to heating, thus pre-heating appears a necessary step in preventing or minimizing involvement of whey proteins in protein aggregation. However, other possibilities should also be considered such as manipulating the native pH of concentrated milk before heat treatment as it may promote greater stability during heat treatment.

Chapter 5: Impact of pH adjustment on properties of concentrated raw milk during heating

5.1 Introduction

Heat treatment of concentrated milk culminates in several problems including partial or complete coagulation of milk proteins, which consequently results in destabilization of the system and time dependent gelation during storage. As indicated by past studies, heat stability of milk depends on many factors including solids concentration, pH, processing conditions, and seasonal variations, all of which govern how the milk proteins would change during processing (Walstra et al., 2005). Milk with increased solids concentration is less stable upon heat treatment in comparison to unconcentrated milk and exhibits maximum stability in pH range between 6.4-6.6 (O'connell and Fox, 2003). Any alteration of milk pH prior to heat treatment affects the system stability leading to intense coagulation and gel formation of proteins. Particles in concentrated milk are found in a new equilibrium with closely packed molecules characterized by lower electrostatic repulsions thus allowing other attractive forces to prevail and induce new interactions when high temperature is applied. Whey proteins appear instrumental in this destabilization, thus in order to prevent this, concentrated milk is prewarmed in order to denature whey proteins and minimize their involvement in polymerization reactions. This was confirmed in Chapter 4 as a necessary step required during processing of concentrated milk with unaltered pH. Since pH decline is observed during concentration (Chapter 3), which apparently influences heat stability of concentrated milk systems, it is still largely unknown, which molecular changes of the caseins and whey proteins take place during heating of non-preheated raw concentrated milk with adjusted pH.

Unconcentrated milk has the maximum heat stability in its native state at pH 6.7. During concentration of milk, pH declines by 0.2 or 0.25 units for concentration factor of 1.9 and 2.8, respectively (Chandrapala et al., 2010). Heat treatment of concentrated milk further reduces pH due to presence of organic acids generated from lactose degradation and intensive shifting of calcium and phosphate into the micelle, all of which destabilize the micelle and lead to protein aggregation (Nieuwenhuijse et al., 1988, Singh, 2004). Since the pH continues to decrease with heat treatment, its slight adjustment to 6.7 (native pH of unconcentrated milk) before sterilization may be important in shifting the protein distribution and stabilizing their molecular conformation that would resemble that of the native pH.

Addition of alkaline solution in the system usually leads to enhanced electro negativity of the caseins and consequently larger electrostatic repulsions, which eventuates in disintegration of salt bridges and loosening of the micellar structure (Liu and Guo, 2008). This alkaline induced disruption of the casein micelle is less progressive at higher solids concentration but is intensified by heating (Vaia et al., 2006). Under these conditions, stability of the casein micelle is compromised by diminishing of hydrophobic attractive forces that would induce dissociation of individual caseins from the micelle (Madadlou et al., 2009), which is also intensified upon heat treatment. Extent of dissociation of κ-, αs- and β- casein appears pH, concentration and temperature dependant (Anema and Klostermeyer, 1997a, Anema, 1998, Anema and Klostermeyer, 1997b, Dalgleish and Law, 1988, Singh and Creamer, 1991a). Moreover, liberated caseins in the serum form aggregates through disulfide interchange reactions and thiol-disulfide interactions with heat denatured whey proteins (Huppertz, 2013). These observations have been mainly obtained in studies that used pre-treated or reconstituted milk, which certainly confounded these effects with the main independent variables applied in these studies.

Understanding the basis of the changes in raw concentrated milk in such an altered environment is thus lacking in the literature. In Chapter 4, it was reported how milk proteins and overall equilibrium have changed during heating of concentrated milk in its unaltered pH environment. This chapter would expand these findings to compare how slight (6.7) and greater (7.5) pH adjustment affected properties of raw skim milk concentrated systems.

5.2 Material and methods

5.2.1 Sample preparation

Preparation of concentrated milk solution from raw milk as a base was performed as described previously in section 3.2.1. Three different solids concentrations (9, 17 and 25% TS) were used for the study. The pH of each sample was measured using a Metrohm pH meter (Metrohm AG, Oberdorfatrasse, Herisau, Switzerland) and further pH adjustment was performed by drop wise addition of 6M NaOH up to preferred value with continuous stirring. Since unconcentrated sample (9% TS) had a pH of 6.7 only one level of adjustment was completed (7.5), in contrast to concentrated samples (17 and 25% TS), the addition of alkaline was conducted up to two pH values of 6.7 and 7.5. The concentrated samples with unaltered pH served as the controls.

After pH adjustment, all samples were heat treated in a hot oil bath. The treatment followed the same procedure as described in section 4.2.1. After treatment, all samples were centrifuged at 100,000 g at 21°C for 1 hour using a Beckman Ultra L-70 centrifuge (Beckman Coulter, Australia Pty. Ltd, Gladesville, NSW, Australia) with an aim to separate the supernatant needed for further analysis.

5.2.2 Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Since the pH adjustment of samples with different solids concentration and further sequential heat treatment would affect the protein distribution among the serum and casein micelle, the SDS-PAGE was used for separation of individual proteins in the serum. The method was described in more detail in Chapter 3, section 3.2.4.

5.2.3 Fourier transform infrared (FTIR) spectroscopy

Changes in secondary structure of proteins that appeared after pH adjustment and sequential heat treatment were established using FTIR spectroscopy. Preparation of the samples and the measurement were performed as described in a previous chapter (3.2.6). The measurements were conducted in duplicates and obtained spectrum was analysed in a region between 1700-1600 cm^{-1} (Amide I) with the application of the second derivative in order to enhance the resolution of the spectra and assign peak positions to relevant components of the secondary structure.

5.2.4 Statistics

The FTIR spectra of all samples were exported to the optical spectroscopy software (Spectrograph, version 1.2.7, Oberstdorf, Germany), statistically smoothed and averaged.

5.3 Results

The concentrated samples with pH adjustment to 6.7 or 7.5 with different solids content were studied for rearrangement in their secondary structure and dissociation and aggregation behaviour of proteins when they were subjected to heat treatment. The results depict substantial modifications in the molecular structures of proteins that were dependent of concentration factor, applied temperature and pH level. In addition, these variations in the observed spectra obtained

from FTIR analysis were related to cleavage of native and formation of new bonds, which led to formation of new structures in the system that were studied by SDS PAGE electrophoresis.

5.3.1 Impact of pH adjustment on protein partitioning

Protein behaviour in their native environment (9% TS at pH 6.7) during heat treatment was reported in Chapter 4. Raising pH of unconcentrated milk up to 7.5 was performed in order to establish how more alkaline environment would affect the system in regards to protein modifications after heat treatment at defined temperatures. When skim milk with pH 7.5 was heated up to 121°C, whey proteins started to change their native concentration in the serum following sequential intensity reduction, which intensified at the final temperature. The SDS gels presented in Figure 5.1A illustrate intense aggregation between β-LG and α-LA indicated by the presence of soluble aggregates on the top of the stacking gels. The intensity of these bands increased concomitantly with the rise in temperature. In the previous chapter, it was observed that denaturation levels of whey proteins increased slightly as the temperature raised. At elevated temperatures, these proteins were found more attached to the micelle and less in a form of soluble aggregates in the serum. Contrary to this observation, in the alkaline environment (Figure 5.1.A,A1) prolonged heating at 121ºC induced intense aggregation of α-LA and β-LG resulting in formation of more small aggregates, which remained in the serum phase. Concurrently, less of these aggregated whey proteins were attached to the micelle surface.

Heating raw skim milk with pH 7.5 affected partitioning of the caseins in the system. At 75ºC all caseins (αs-, β- and κ-) were greatly affected by pH and found soluble in great proportion in the serum. Consequent increase in temperature by 10ºC had no effect on the concentration of αs- or β-caseins in the serum, however κ-casein concentration in the serum increased. As reported in the previous chapter, heat treatment of skim milk with native pH at 121ºC affected the soluble

caseins by moving them into the micelle and thus decreasing their concentration in the serum. On the other hand, increasing pH to 7.5 resulted in similar dissociation of the caseins from the micelle regardless the temperature. Moreover, κ-casein appeared to be involved more readily in covalent interactions with whey proteins by forming small aggregates which remained soluble in the serum as detected on the top of every line of SDS stacking and resolving gel (Figure 5.1A). The intensity of these bands increased concomitantly with temperature.

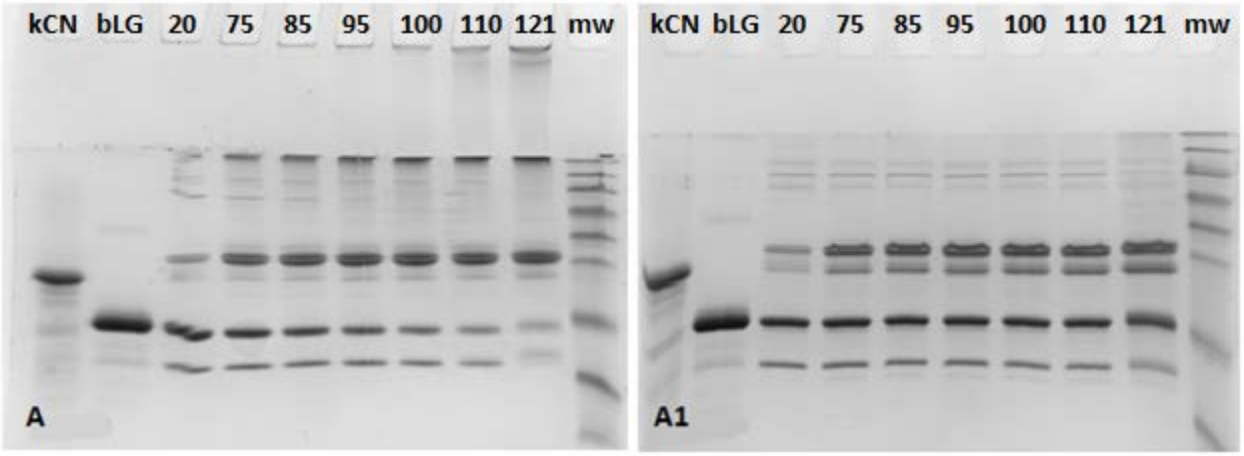

Figure 5.1: Non- reducing (A) and reducing (A1) SDS PAGE of serum of raw milk containing 9% total solids and pH adjusted to 7.5. kCN is κ-casein and bLG is β-LG. The numbers indicate the temperature at which the samples were taken, mw – molecular weight markers.

When a more concentrated sample (17%) had its pH adjusted to that of the starting raw skim milk prior heat treatment it resulted in great variations in protein distribution during heat treatment as presented in the SDS gels (Figure 5.2 A,A1). Additionally, in the previous chapter it was observed that denaturation of whey proteins in concentrated milk at its native pH (6.5) was hindered to a certain extent at temperatures below 100°C. At this temperature, intensive denaturation started to take place and by the time the temperature reached 121°C all of the serum proteins were involved in the covalent interactions. However, upon slight pH adjustment this

retardation of protein denaturation was not observed. Moreover, α-LA and β-LG started to form aggregates through covalent interactions even at 75ºC. Raising temperature by 10ºC had a great impact as these proteins appeared more activated and engaging in the reaction. At the maximum heating temperature of 121ºC, almost 100% of α-LA and β-LG were denatured and present in the form of large aggregates.

Samples heated at pH 7.5 experienced intense denaturation of whey proteins, however the process was not hindered as previously shown in chapter 4 and the maximum aggregation was observed at 121°C. Hence, when concentrated sample with 17% TS adjusted to pH 7.5 was heated, denaturation and aggregation of whey proteins intensified and proceeded faster than the samples at lower pH. In both milk samples at pH 6.7 and 7.5, denatured whey proteins created soluble aggregated complexes with caseins in the serum during heating. Upon heating at 121ºC, the intensity of the bands representing β-LG and α-LA in the reducing SDS gels (Figure 5.2,A1,B1) slightly faded which likely indicates interactions of these proteins with the casein micelle, creation of insoluble aggregates which precipitated upon centrifugation.

In 17% concentrated samples with unadjusted pH, heat treatment affected levels of caseins in the serum phase which were in a dynamic equilibrium with those in the micelle as the levels fluctuated with the temperature (chapter 4). In the samples with adjusted pH to 6.7, the casein distribution in the serum fluctuated even more, indicating that pH adjustment augmented the effect of heating resulting in intensified dissociation of caseins from the micelle. This is clearly visible in Figure 5.2A1 as the band intensity of the control samples at 20ºC assigned to αs-, β- and κ- casein increased following the pH adjustment. At this pH, when temperature raised from 75ºC to 95ºC, concentration of αs- and β- caseins in the soluble phase increased, while further heating at 100ºC or 110ºC resulted in reduced concentration in the serum. This reduction was

more prominent for αs-casein, which would indicate re-association of this protein with the micelle. However, it still needs to be resolved which proteins are directly involved since as_1- and as_2-casein cannot be clearly separated by this method. At final heating temperature (121ºC), the intensity of bands that assign αs- and β- casein again increased in the serum (Figure 5.2A1). Contrary, concentration of κ-casein in the serum increased consistently following the temperature rise and reaching the maximum dissociation from the micelle at 121ºC. In heated samples with the unadjusted pH, κ-casein was found in the serum mainly in aggregated complexes with the whey proteins (chapter 4). Adjusting pH to 6.7 apparently did not change the trend as κ-casein was again engaged in the aggregation with whey proteins and created soluble complexes in the serum; on the other hand, the bands representing αs- and β-casein also slightly decreased at particular temperatures denoting their involvement in the aggregate formation. Most likely αs_2- casein created aggregates via disulfide interactions with aggregated whey proteins forming soluble complexes as well as with the casein micelle along with β-casein, which might have been involved in the aggregation through weak interactions since β-casein does not possess thiol group for disulphide bridging (Huppertz, 2013).

In more alkaline environment (7.5), heating of the concentrated sample resulted in a greater presence of αs- and β-caseins in the serum. Concentration of these proteins was relatively high immediately after temperature reached 75ºC and remained constant up to 121ºC, at which point the band intensity became only slightly reduced (Figure 5.2B1). Similarly, the concentration of κ-casein in the serum was greater than that at 6.7 and continuously increased during heating.

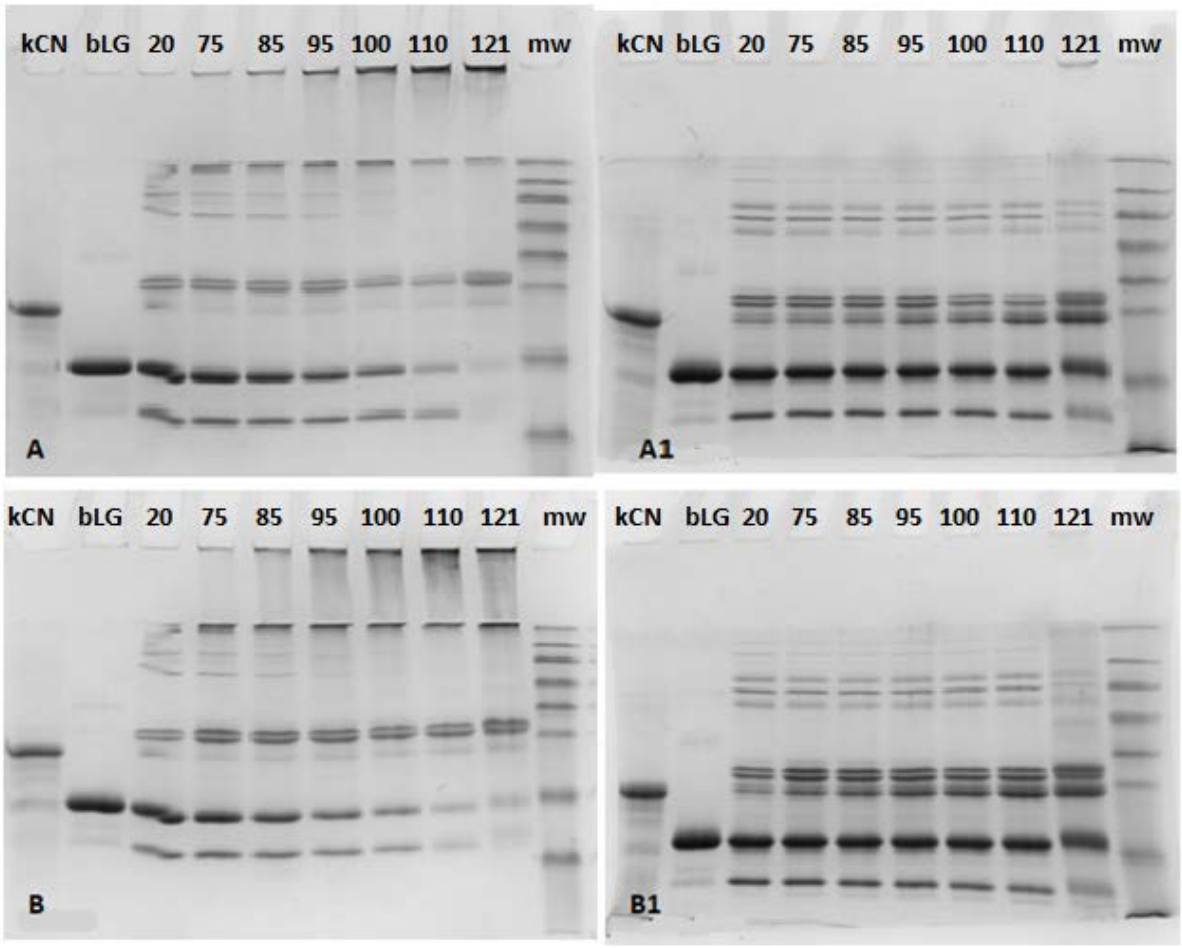

Figure 5.2: Non-reducing (A, B) and reducing (A1, B1) SDS PAGE of supernatants obtained by ultracentrifugation of concentrated raw milk containing 17% TS and adjusted to pH 6.7 (A) or 7.5 (B). kCN is κ-casein and bLG is β-LG. The numbers indicate the temperature at which the samples were taken, mw – molecular weight markers.

Concentration driven hindrance of whey protein denaturation in concentrated raw milk containing 25% TS as observed in chapter 4 was not obvious when pH was readjusted. Heat treatment of the sample (25%TS) at pH 6.7 induced immediate denaturation of α-LA and β-LG when temperature reached 75ºC and only intensified as the temperature was raised (Figure 5.3A,A1). At pH 7.5, this reaction appeared even more intense proceeding at a greater rate than

previously observed as such that at 100, 110 and 121ºC almost all of these whey proteins not only unfolded but also aggregated (Figure 5.3B,B1). Moreover, it appeared that whey proteins were involved in formation of soluble aggregates among themselves and/or dissociated caseins that remained in the serum and did not interact with the micelle during heating from 75 to 110ºC. In contrast, prolonged heating at 121ºC resulted in a slight reduction of the band intensity assigned to α-LA and β-LG in the reduced gels (Figure 5.3A1), which confirmed that part of these proteins created new interactions with the casein micelle and separated in the pallet after ultracentrifugation.

Casein distribution between the serum and colloidal phases during heating of the concentrated raw milk containing 25% TS with unadjusted pH is presented in the preceding chapter. By heating 25% concentrated skim milk with unadjusted pH, dissociation of κ-casein from the micelle was initially low at 75ºC and 85ºC, which was followed by an intense dissociation above that temperature. In comparison, in pH readjusted samples, κ-casein content in the serum increased substantially as soon as the temperature reached 75°C and continued to rise in the serum phase concomitantly with the rise in temperature. Content of other caseins in the serum appeared to vary with temperature; for example, in samples at pH 6.7, αs- and β- caseins in the serum were found in a high concentration, which remained fairly consistent during the initial heating; however the intensity of the bands was lowered between 95 ºC and 110 ºC, which may indicate their re-association with the casein micelle. Similar observation was made with the samples containing 17% TS and the same pH, however this reaction was delayed until the temperature reached 100ºC. In addition to these interactions with the micelle, all caseins also participated in formation of soluble aggregates, which appeared on the top of the gel for every observed temperature in the non-reducing SDS gels (Figure 5.3 A). Prolonged heating at 121ºC

resulted in re-dissociation of αs- and β- casein from the micelle, similar to the samples with 17% total solids.

Heat treatment of samples with high pH (7.5) gave consistent level of dissociated caseins as temperature of heating increased up to 110 ºC. Moreover, similarly as in 17% concentrate with pH 7.5, concentration of αs- and β-caseins in the serum was comparatively high in the temperature range from 75 to 110ºC to that at high temperature (121ºC), which was slightly reduced. κ-Casein, on the other hand, consistently increased in the serum concomitantly with the temperature. All caseins were again present as soluble aggregated complexes observed at the top of the stacking gel in non-reducing SDS PAGE (Figure 5.3B). However, the bands assigned to αs- and β- casein were slightly reduced after heating at 121ºC, similarly as in samples with pH 6.7.

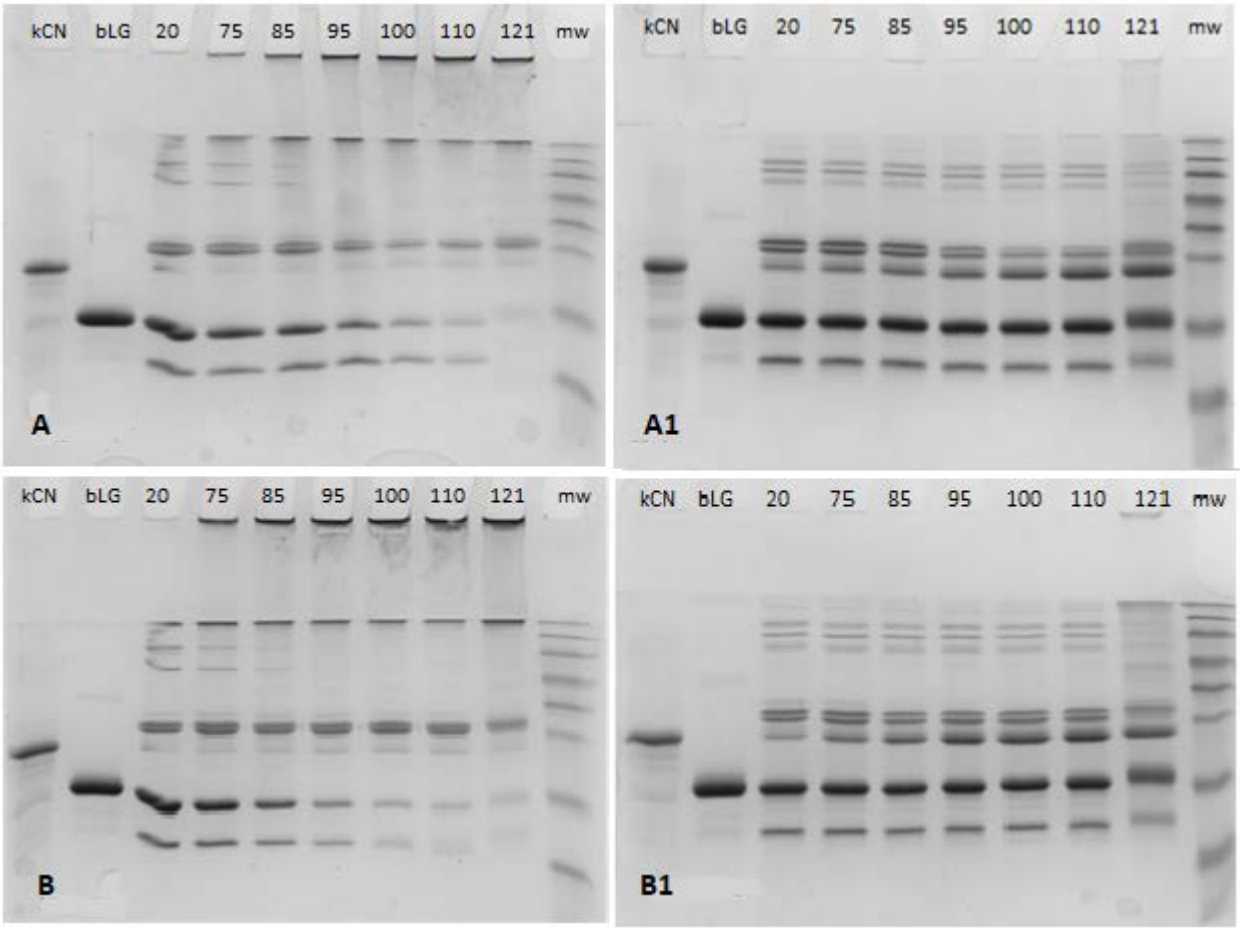

Figure 5.3: Non-reducing (A, B) and reducing (A1, B1) SDS PAGE of supernatants obtained by ultracentrifugation of concentrated raw milk containing 25% TS and adjusted to pH 6.7 (A) or 7.5 (B). kCN is κ-casein and bLG is β-LG. The numbers indicate the temperature at which the samples were taken, mw – molecular weight markers.

5.3.2 Effect of pH adjustment on changes of the secondary structure of proteins in concentrated raw skim milk

Alkalization of unconcentrated skim milk to 7.5 resulted in slight variations in the secondary structure of proteins observed by FTIR spectra compared to concentrated skim milk at their initial pH. Identification of the peaks was performed accordingly to the observations reported by (Grewal et al., 2017a). Moreover, in unconcentrated skim milk increase in pH up to 7.5 increased the intensity of a peak at 1632 cm^{-1} related to anti-parallel β sheets, a peak at 1645 cm^{-1} assigned to appearance of a random structure and a peak at 1697 cm^{-1} related to aggregated β sheets, what appeared on the expense of slightly reduced α-helix (1653 cm^{-1}) (Figure 5.4.)

It is known that during concentration step, pH of milk declines (Nieuwenhuijse et al., 1991a), which was also confirmed in Chapter 3 by observing a pH reduction of 0.2 and 0.25 units for 17 and 25 % TS concentration, respectively. The hypothesis of this work was based on the fact that readjusting pH to its initial value would likely affect milk proteins in such a way that would provide addition stability during downstream processing. It was expected that further alkaline addition to increase pH of concentrated milk would have more profound effect on properties of the system.

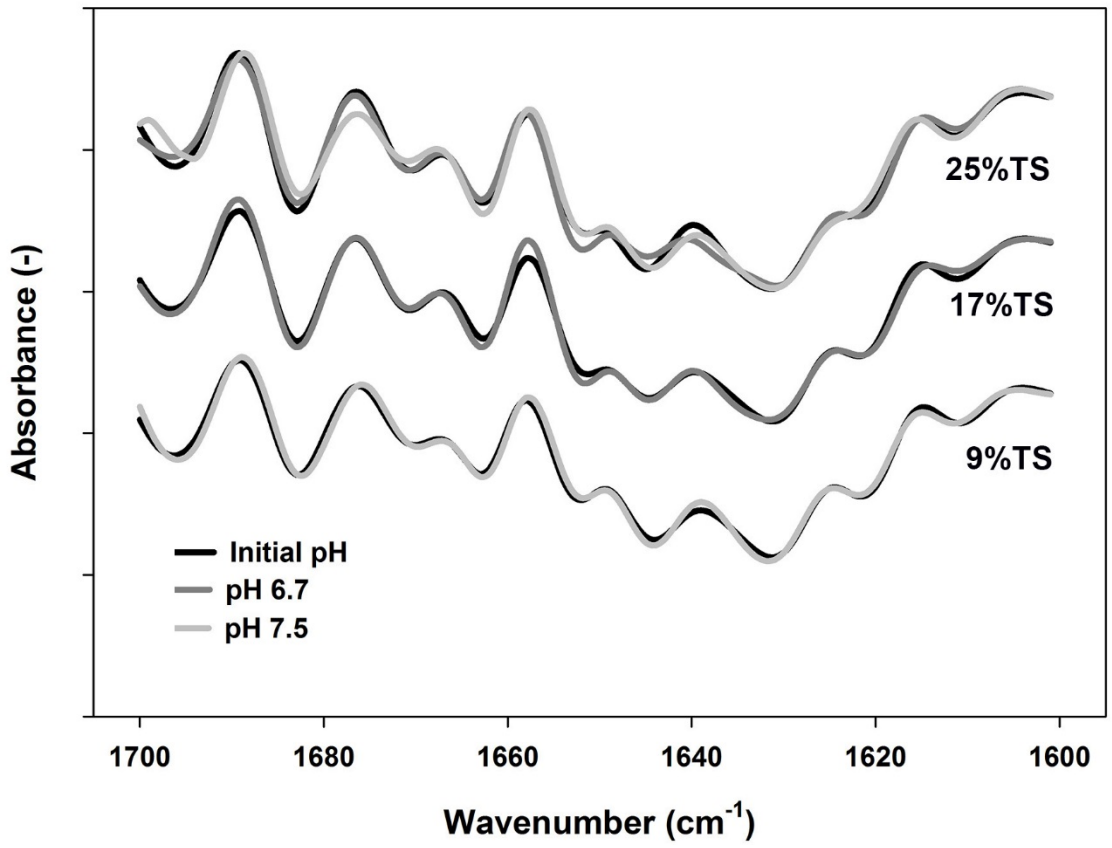

Figure 5.4: Second derivative of raw milk samples containing 9, 17 or 25% TS with initial and altered pH to 6.7 and 7.5.

In the sample containing 17% TS, adjustment of pH 6.7 resulted in noticeable increase of peaks at 1610 cm^{-1} (side chains of the protein molecules), 1622 and 1632cm^{-1} (antiparallel β sheets), 1645cm^{-1} (unordered structures), 1653cm^{-1} (α-helix/loop structure), 1663 and 1670cm^{-1} (β turns) and 1694 cm^{-1}(aggregation of β sheets). For the samples with 25% TS, the peaks with high intensity were 1610 cm^{-1} (side chains), 1622, 1683 and 1630 cm^{-1} (antiparallel β sheets), 1653 cm^{-1} (α helix/loop), 1663 and 1670 cm^{-1} (β turns), 1698 and 1694 cm^{-1} (aggregated β sheets).

In 17% concentrated samples, pH readjustment increased the intensity of a peak associated with α-helical structure. On the other hand, β-sheets were more affected with more alkaline

environment (7.5) accompanied with reduction of side chains and antiparallel β-sheets and simultaneous intensification of random structure. At both pH levels, observed high peak intensity at 1663 cm^{-1} was related to predominance of β-turns indicating structural rearrangements (Figure 5.4). The samples containing 25% TS were characterized at pH 6.7 with an intensive peak assigned to α-helical structure accompanied with simultaneous reduction in intensity of antiparallel β sheets, random structure and side chains (Figure 5.4). At pH 7.5 intensity of α-helical structure was reduced compared to that of the sample at pH 6.7. This structural change was followed with appearance of new turns (1676 cm^{-1}) and large transformation of β sheets with appearance of a high intensity peak related to aggregation of antiparallel β sheets (1698 cm^{-1}). In addition, a peak at 1623 cm^{-1} depicting β-sheet structure almost disappeared, which may indicate large structural modifications.

5.3.3 Behaviour of concentrated systems during heating

Heating unconcentrated milk adjusted to pH 7.5 induced substantial modifications of the secondary structure of proteins (Figure 5.5). Heating to 75ºC resulted in enhanced intensity of α-helix (1653 cm^{-1}) with simultaneous reduction of antiparallel β-sheets (1632 cm^{-1}). Further increase in temperature up to 85, 95 or 100ºC affected mainly β-turns (1663 cm^{-1}), which slight reduction may indicate subtle structural rearrangements. Major structural changes were observed when heating temperature was increased up to 110 ºC by large reduction of β sheets (peaks 1632 and 1683 cm^{-1}), slight reduction of α-helix (1653 cm^{-1}) and a greater presence of randomly distributed structures (1645 cm^{-1}). Finally, prolonged heating at 121 ºC resulted in a great reduction of peak assigned to α-helical structure (1653 cm^{-1}) and slight increase of random structures (1645 cm^{-1}).

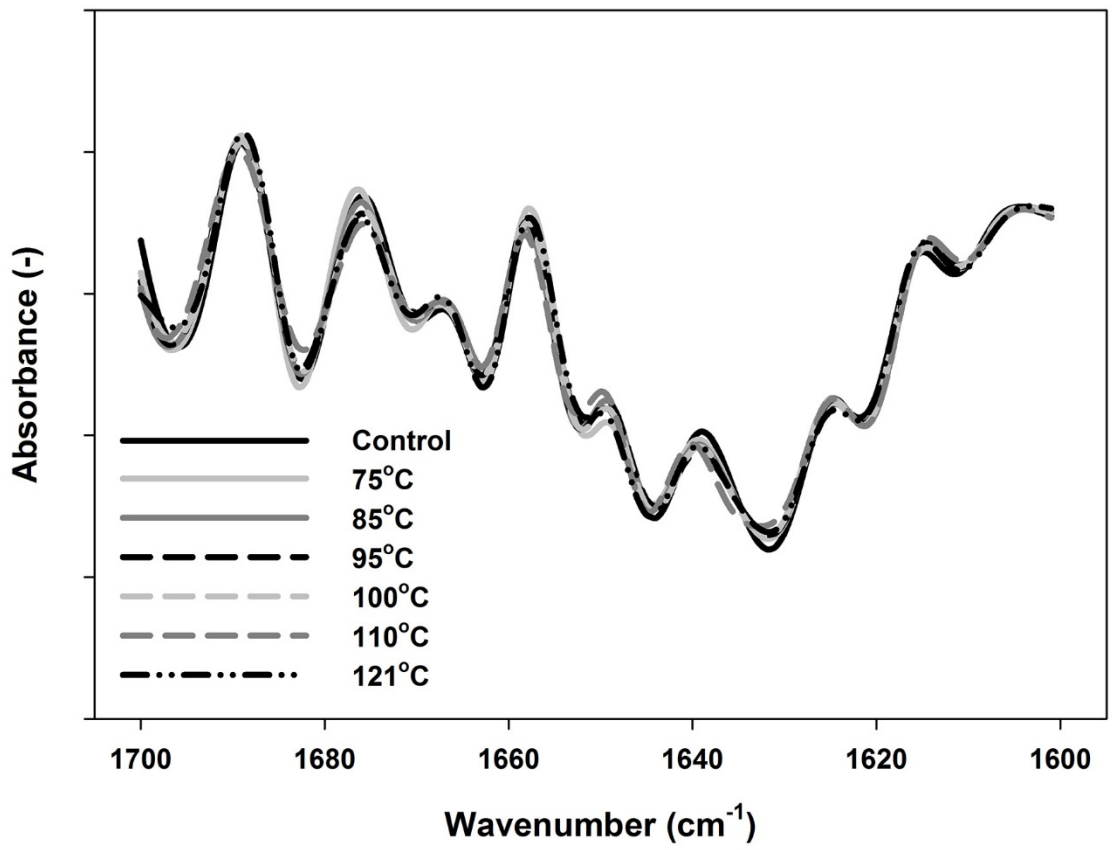

Figure 5.5: FTIR spectra of unconcentrated milk with pH 7.5 with control sample and samples heated at temperature range from 75, 85, 95, 100, 110, 121ºC.

From the FTIR spectra presented in Figure 5.6, it appears that combining concentration factor with slight alkalization and heat treatment would bring about variable impact on the secondary structure of the proteins in the system. As reported above, increasing pH slightly to 6.7 in the sample containing 17% TS (Section 5.3.2.) resulted in minor modifications of protein conformation mainly by increased helical structure, reduced side chains and greater presence of turns in the system. However, consequent heating produced larger variations in peaks distribution (Figure 5.6). Increasing temperature to 75 ºC resulted decline of specific (1663 cm^{-1}) and increase (1670 cm^{-1}) of turns accompanied with increased intensity of a peak at 1620 cm^{-1}

related to antiparallel β sheets with simultaneous shifting of a peak at 1695 to 1698 cm^{-1} (aggregated β sheets).

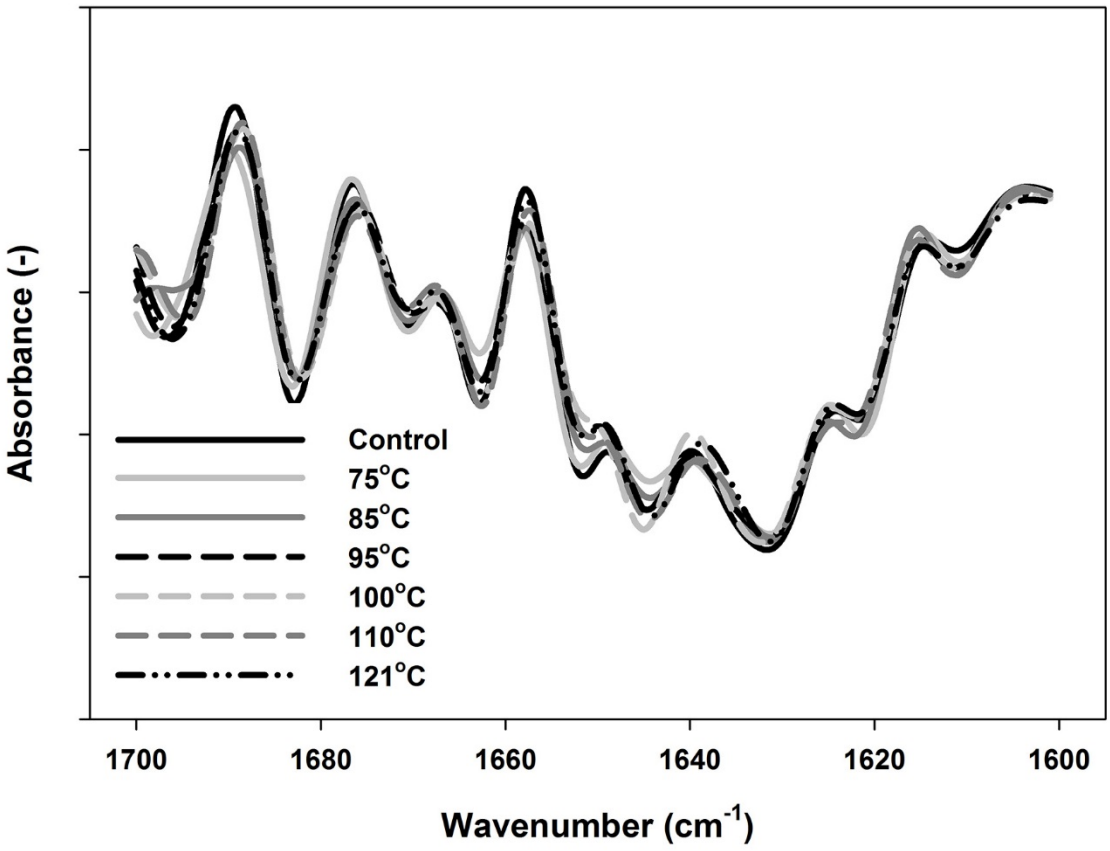

Figure 5.6: FTIR spectra of milk (17% TS) with pH 6.7 with control sample and samples treated at sequential heat treatment at temperatures of 75, 85, 95, 100, 110 and 121ºC

Further temperature increase to 85ºC slightly reduced α- helical structure in the system and promoted intense reduction of aggregated β-sheets followed with an increase in anti-parallel β-sheets. Raising temperature from 85 to 95ºC decreased the α-helical structure in the system and at 100 ºC this peak was completely lost. Moreover, at temperature range from 85-100ºC was observed increased presence of randomly distributed structures (1645 cm^{-1}). Further rise in temperature to 110 ºC resulted in a loss of a peak at 1620 cm^{-1} (antiparallel β-sheets) followed by

simultaneous appearance of a peak at 1695 cm^{-1} (aggregated β sheets). Interestingly at the final temperature (121 ºC), the peaks assigned to β-sheets and α-helix reappeared while intensity of a peak indicating aggregation (1697 cm^{-1}) was also enhanced.

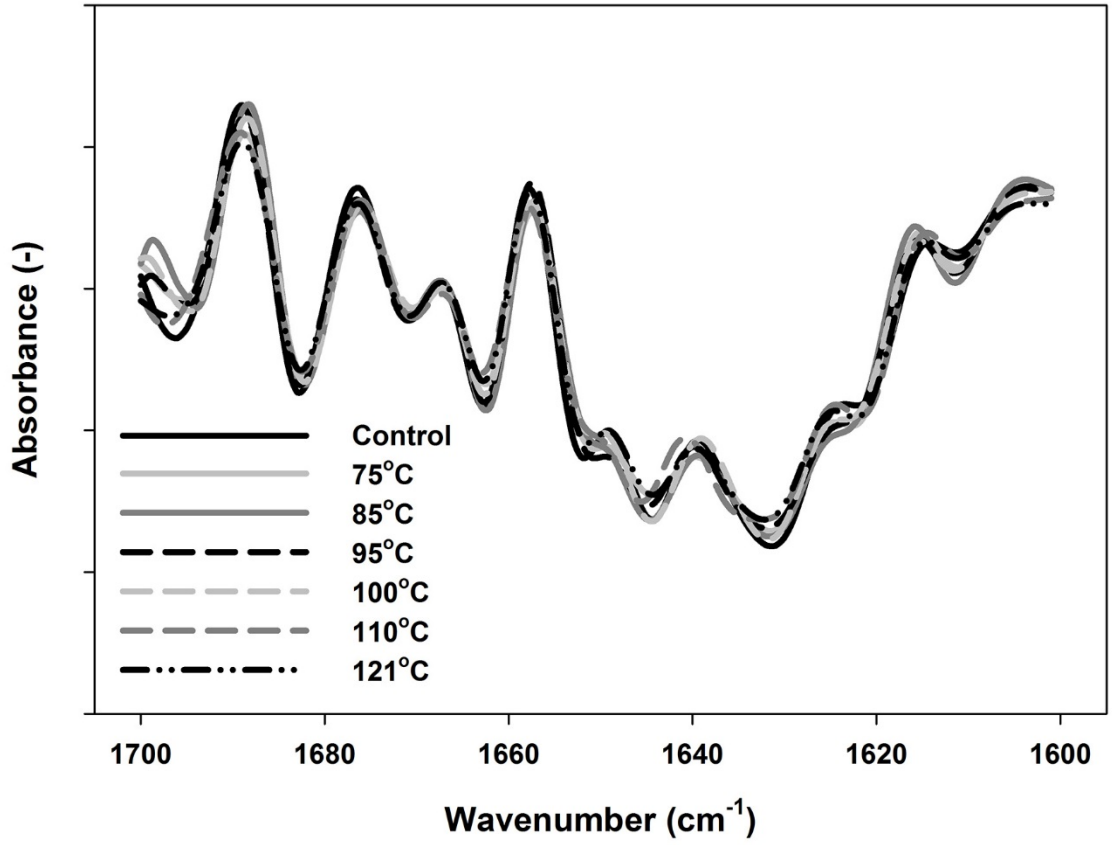

Figure 5.7: FTIR spectra of milk with 17% TS and pH 7.5 with control and heated samples at temperatures of 75, 85, 95, 100, 110 and 121ºC

Alkalization of the concentrated sample with 17% TS up to 7.5 slightly reduced β-sheets and increased random structure. Intensity of peaks assigned to aggregated β sheets (1695 cm^{-1}) and α-helical structure was minimized upon heating to 75ºC. Further temperature rise was characterized by a continuous decline of α-helix followed by a slight reappearance at 121ºC. Antiparallel β

sheets observed at 1620cm^{-1} disappeared as temperature increased from 75ºC to 100ºC; however this peak reappeared at 110ºC and 121ºC (Figure 5.7).

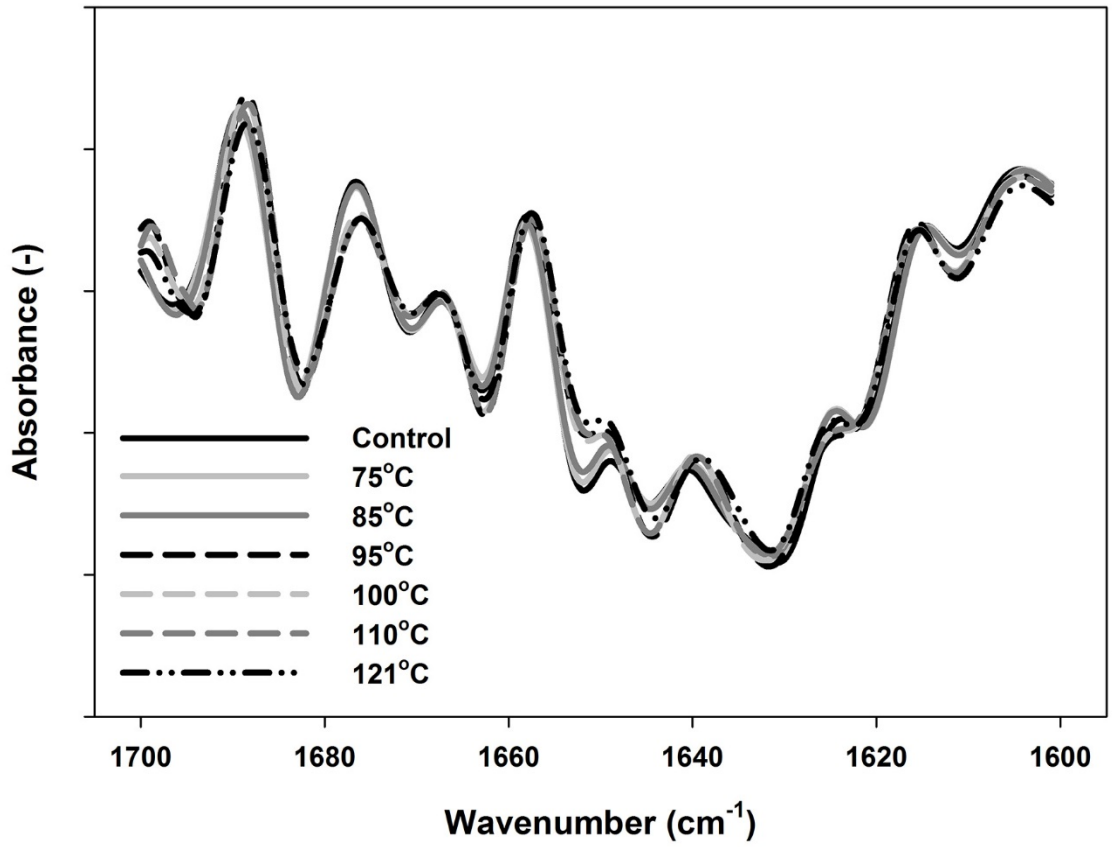

Figure 5.8: FTIR of milk with 25% TS and pH 6.7 with control sample and samples heated at 75, 85, 95, 100, 110 and 121ºC

Temperature treatment of 25% concentrated milk with altered pH to 6.7 produced only slight structural modification at 75ºC and 85 ºC, outlined by a minor reduction in α-helix (1653 cm^{-1}), slight increase in aggregated structures (1697 cm^{-1}) and a greater presence of turns in the structure (1663 cm^{-1}). Further temperature rise, all the way to 121 ºC, induced more intense reduction of α-helix, complete loss of β-sheets observed at 1620 cm^{-1} accompanied with intensification of side chains (1610 cm^{-1}) leading to new aggregated structures presented by

shifting of peaks from 1698 to 1695 cm^{-1}. Moreover, at these high heating temperatures, the milk sample with pH 6.7 was characterized with more random structures (1645 cm^{-1}) and complete disappearance of α-helix and antiparallel β-sheets (Figure 5.8).

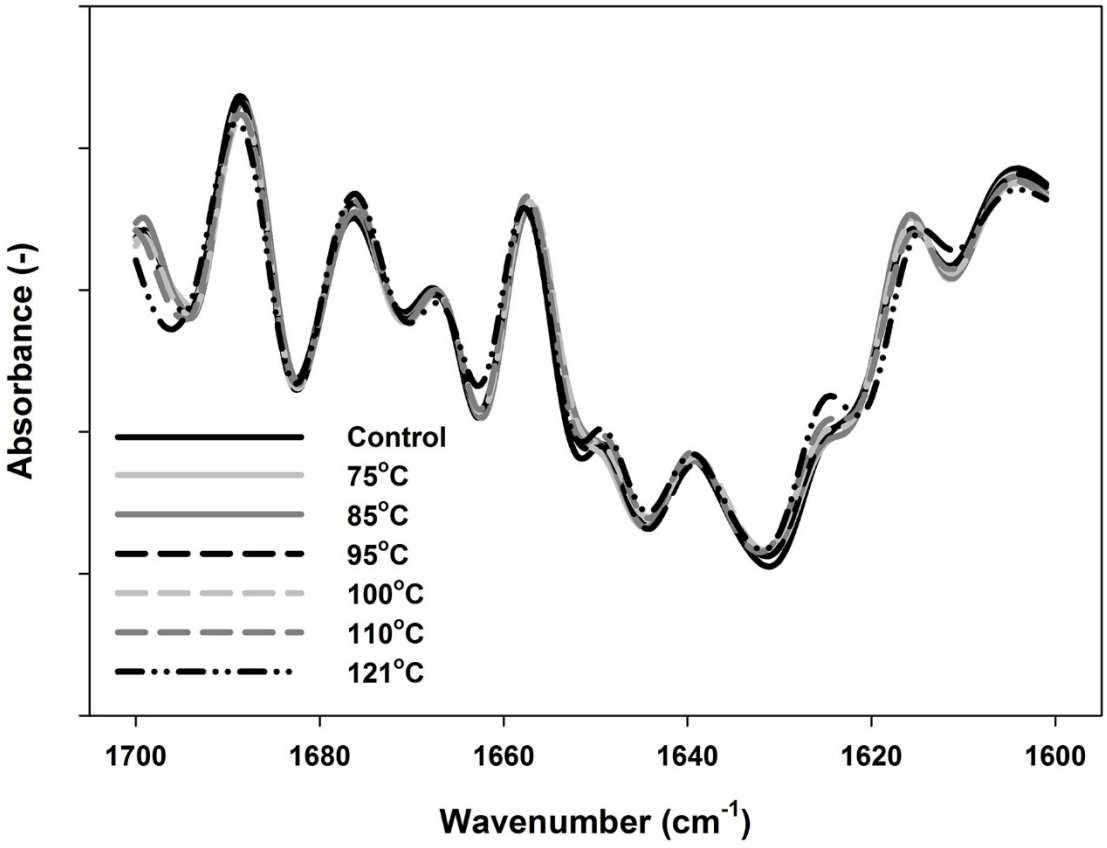

Figure 5.9: FTIR of milk with 25% TS and pH 7.5 containing control sample and samples heated at 75, 85, 95, 100, 110 and 121ºC.

Adjusting pH to more alkaline levels of milk system containing greater total solid content appears to induce greater structural changes of proteins in the system (Figure 5.9). Slight pH adjustment of the system containing 25% TS induced large modifications in the secondary structure mainly characterized by a disappearance of antiparallel β sheets. This occurrence, described as a loss of a peak at 1620 cm^{-1}, remained more or less similar as the temperature

increased from 75 to 110 ºC, after what prolonged heating at 121 ºC induced reformation of β-sheet structure. On the other hand, α-helical structure almost disappeared at temperatures as low as 75 or 85 ºC, however the intensity of this peak was somewhat reintroduced but to a lesser extent than the control. At 121 ºC, the sample experienced greatest changes depicted by reduction of turns and side chains of the molecules and intensification of peaks assigned to aggregated structures.

5.4 Discussion

Milk proteins experience very unpredictable behaviour when milk concentration is increased and pH level is moved towards more alkaline region before heat processing. This diversity of observed changes stems from various proteins interactions impacted by modifications of the native secondary structure of the protein system in the milk. When skim milk with different solids concentration is alkalized, the electrical conductivity of the medium increases, which may lead to disruption of the micellar integrity by cleavage of existing and formation of new hydrogen bonds (Madadlou et al., 2009). A pH dependent dissociation of the micellar caseins during heat treatment has been reported by many authors (Anema and Klostermeyer, 1997a, Anema and Li, 2000, Anema, 1998, Nieuwenhuijse et al., 1991b, Singh and Fox, 1987, Anema and Li, 2003b, Singh and Creamer, 1991a). During heating of concentrated milk at elevated pH, whey proteins form aggregates with liberated caseins that remain soluble in the serum (Anema and Li, 2003b, Singh and Creamer, 1991a, Vasbinder and de Kruif, 2003). Adjusting pH to more alkaline values leads to alkaline disruption of the casein micelle, as an outcome of an increase of net-negative charge of the micelle and intense shifting of calcium and phosphate into the micellar phase, which is even more enhanced by rise in temperature that leads to improvement of the solvent quality of milk (Vaia et al., 2006). Under these conditions, κ-casein is separated from the

micellar surface and found in elevated concentration in the serum while αs- and β-caseins dissociate slowly due to their location within the micelle (Huppertz, 2013). The current study for the first time provided evidence of the dynamics of these changes impacted by solids concentration, pH adjustment and temperature changes and how all these factors impact the secondary structure of the milk proteins and consequently their interactions.

Upon heating of raw skim milk containing standard solids concentration (9%) at pH 7.5, it would be expected to observe that most of the whey proteins would undergo unfolding and consequently be involved in new interactions; on the other hand, concentration of dissociated caseins in the serum phase would increase (Singh and Fox, 1986). In the current study, it was confirmed that whey proteins undergo intense aggregation with dissociated caseins creating soluble aggregates that remain in the serum in a temperature dependant manner. Moreover, denaturation of the whey proteins started at first observed temperature (75ºC), when more α-helical structure and less β-sheets were present in the system. α-LA is characterized by more α-helical structure (Brew, 2003) in comparison to β-LG, which contains greater proportion of β-sheets (Sawyer, 2003). Thus, at this temperature α-LA is present at a high concentration in the serum as an unchanged globular protein and only β-LG opens up the structure and forms soluble aggregates with dissociated κ-casein in the serum. Moreover, unfolding of the β-LG molecule would assist in facilitating intra- and intermolecular associations via hydrophobic and electrostatic interactions that can be easily transferred into disulphide bonding (Creamer et al., 2004, Liyanaarachchi et al., 2015). Similar behaviour was observed when temperature increased to 85, 95 or 100ºC and intensity of β-sheets and β-turns continued to decline denoting more intense unfolding of β-LG and its involvement in new interactions. However, at 110ºC, proportion of α-helical structure starts to decline accompanied with diminishing of β-sheets and

appearance of more randomly distributed structures. The process is further intensified with continuation of heating (121ºC). Reduction in α-helical structure in the system denotes that α-LA becomes involved in the aggregation process together with already denatured β-LG (Figure 5.1A).

Extent of formation of the soluble aggregates in the serum phase is governed by the levels of the dissociated κ-casein from the micelle surface (Anema, 2007). Moreover, κ-casein dissociated more intensively from the micelle surface during heating when pH of milk was adjusted to pH 7.5. Change of pH also induced dissociation of β- and αs-caseins, however their concentration remained rather constant during temperature increase. Dissociated caseins in the serum form soluble aggregates with whey proteins. Apparently, at temperatures up to 100ºC preferably β-LG reacts via its reactive thiol group (Cys_{121}) and its two disulphide bonds (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}) through intramolecular thiol-disulphide interactions with the serum caseins. β-LG is found in the serum as an equilibrium between dimeric and monomeric forms; however, during heating dimmers dissociate into monomers, which leads to exposure of their free thiol groups for sulfhydryl-disulphide interactions (Sawyer, 2003). Among dissociated caseins, κ-casein presents the most available serum casein for interactions with two cysteine residues (Cys_{11} and Cys_{88}), while αs_2- casein also has two cysteine residues (Cys_{36} and Cys_{40}). On the other hand, β- and αs_1-casein do not engage in disulphide interchange reactions due to lack of cysteine in their structure (Huppertz, 2013).

In the temperature range from 75 to 110ºC, β-LG-κ-CN-αs_2CN complexes are formed in the serum through disulphide interactions. a-LA retains its native structure up to 110ºC when it starts interacting through disulphide interchange reactions with β-LG via its available disulphide bonds (Cys_6-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{60}-Cys_{77} and Cys_{73}-Cys_{90}) (Huppertz, 2013). However, due to

lack of free cysteine residue in its structure, this protein is proportionally less involved in aggregation in comparison to β-LG (Mediwaththe et al., 2018). Upon prolonged heating at 121ºC, aggregated complexes are created in the serum, which consequent the micelle. Moreover, Figure 5.10A,B illustrates possible mechanisms involving milk proteins during heating of milk at temperatures below 110 and above 110°C at alkalized pH.

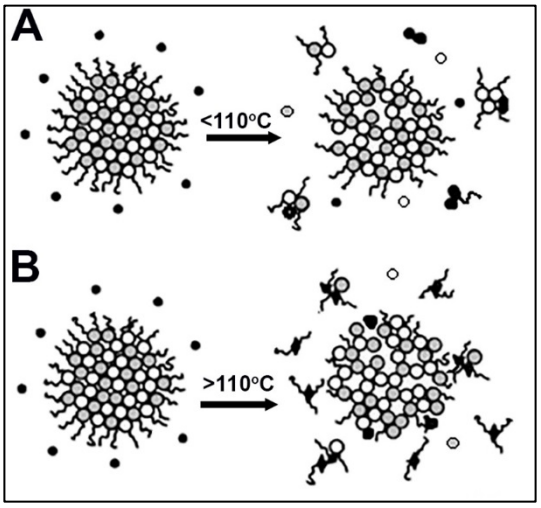

Figure 5.10: Changes in the casein micelle and whey proteins in milk and concentrated milk with altered pH >6.7 heated at temperature below 110ºC (A) and above 110ºC (B). Where ● are whey proteins; ⸗ is κ- casein; ◍ is αs - casein and ○ is – β casein

However, when solids content is increased before alkalization, heat treatment induced large changes in proteins through formation of new structures and interactions. Slight alkalization of concentrated milk to 6.7 resulted in modifications of the protein conformation in the system. Increase of pH in the system leads to deprotonation of carboxylic groups of aspartic acid (Asp) and glutamic acid (Glu), which loosens the structure of the casein micelle structure due to destruction of salt bridges, electrostatic repulsions and formation of buried isolated charges (Liu and Guo, 2008). Weakening of the micellar structure is further promoted when heat treatment is

applied. From the observation of the changes in the secondary structure of concentrated milk, slight alkalization increased the presence of helical structure and turns. In addition, from SDS gels it cannot be clearly identified, which proteins are responsible for these changes that could probably relate to subtle variations in the molecular conformations. It was previously confirmed that pH adjustment would result in shifting of soluble calcium and phosphorus into the micellar state (Sinaga et al., 2017). Moreover, these changes lead to swelling of the micelle due to extension of polypeptide chains resulting from attachment of calcium on phosphate side chains in the solution, which may be the reason for extended loop and turn structures (Curley et al., 1998). The same concentrated system with adjusted pH up to 7.5 was characterized with more random structures due to more intense loop alterations resulting from higher bond energies due to extended molecular chains.

Heating of 17% concentrated milk adjusted to pH 6.7 resulted in structural variations that are temperature dependant. At 75ºC, α-helical structure remains more or less stable; however, β-turns rearranged into new conformations of β-sheets in the system following diminishing of antiparallel β-sheets and formation of aggregated structures. Moreover, at this temperature, it may be expected to observe reversible denaturation of the β-LG; however, in a concentrated environment denaturation occurs through creation of disulphide bridges, which are irreversible. Aggregated structures as identified by the FTIR at this temperature appear to originate from β-LG aggregation. During heating of unconcentrated milk with pH 7.5, α-LA exhibits high heat stability up to 110ºC, however, in a concentrated system with slightly altered pH to 6.7, this stabilization becomes compromised when temperature of 85ºC is reached. Moreover, further heating to 95 and 100ºC resulted in intense reduction of α-helical structure, which subsequently completely diminished above these temperatures. This was apparently a consequence of

disulphide bridging between α-LA and β-LG along with the serum caseins resulting in reduction of a peak associated with α-helical structure. These interactions lead to more randomly distributed structures observed in the spectra obtained at given temperatures. In addition to disappearance of helical structure at 110ºC, anti-parallel β sheets, identified by a peak at 1620 cm^{-1}, also diminished followed by a proportional rise of aggregated β-sheets. The secondary structure of β-LG is greatly affected by heat treatment at high concentrations (Lefèvre and Subirade, 1999). Thus, initial loss of the peak at 1620 cm^{-1} is associated with an appearance of only one component in the region between 1620 and 1635 cm^{-1} (1632 cm^{-1}), which characterizes dimmer to monomer transition (Lefèvre and Subirade, 1999). As observed by the PAGE analysis, at this temperature β-LG undergoes intense denaturation outlined with almost complete dissociation into monomers and engagement in covalent aggregation through thiol disulphide interchange reactions with α-LA and κ- and αs_2-casein. Moreover, serum αs- and β-caseins are also involved in aggregation with the micelle, likely with remaining κ- casein on the micelle surface, which is confirmed by their reduction in the serum. Prolonged heating at 121ºC affected the secondary structure of milk proteins by reappearance of the α-helical structure and greater presence of aggregates in the system. At this temperature, dissociation of caseins from the micelle is also enhanced, which may be responsible for restoration of α-helix. Furthermore, new loop structures are also created from extended polypeptide chains due to intense shifting of calcium and phosphate into the micelle, which associate with CCP. This occurrence destabilizes the micelle due to inability of newly formed CCP to hold its structure (Aoki et al., 1990). Similar to unconcentrated milk at pH 7.5, at this solid concentration heated at same temperature, whey proteins aggregate with the serum caseins and form aggregates that are partially soluble and partially attached to the micelle surface (Figure 5.10B).

Heat treatment of 17% concentrated milk under greater alkaline environment induced further casein dissociation and intense aggregations of whey proteins. Previously observed hindrance of α-LA involvement was not apparent, thus at 75ºC, α-LA and β-LG are both involved in aggregation. Antiparallel β-sheets represented by the peaks distributed in region from 1620-1635 cm^{-1} were substantially reduced while loss of a peak at 1620 cm^{-1} confirmed transition of β-LG from its dimeric to monomeric form. Greater exposure of reactive sites of β-LG and apparent activation of α-LA accompanied with greater concentration of soluble κ- casein and αs_2-casein in the serum phase cumulated in formation of larger soluble complexes through disulphide bonds or interchange reactions containing combinations of these proteins in the structure. Moreover, continuous increase in temperature induces intense reduction of α-helical structure and antiparallel β-sheets indicating unfolding of α-LA and likely involvement in thiol-disulphide interchange reactions with β-LG. However, slight reformation of α-helical and loop structure at 121ºC may be related to intense concentration of αs- and β-casein in the serum, which may have extended polypeptide chains and loop structure. On the other hand, at this temperature α-LA appears completely unfolded and involved in aggregation. Formation of soluble aggregates continuously progressed over the observed temperature range and only at the end of heat treatment, concentration of whey proteins in the serum declined due to attachment of formed aggregates to the micelle surface (Figure 5.10A,B).

Heating of 25% concentrated sample with pH adjusted to 6.7 changed the conformation of proteins greatly. Interestingly α-helical structure appeared more stable at this concentration and given pH in comparison to less concentrated sample. While opening of α-helix was noticeable and consistent over the temperature range, it did not completely diminish at the final heating temperature. Similarly, during temperature rise from 95 to 110ºC a substantial change of β-sheets

was noted, which presented dimmer to monomer transition of β-LG followed by aggregation with α-LA and/or serum caseins and creation of soluble complexes that would finally interact with the micelle. While main driver of the aggregation is creation of disulphide bridges among these proteins (Mediwaththe et al., 2018), αs_1- and β-casein may associate with soluble aggregates and the micelle via hydrophobic attraction (Walstra et al., 2005). Prolonged heating at 121ºC of this sample intensified dissociation of all caseins from the micelle likely due to shift in a mineral equilibrium and creation of an alternative form of CCP (Aoki et al., 1990, O'mahony and Fox, 2013). Similar to the sample containing 17% solids, the aggregations involved two steps – first creation of soluble aggregates and then second either their growth or attachment to the micelle surface (Figure 5.10A,B).

Further alkalization to pH 7.5 of the concentrated samples with 25% TS initially reduced the intensity of β-sheets in the system, however this structural character did not appear further affected to a great extent as the temperature increased to 110ºC. Moreover, loss of intramolecular hydrogen-bonded β-sheet structure indicates presence of monomeric β-LG with disrupted hydrogen bonds in the structure and creation of new aggregates (Ngarize et al., 2004). In addition, prolonged heating at 121ºC induced reappearance of the same peak that could relate to formation of new stronger intramolecular hydrogen bonds, which subsequently converted into stronger disulphide bonds. Even at initial heating temperatures (75 and 85ºC), changes in β sheets were concomitant to unfolding of α-helix which assisted in opening the structure and exposing burred hydrophobic sides and active cysteine residues required for interactions (Nieuwenhuijse et al., 1991b). As it appears under these conditions, all proteins including dissociated caseins and whey proteins are greatly activated and participate in the aggregation process through the interchange reactions between free sulfhydryl groups and disulphide bonds

or disulphide-linked interchange interactions. In addition, β-casein can also engage in hydrophobically driven attractions and create between the micelle surface and soluble complexes, which would also reduce the concentration of these proteins in the serum.

5.5 Conclusion

Increasing pH of concentrated raw milk results in definite conformational rearrangement of the proteins in the system. This is likely reflected in cleavage of existing and formation of new hydrogen bonds within and among the proteins, which induce substantial changes in the micellar organization and liberalization of individual caseins into the serum. Concentration factor appears to play an important role in relation to partitioning of whey proteins and the caseins in the system when samples with altered pH are heat treated. Thus, in unconcentrated skim milk with pH 7.5 whey proteins are more stable on heating while αs- and β-caseins remain at a constant level in the serum without large variations upon temperature increase; however, concentration of κ-casein consistently increases in the serum under these conditions. Also, formed aggregates at all temperatures are distributed as soluble particles in the serum with only slight attachment to the micelle at high temperature. Heat treatment of concentrated milk with 17 or 25% TS with slightly altered pH to 6.7 results in similar aggregation pattern, which was substantially different from that of unconcentrated skim milk. The level of formed aggregates increases as concentration and pH increase before heating. In concentrated samples with pH 6.7 and 7.5 at the final heating temperature, the aggregates appeared in two forms - small soluble aggregates and large aggregated structures attached to the casein micelle similar to that of unconcentrated milk at pH 7.5. Raising pH of concentrated milk slightly up to 6.7 changed behaviour of dissociated caseins as they interacted with the micelle, which consequently declined their levels in the

serum. Moreover, this occurrence was more apparent in 25% TS as it eventuated at a temperature as low as 95ºC, while it took place at or above 100ºC in the sample containing 17% TS.

In general, this study showed that pH adjustment may be efficient up to a certain temperature (at and below 110ºC) as it provided some stability to the system by directing interactions between the caseins in the serum with the micelle and minimizing their availability in the serum for further interactions. It appears that by minimizing involvement of dissociated caseins in interactions with whey proteins may provide stabilization during heating, therefore finding approaches to stabilize the casein micelle within a narrow pH range may be one of the possibilities to prevent problems that the producers are facing during downstream processing and storage of concentrated milk.

Chapter 6: Conclusions and Future Directions

6.1 Conclusions

Downstream processing of concentrated milk into a stable product without induction of undesirable aggregation and coagulation has long been a problem in the dairy industry. In addition to heat treatment, pre-heating and addition of stabilizers are frequently part of industrial practices as required units of operation in manufacturing of commercially sterile products with long shelf life. However, a limited number of studies have been concerned with the basics of the changes, which occur in milk systems when the water content is reduced and further heating is applied. Moreover the requirement of above mentioned stabilization of concentrated milk also needs greater understanding and knowledge. Thus, the main objective of proposed work was to identify the changes in the milk when water was reduced in the system, how that new re-established equilibrium would respond to a high temperature treatment and elucidate on the role of pH on the overall stability of concentrated milk.

During this research, physiochemical properties of milk were stepwise analysed using the total solids level as the main factor of variation. Thus, decreasing water content in skim milk to produce a concentrated milk containing 17% TS resulted in a slight rearrangement of the secondary structure of proteins, which were further intensified as the solid concentration was increased to 25%. Moreover, the natural balance in the system was disrupted by shifting of minerals into the micelle, which apparently destabilized its structure and resulted in liberalization of individual caseins into the serum. Hence, αs- and β- casein were found more prominent in the serum at 17% TS while κ- casein was present in a greater concentration in the sample containing 25% TS. In the latter, β-LG denatured and was involved in the interactions with the casein micelle, which did not have a major effect since the oversize of the micelle did not change substantially. Concentration of skim milk to a certain level of total solids induced close packing

of the molecules, which found themselves in a new equilibrium that was highly prone to destabilisation during further processing. This would therefore require stabilization of the milk prior to concentration process and downstream treatment. Even though it appears that preheating milk prior concentration indeed is required for stabilization of milk concentrates some alternative approaches may also be investigated.

The concentrated skim milk products were further studied for changes in the system when the heating was applied. Aggregation of whey proteins was retarded at low heating temperatures and as concentration increased aggregation was delayed and shifted towards higher temperatures. Moreover, at heated 17% TS formed aggregated were with low diameter size which grew into large particles. On the other hand, in 25% TS the observed aggregation was concomitant with immediate formation of large aggregates. Applied heat treatment induced shifting of minerals into a colloidal state that promoted destabilization of the casein micelle and dissociation of caseins in the serum. This casein partitioning was apparently concentration and temperature dependant; hence most frequent casein liberated from the micelle was κ-casein, followed to a lesser extent with αs- and β-casein. Observation of the secondary structure of proteins confirmed rearrangement of the native structure as a result of increased solids level. Therefore, it was concluded that raw milk indeed needs stabilization prior concentration process that would minimise the changes of the native structure of the molecules. Thus, preheating appeared as necessary step in production of concentrated milk.

The above explained observed changes during heat treatment were moderated by alteration of pH to 6.7 or 7.5. Since pH of 6.7 was the starting pH of observed milk prior to the concentration process, its alteration to readjusted native state was performed to assess a possible stabilisation during heat treatment. Interesting behaviour of αs- and β- caseins was observed by their return to

the micellar state, which started at lower temperature of heating for 25% TS and later for 17% TS and concluded at 110ºC. On the other hand, denaturation of whey proteins was not hindered in concentrated samples as it appears that they were involved in denaturation immediately when heating temperature reached that of denaturation. In highly alkalized concentrated samples individual caseins remained in solution and did not return in the micelle, moreover they continued to dissociate as temperature increased and were involved in the aggregation. In summary, pH may have some stabilizing effect during heating of concentrated samples; however this stabilizing effect is highly dependent on pH and extent of heating.

6.2 Future directions

Present study has expanded the existing knowledge of heat stability of concentrated milk involving pH as an important factor for producing a product with less variations of the native structure. Based on these findings, processing techniques such as pre-heating may be required to stabilize whey proteins aggregation prior concentration process, however further studies are needed to evaluate the effect. Moreover, in milk systems with increased solids content returning native pH environment need to be taken as an alternative for manipulation with heat stability which again need to be taken with precaution to avoid even higher destabilization. Thus, the findings revealed a major area of alternatives that should be investigated.

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