EFFECT OF CONCENTRATION, pH AND ADDED CHELATING AGENTS ON THE COLLOIDAL PROPERTIES OF HEATED RECONSTITUTED SKIM MILK

By

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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December 2008

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ABSTRACT

The thermal processing of milk changes the composition and surface properties of the colloidal particles present and alters the physical properties of the milk. Whilst some changes such as those used to improve the texture of products such as yoghurt and are desirable, others such as gel formation during the manufacture of *Ultra-High Temperature* milk are highly undesirable. This work aims to characterize the effects of milk composition and pH on the chemical and physical changes that occur when milk is heated in order to understand and control the effect of thermal processing on the functional properties of the milk. Particularly important are:

(i) the changes to the integrity of the casein micelles and the extent to which they are reversible on cooling of the heated milk,

(ii) the changes to the speciation of the components of the serum as they re-equilibrate in response to the changed environment during heating and on cooling,

(iii) the heat-induced denaturation of the whey proteins,

(iv) the interaction between the components of the micelles and those in the milk serum, particularly those interactions that lead to aggregation or other changes that affect the functional properties of the milk on heating.

This project includes thermal treatment (90°C/10 min) of control skim milk solutions (9% Milk Solids Non Fat) with or without addition of calcium chelating agents (orthophosphate (P_{in}) & Ethylenediaminetetraacetic acid (EDTA)) and concentrated skim milk solutions (up to 21% MSNF). The pH range chosen was 6.2 to 7.2. Almost all of the studies on heat stability to date have been carried out by heating the milk and determining the changes that have occurred after the milk is cooled. This project is focussed on the direct measurements in real time of the changes that occur at the exact

temperature. The experimental techniques included pH, calcium activity and ^{31}P NMR measurements at high temperatures to investigate the consequences to the change in mineral speciation, Size Exclusion Chromatography in combination with SDS-PAGE analysis for protein speciation during heating and Diffusing Wave Spectroscopy and viscosity measurements to determine the heat stability of milk systems.

pH and calcium activity decreased with increase in temperature for all the milk systems studied. These changes were largely reversible as enough time was given for equilibration. pH and calcium activity changes during heating are a function of milk composition. The quantity, size and the composition of the protein aggregates present in the serum phase after mild centrifugation (~33,000g) of heated (90°C/10min) milk solutions were found to be a function of pH and milk composition (including the consequent differences in speciation of the components of milk). DWS and the viscosity measurements showed that pH at the temperature of heating is one of the primary determinants in influencing the aggregation of the proteins, which led to thermal stability of milk systems.

Hence, changing the milk composition resulted in differences in pH at the temperature of heating, which led to different behaviours of heat stability of milk systems.

Careful control of the composition of milk and thereby the pH at the temperature of heating allows a greater control of thermal stability of milk systems.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or in any other institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

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DECEMBER 2008

ACKNOWLEDGEMENTS

Many people have contributed in various ways to this PhD study. First of all; my respectable praise to Lord Murugan, Lord Ganesh and to all other hindu gods & goddesses for giving me this great opportunity of undertaking a PhD research. I would like to express my sincere gratitude to the following people:

My supervisors; Dr. I.R. McKinnon, Prof. Maryann Augustin and Dr. Punsandani Udabage for the support, guidance, encouragement and patience shown to me throughout the course of my study.

The Dairy Innovation Australia and Monash University for providing me with a scholarship.

Dr. Roderick Williams for his tremendous technical assistance and patience with SEC, willingness to help and make the measurements whole lot easier.

Dr. Yacine Hemar for his assistance in Diffusing Wave Spectroscopy (DWS) technique and in interpretation of the DWS results.

Rod Hall, Rod Mackie and Bruce Dobney for their assistance with Atomic Absorption Spectroscopy, X-Ray Diffraction and UV spectroscopy measurements respectively.

My group members for their friendship, advice and encouragement throughout my PhD study.

My aunt and uncle for being like parents for me to comfort and encourage me in certain situations.

Most importantly, to my loving parents for encouraging me to continue studies and for their ongoing patience, support and encouragement.

Abbreviations

AAS - Atomic Absorption Spectroscopy

BSA – Bovine Serum Albumin

CCP – Colloidal Calcium Phosphate

CMD – Casein Micellar Dispersion

CN – Casein Nitrogen

DLS – Dynamic Light Scattering

DWS – Diffusing Wave Spectroscopy

EDTA - Ethylenediaminetetraacetic acid

Eq - Equation

HCT – Heat Coagulation Time

HPLC – High Performance Liquid Chromatography

Ig - Immunoglobulins

LDS - Lithium Dodecyl Sulphate

MES - Morpholino Ethane Sulphonic acid

MSNF - Milk Solids Non Fat

MW – Molecular Weight

NCN - Non Casein Nitrogen

NMR – Nuclear Magnetic Resonance

NPN- Non Protein Nitrogen

ppm – parts per million

TCA – Tri chloro acetic acid

TN - Total Nitrogen

TP - Total Protein

SDS-PAGE – Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

SEC – Size Exclusion Chromatography

SMP - Skim Milk Powder

UHT – Ultra High Temperature

UV- Ultra Violet

WPI – Whey Protein Isolate

w/w - weight to weight

XRD - X-Ray Diffraction

Chapter 1

Introduction

1.1 Background

Milk is a complex mixture of nutrients, which has the primary function of nourishing and providing immunological protection for the mammalian young (Creamer & MacGibbon, 1996; Fox & McSweeney, 1998). Heat treatment of milk lowers bacterial growth and results in an extended shelf life (Law & Leaver, 2000; Singh & Waungana, 2001; Lehmann & Buckin, 2005). Heat treatments that are applied in the manufacturing of various dairy products include thermization, pasteurization, direct and indirect UHT sterilization and in-bottle sterilization (Morales *et al.* 2000). During the heating process, numerous reactions occur that influence the nutritional, physical and functional properties of the milk. The nature and extent of these reactions depend on the temperature, the duration of the heat treatment and milk composition (total solids, mineral composition, protein content and pH) (Parris & Baginski, 1991; Xiong *et al.* 1993; Oldfield *et al*. 2005). It is important to have an understanding of these reactions, so that suitable heating conditions can be applied to achieve the desired functional and nutritional properties for target milk products while keeping undesirable reactions to a minimum (Fox, 1981; Singh, 1994; Augustin & Udabage, 2007). For example, in cheese making the use of heated milk has the desirable effect of achieving higher yield from a given quantity of milk, but the undesirable effect of longer coagulation times and a weaker curd structure (Singh & Waungana, 2001). The manufacture of fermented milk is another example of using heated milk to achieve increased water holding capacity and improved "mouthfeel" (Allmere *et al.* 1997).

The effects of heat treatment on the basic properties of milk are important in terms of the final product. New technical advances are opening doors to some very exciting new product possibilities (Creamer & MacGibbon, 1996). The ability of milk to withstand high temperatures without loss of its stability is unique and makes the production of milk products possible. From an industry point of view, the heat stability of milk at normal concentrations is seldom a problem. However, the stability of concentrated milk is not properly understood. Additives such as calcium chelating agents are commonly used in industry as stabilizers that minimize the changes on heating (Fox, 1981). However, the use of additives requires careful control of the concentrations to achieve the desired heat stability. A greater knowledge and understanding of the chemical and structural changes of milk during heat treatment will be of great benefit with respect to the use of additives and higher milk concentrations.

1.2 Outline of the Chapter

The initial part of this chapter (Section 1.3 – 1.7) focuses on the background in relation to the chemical composition and the physical structure of milk and their changes as a result of increasing temperature. These sections are followed by the hypothesis (Section 1.8) on which, the present work is focussed on. The final section (Section 1.9) gives an overview of the contents of each chapter in the present thesis.

1.3 Milk

Cows milk contains about 87% water, 3.9% fat, 3.5% protein, 4.6% lactose, 0.65% mineral substances and a number of minor constituents such as enzymes (peroxidase, catalase, phosphatase, lipase), gasses (oxygen, nitrogen) and vitamins (A, C, D, thiamine, riboflavin) (Webb *et al.* 1974).

Milk can be considered as an emulsion of fat globules and a colloidal dispersion of particles made up of aggregated proteins cross linked through nano particles of amorphous calcium phosphate called the "casein micelles" dispersed in the serum phase (Ruettimann & Ladisch, 1987; Horne, 2003), as illustrated in Figure (1.1).

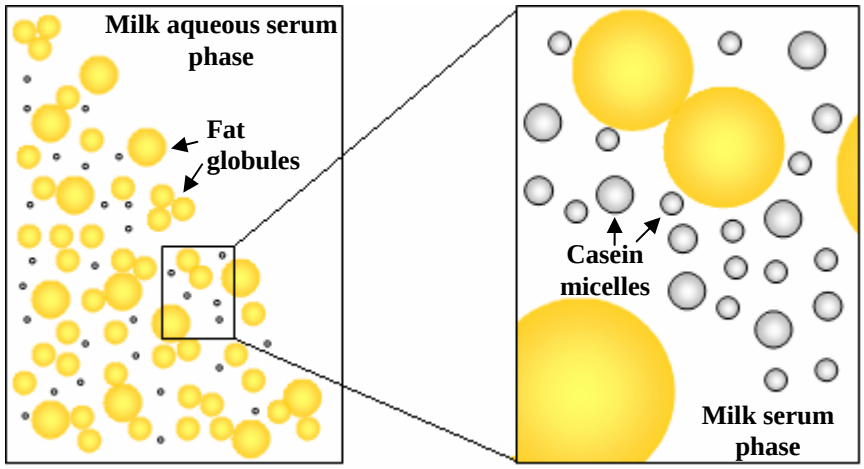

Figure (1.1): Schematic representation of milk structure

Skim milk is prepared by removing fat particles through centrifugation. The speciation of the components in milk is not just merely proportional to the milk concentration. The minerals are in dynamic equilibrium between the colloidal and the aqueous phases of skim

milk which can be separated by centrifugation. For example, when ~ 10mM of Ca^{2+} is added to milk; about 75% of the added Ca^{2+} ions go to the colloidal phase and 20% stay in the aqueous phase at 25°C (Udabage, 1999; Phillippe *et al.* 2003). Once the amount of added Ca^{2+} ions increases up to ~30mM, the amount of Ca^{2+} ions goes in to the colloidal phase decreases (Udabage, 1999; McKinnon & Chandrapala, 2006). On addition of ~10-30mM of P_{in} approximately 15% of the added P_{in} goes in to the colloidal phase whereas almost 85% stays in the aqueous phase (Udabage, 1999; McKinnon & Chandrapala, 2006). However, the additions not only change the concentration and speciation of the added mineral salt, but also influence the speciation of other minerals present in the milk system.

1.4 The Milk Proteins

There are two main protein fractions in milk. One fraction, the "Caseins", comprises the proteins precipitated at pH 4.6. The caseins make up 80% of the total protein content. The caseins are in dynamic exchange between the colloidal and serum phases. Many technological applications that change the milk composition, temperature and pH result in a redistribution of caseins, which will be discussed in Section (1.7.1). The other fraction, which makes up about 20%, is collectively referred to as "Whey Proteins" (Bloomfield & Morr, 1973; Webb *et al.* 1974; Ruettimann & Ladisch, 1987; Creamer & MacGibbon, 1996; Fox & McSweeney, 1998; Bordin *et al.* 2001). They are found primarily in the serum phase. The whey proteins are globular proteins (Creamer & MacGibbon, 1996). They denature on heating (Fox & Morrissey, 1977; Corredig *et al.* 2004). As will be discussed in Section (1.7.1.3), their denatured state has a major impact on the functionality of the milk systems.

1.4.1 Whey Proteins

Whey proteins are a heterogeneous group of proteins (Havea *et al.* 2001). The four major types of whey proteins are β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), Bovine Serum Albumin (BSA) and Immunoglobulin (Ig) with approximate percentages of 57%, 27%, 6% and 10% respectively (Ruettimann & Ladisch, 1987; Creamer & MacGibbon, 1996; Jussila *et al.* 1997; Bordin *et al.* 2001). They have a high hydrophobicity and are densely folded peptide chains. They are heat sensitive and denature upon heating at temperatures > 60°C (Dalgleish, 1990; Patel *et al.* 1990; Corredig & Dalgleish, 1996; Creamer & MacGibbon, 1996; Law & Leaver, 1997; de Jong & van der Linden, 1998; Fox & McSweeney, 1998; Oldfield *et al.* 2000; Jimenez-Guzman *et al.* 2002; Cho *et al.* 2003; Livney *et al.* 2003; Considine *et al.* 2007). The order of sensitivity to heat of the various whey proteins in milk has been reported to be Immunoglobulin > Bovine Serum Albumin > β-lactoglobulin (β-Lg) > α-lactalbumin (α-La) (Law & Leaver, 1997, 2000; Singh, 2001).

β-Lg is a globular protein with a net negative charge at neutral pH. β-Lg normally exists as a dimmer. It's monomeric form has a molecular mass of 18 kDa (Creamer & MacGibbon, 1996). It consists of 162 amino acid residues and contains two disulfide bonds (Cys66–Cys160 & Cys106–Cys119) and a free thiol group (Cys121) (Appendix A) (Sakai *et al.* 2000; Sakurai *et al.* 2001; Yagi *et al.* 2003; Considine *et al.* 2007). β-Lg has an 8-stranded β-barrel and a 3-turn helix that lies parallel to the three β-strands. The reactive thiol group is situated within the group of hydrophobic residues between this helix and the β strands (Creamer & MacGibbon, 1996; Sakai *et al.* 2000; Sakurai *et al.* 2001). At around 60–65°C, a critical change in the conformation of the molecule occurs, which exposes the buried -SH

group of Cys121 and initiates sulfhydryl/disulfide (SH/S-S) interchange reactions, leading to irreversible aggregation/polymerization (Shimada & Cheftel, 1989; Dalgleish, 1990; Monahan *et al.* 1995; Dalgleish *et al.* 1997; Law & Leaver, 1997; Fox & McSweeney, 1998; Corredig & Dalgleish, 1999; Fairise *et al.* 1999; Galani & Apenten, 1999; Oldfield *et al.* 2000; Sakai *et al.* 2000; Havea *et al.* 2001; Singh & Waungana, 2001; Bikker & Anema, 2003; Cayot *et al.* 2003; Cho *et al.* 2003; Livney *et al.* 2003; Yagi *et al.* 2003; Alting *et al.* 2004; Creamer *et al.* 2004; Considine *et al.* 2007).

α-La is a smaller globular protein with a mass of 14.2 kDa consisting of 123 amino acid residues. α-La is relatively stable compared to other whey proteins present (Wehbi *et al*. 2005; Considine *et al*. 2007). The primary structure of α-La contains four disulfide bonds and no free thiol groups (Permyakov & Berliner, 2000; Livney *et al.* 2003). As a consequence, the denatured α-La can be incorporated in to aggregate structures only via thiol-disulfide exchange reactions with denatured proteins that contain free sulphydryl groups (Dalgleish *et al.* 1997; Fairise *et al.* 1999; Oldfield *et al.* 2000; Bikker & Anema, 2003; Hinrichs & Rademacher, 2005; Oldfield *et al.* 2005). Hence α-La does not associate with the casein micelle on its own, but associates with the micelle via aggregates of β-Lg with α-La. Thus, the association behavior of α-La is likely to be influenced by that of β-Lg (Corredig & Dalgleish, 1996; Oldfield *et al.* 1998, 2000). Native α-La consists of two domains; a large α-helical domain and a small β-sheet domain, which are connected by a calcium binding loop. It is a strong binder of calcium and other ions and changes conformation markedly on calcium binding (Creamer & MacGibbon, 1996; Permyakov & Berliner, 2000; Considine *et al*. 2007). The apo-form (calcium free form) of α-La is much

more heat labile than the holo-form (calcium saturated form) (Permyakov & Berliner, 2000; Webhi *et al.* 2005). The binding of cations increases protein stability against action of heat and various denaturing agents (Permyakov & Berliner, 2000).

1.4.2 The Caseins

Caseins are unique proteins with respect to both their structure and function. There are four main types: α_{S-1}, α_{S-2}, β and κ-casein (Bloomfield & Morr, 1973; Horne, 1986; Ruettimann & Ladisch, 1987; Creamer & MacGibbon, 1996; Jussila *et al.* 1997; Fox & McSweeney, 1998; Bordin *et al.* 2001). The four types of caseins differ in primary structure and have several genetic variants (Creamer & MacGibbon, 1996). Some of the properties of the four types of caseins are listed in Table (1.1).

Table (1.1): Properties of four types of casein in milk

	α_{S-1}	α_{S-2}	β-casein	κ-casein
Concentration (mg/100ml)	12-15	3-4	9-11	2-4
No. of amino acid residues	199	207	209	169
MW	23600	22500	24000	19000
Relative Abundance (%)	38	10	36	13
No. of phosphorylated residues	8-9	10-13	5	1-2
No. of cysteine residues	0	2	0	2
****No. of phosphate centres**	2	3	1	0

*Primary structures of the four types of casein are in Appendix B

**Phosphate centre comprises of at least three phosphoseryl residues

(Data taken from Ruettimann & Ladisch, 1987; Creamer & MacGibbon, 1996; Holt, 2004)

Caseins have open and flexible conformations (Fox & McSweeney, 1998; Horne, 2002; Smyth *et al.* 2004). In contrast to whey proteins, caseins have high surface hydrophobicity. They have a high proline content resulting in a very low content of α-helix or β-sheet structures (Creamer & MacGibbon, 1996; Fox & McSweeney, 1998; Horne, 2002). An interesting feature of the primary structure of all caseins is that the polar and apolar residues are not uniformly distributed but occur in clusters giving hydrophobic and hydrophilic regions (Slattery & Evard, 1973; Creamer & MacGibbon, 1996). β-casein is the most hydrophobic of the caseins and α_{S-2} is the most hydrophilic. The C-terminal region of κ-casein is strongly hydrophilic while N-terminus is hydrophobic which is important to micelle stability (Slattery & Evard, 1973; Creamer & MacGibbon, 1996; Fox & McSweeney, 1998; Creamer *et al.* 1998). κ-casein and β-casein have high voluminosities as monomers (Holt & Horne, 1996; Smyth *et al.* 2004).

Caseins exhibit strong tendency to self-associate (Azuma *et al.* 1994; Creamer *et al.* 1998). The type of association is mainly hydrophobic and is quite specific. This association is dependent on protein concentration and temperature (Fox & McSweeney, 1998; Bouguyon *et al.* 2006).

A characteristic of caseins is their post translational modification resulting in the phosphorylation of the Seryl residues (Holt *et al.* 1982; Creamer & MacGibbon, 1996; Horne, 2002). The pK_a's of the phosphoserine residues of caseins range from 6.4 to 7.2 (Baumy *et al.* 1989). The caseins contain a variable amount of phosphoserine residues, which are responsible for their potential to interact with calcium and calcium phosphate

(Fox & McSweeney, 1998). α_{S-1}, α_{S-2} and β-casein precipitate in the presence of Ca^{2+} ions, whereas κ-casein does not (Bloomfiled & Morr, 1973). It is because κ-casein does not contain a phosphate centre, which contains three phosphoserine residues in order to bind calcium phosphate. The clustering together of phosphorylated residues in the primary structure to form the phosphate centres appears to be crucial in calcium phosphate sequestration (Holt & Hukins, 1991; Smyth *et al.* 2004). The cluster sequence of casein, which binds the calcium or calcium phosphate is SerP-SerP-SerP-Glu-Glu (Cross *et al.* 2004). Although, the principal binding site for Ca^{2+} ions within the micelle are phosphoseryl residues (Aoki, 1989; Aoki *et al.* 1990), the other acidic sites such as carboxyl groups of glutamic acid and aspartic acid residues are also involved, since caseins can bind more Ca^{2+} ions than there are phosphoserine residues (Bingham *et al.* 1972).

β-casein and α_{S-1} casein do not contain cysteine, but κ-casein and α_{S-2} casein can form inter molecular disulfide linkages with whey proteins (Slattery & Evard, 1973; Jang & Swaisgood, 1990; Rasmussen *et al.* 1992*a,b*; Azuma *et al.* 1994; Creamer *et al*. 1998; Rasmussen *et al.* 1999; Farrell Jr. *et al.* 2006; Considine *et al*. 2007). κ-casein in its native state can range in size from a monomer to multimeric disulphide linked structures larger than a decamer (Creamer & MacGibbon, 1996; Bouguyon *et al.* 2006). The multimeric nature of κ-casein is said to facilitate its covering the micelle surface, thereby stabilising the micelle structure (Rasmussen *et al*. 1992*b*).

1.5 The Milk Salts

Milk contains a large number of different salts. The salt composition is given in Table (1.2). Among these salts, calcium and phosphate are of importance in maintaining the casein micelle structure (Schmidt & Poll, 1986; Walstra, 1999). The salts which can diffuse through a membrane (with a MW cut off inferior to 10,000-15,000Da) are defined as diffusible salts (Gaucheron, 2005). The salts, which are retained by the membrane are considered to be associated with the casein micelles.

Table (1.2): The composition of salts in milk

Component	Total (mM)	Diffusible(mM)
Calcium	29.0	10.2
Magnesium	4.9	3.4
Phosphate	20.6	12.4
Citric acid	9.5	9.4
Chloride	30.4	30.3
Sodium	22.0	22.0
Sulphate	1.2	1.2
Potassium	38.3	38.0

(Figures taken from Holt, 2004)

There is about 29 mM of calcium in milk, of which 18.8 mM is colloidal and 10.2 mM is in the aqueous phase. Of the latter, free ionic calcium is about 2 mM and 6.97 mM is

coordinated to citrate and 0.67 mM to phosphate. Similarly, there is approximately 20.6mM of phosphate in milk. Of this, about 8.2 mM is colloidal, 0.67 mM is bound to calcium in the aqueous phase and 10.15 mM is free inorganic phosphate present as HPO_4^{2-} and $H_2PO_4^-$ (Holt, 2004). In general, 65% calcium and 51% phosphate are found within the micelle whereas the remainder is solubilized in the serum (Holt, 2004). Other authors confirmed the distribution of calcium and phosphate ions similar to stated above (van Hooydonk *et al.* 1986; Zhang & Aoki, 1996; Udabage, 1999; Vyas & Tong, 2004; McKinnon & Chandrapala, 2006).

The mineral equilibria between the colloidal and aqueous phases of milk, particularly the calcium and phosphate equilibria, which have an important role in maintaining the integrity of casein micelles (Aoki *et al.* 1990) are significant. Hence, changes in composition of milk with respect to Ca^{2+}, phosphate and pH result in changes in the chemical equilibria, the structure, processability and the functional properties of milk such as solubility, gelation, viscosity and emulsification (Zhang & Aoki, 1996; Augustin & Udabage, 2007). However, some of the interactions of salts with proteins are complex and are still not completely understood.

$$Ca^{2+}{}_{(aq)} + 0.7\,HPO_4^{2-}{}_{(aq)} + 0.2PO_4^{3-}{}_{(aq)} \leftrightarrow Ca\left(HPO_4^{2-}\right)_{0.7}\left(PO_4^{3-}\right)_{0.2(s)} \qquad \text{Eq 1}$$

$$\text{At } 25°\text{C}, \qquad K_{sp} = a_{Ca^{2+}}\, a^{0.7}_{HPO_4^{2-}}\, a^{0.2}_{PO_4^{3-}} = 1.6*10^{-7}$$

$$HPO_4^{2-}{}_{(aq)} + H^+{}_{(aq)} \leftrightarrow H_2PO_4^-{}_{(aq)} \qquad \text{Eq 2}$$

$$HPO_4^{2-}{}_{(aq)} \leftrightarrow H^+{}_{(aq)} + PO_4^{3-}{}_{(aq)} \qquad \text{Eq 3}$$

$$Ca^{2+}_{(aq)} + PO_4^{3-}{}_{(aq)} \leftrightarrow CaPO_4^{-}{}_{(aq)} \quad \text{Eq 4}$$

$$Ca^{2+}_{(aq)} + HPO_4^{2-}{}_{(aq)} \leftrightarrow CaHPO_{4(aq)} \quad \text{Eq 5}$$

$$Ca^{2+}_{(aq)} + cit^{3-}{}_{(aq)} \leftrightarrow Cacit^{-}{}_{(aq)} \quad \text{Eq 6}$$

(*Eq 1 taken from Holt, 2004)

The solubility of calcium phosphate decreases markedly with increase in temperature (Morr, 1985; Castro *et al.* 1986; Eldin & Aoki, 1993; Zhang & Aoki, 1996; O'Connell & Fox, 2001; de la Fuente *et al.* 2002; Havea *et al.* 2004; Singh, 2004). Hence, on increasing the temperature, precipitation of calcium phosphate occurs until the equilibrium is re-established (Eq 1). Removal of HPO_4^{2-} and PO_4^{3-} from the serum phase requires re-equilibration of equation 2 & 3. Hence, a decrease in pH results as a consequence. Also calcium forms complexes with phosphates in the serum phase as illustrated in equation 4 & 5. According to Holt (2004), there are significant amounts of calcium ions in the serum phase bound to citrate ions, which may highlight the importance of the citrate and calcium equilibria (Eq 6) prevailing in the serum phase.

1.6 The Casein Micelles

In normal milk, almost 95% of the caseins are present as spherical colloidal particles termed "Casein Micelles"(Bloomfield & Morr, 1973; Horne, 1986; Rasmussen *et al*. 1992*a*; Azuma *et al.* 1994; Dalgleish, 1998; Fox & McSweeney, 1998; de Kruif, 1999; Holt *et al.* 2003; Smyth *et al.* 2004). The micelles are approximately spherical and show considerable variation in composition, structure and size distribution as well as in salt composition (Walstra, 1990; Holt & Horne, 1996; Walstra, 1999; Udabage *et al.* 2003). They are

chemically heterogeneous consisting of the four types of caseins and amorphous calcium phosphates. They vary in size from 50-300 nm (Reuttimann & Ladisch, 1987; Fox & McSweeney, 1998; Holt *et al.* 2003; Horne, 2003; Smyth *et al.* 2004). The hydrated density of the particles ranges from 1.06-1.11 g/cm with a voluminosity of 4.0-4.4 ml/g of casein and contain ~3.3-3.7 g water per gram of casein (Bloomfield & Morr, 1973; Ruettimann & Ladisch, 1987). On a dry weight basis, the micelles consist of 94% protein and 6% small ions principally calcium, phosphate, magnesium and citrate. The presence of ions is essential for the maintenance of the micellar integrity under certain conditions of study (Horne, 1986; Walstra, 1990; Rasmussen *et al.* 1999; Holt *et al.* 2003; Horne, 2003; Udabage *et al.* 2003; Smyth *et al.* 2004). The composition of the casein micelles varies with temperature, pH, ionic strength and water activity (Ruettimann & Ladisch, 1987; Walstra, 1999). The thermal stability of the structure of the casein micelles is remarkable (Fox & Morrissey, 1977; Walstra, 1990; De Kruif, 1999). They can be frozen, heated and dried with out affecting their colloidal stability after reconstituted to normal conditions. At 32°C almost 90% of caseins are present in casein micelles, but at 4°C up to 50% may be in the serum phase indicating that casein micelles are dynamic objects (de Kruif, 2003).

1.6.1 The Structure of the Casein Micelle

Detailed knowledge of the casein micelle structure is important because the behaviour, particularly the stability of the micelles is central to many dairy processing operations. Without adequate knowledge of its structure and properties, attempts to solve many technological problems faced by dairy industry will not be successful. Despite extensive studies, the exact structure of the micelle is still unknown because of its complexity (Dalgleish, 1998). Several models for the structure of the casein micelle have been

proposed. Earlier ones include the coat core model, the internal structure model and the sub micellar model (Ruettimann & Ladisch, 1987; McMahon & McManus, 1998), which are no longer in use.

The currently most accepted model was put forward by Holt in the early 1990's and was refined by Horne in late 1990's with respect to the bonding within the micelle and by Holt with respect to the nature of colloidal calcium phosphate. The model describes the casein micelle as an open rheomorphic structure (Figure 1.2). Caseins undergo indefinite self-association, particularly in the presence of calcium to form a micro-gel like structure with a very high voluminosity. Within the micro-gel the proteins are associated through hydrophobic interactions and cross linked through incorporation of phosphate centres of the proteins in to amorphous nano particles of calcium phosphate (Holt & Horne, 1996). The hydrophilic C-terminal of κ-casein (which does not contain a phosphate centre) projects out from the surface of the micelle to form a hydrophilic and negatively charged diffuse outer layer, which causes the micelles to repel each other on close approach. The hydrodynamic radius thickness of this hairy layer is about 7nm (Horne & Davidson, 1986; Walstra, 1990; Holt & Horne, 1996; Dalgleish, 1998; de Kruif, 1998; de Kruif, 1999; Horne, 2003). The incorporation in to and removal of casein and minerals from micelles take place without changing the hydrodynamic radius of the micelles, indicating that the framework can accept the soluble caseins within the interstices (Bloomfield & Morr, 1973; Udabage, 1999).

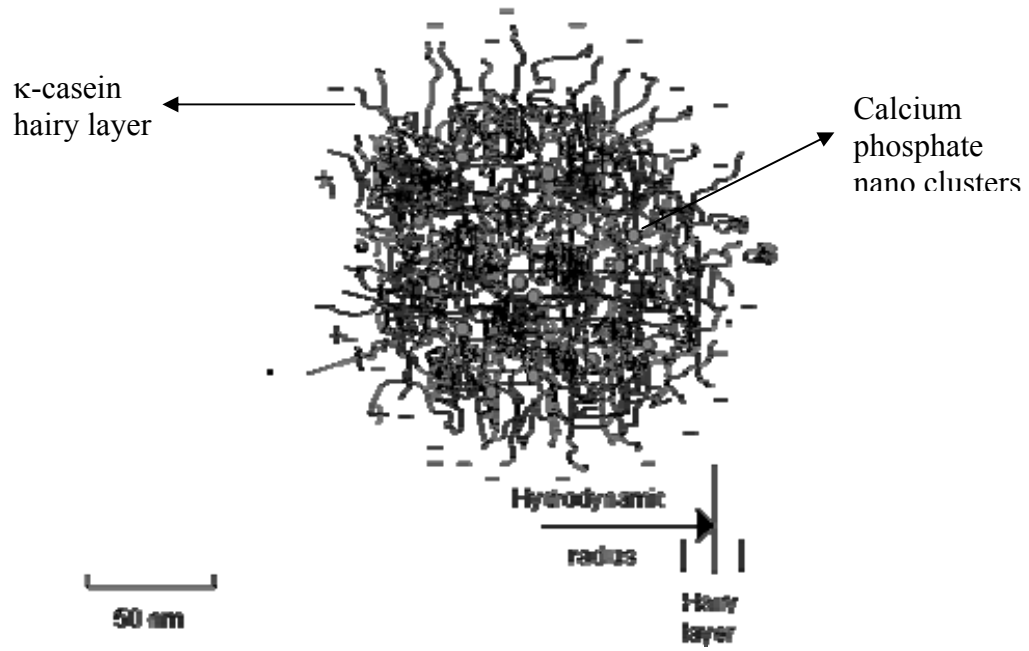

Figure (1.2): The hairy casein micelle model

(Figure taken from Holt & Horne, 1996)

According to Horne's "dual binding model", the amphiphilic caseins may be considered as block co-polymers of alternating charged and hydrophobic segments and the micellar assembly of polymers together through hydrophobic interactions and through calcium phosphate nano clusters as depicted in Figure (1.3). α_{S-1} is depicted as a tri block polymer with a hydrophobic region at each end and a hydrophilic central loop consisting of phosphoserines between them. β-casein is depicted as a di block polymer with a hydrophobic region at one end and a hydrophilic region at the other end. It is speculated that the hydrophobic regions interact intermolecularly in solution rather than compact themselves in to a folded form and the hydrophilic N-terminal peptide containing the phosphoseryl residues extend in to the aqueous phase. In solutions of β-casein, such intermolecular hydrophobic interaction leads to detergent like micellar structures with a

hedgehog like external coating. Also, under certain conditions of pH, ionic strength and calcium concentration, β-casein aggregates in a loose flower like petal arrangement. The self-association is driven by hydrophobic interaction but the size of the polymers and the degree of association is limited by localized electrostatic repulsion. In the casein micelle, κ-casein interacts through hydrophobic interactions, but further growth is not possible because of the absence of phosphoserine residues in the hydrophilic segment. Therefore, κ-casein acts as a terminator of the micellar structure (Horne, 1998; 2002; 2003).

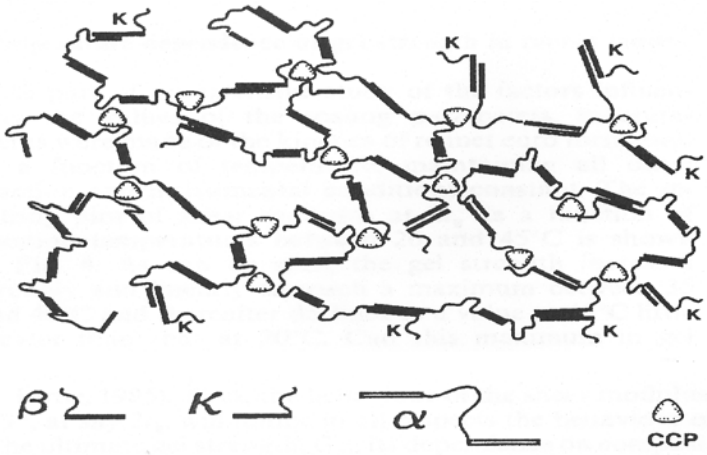

Figure (1.3): The dual binding model

(Figure taken from Horne, 2003)

Holt *et al.* (1982; 1989; 1991; 1998; 2003) had prepared calcium phosphate nanoclusters from phosphopeptides obtained by residues of β-casein and proposed a model of the nano clusters of amorphous calcium phosphate present in the casein micelles. Highly phosphorylated phosphopeptides derived from the calcium sensitive caseins will combine

with amorphous calcium phosphate to form heterogeneous chemical complexes called calcium phosphate nano clusters (Holt *et al.* 1989; Holt *et al.* 1998; Holt *et al.* 2003; Little & Holt, 2004; Smyth *et al.* 2004). Each nanocluster incorporates three or more phosphoserine clusters from different casein molecules as can be seen in Figure (1.3) (Horne, 1998; 2002). A typical casein micelle contains around 800-1000 CCP nano clusters in a disordered array. Each has a core mass of 61 kDa and a core radius of 2.4 nm (Holt *et al.* 1998; Holt *et al.* 2003; Holt, 2004; Smyth *et al.* 2004). Little & Holt, (2004) suggested that calcium phosphate nano particles are not kinetically stabilized particles, but a complex with thermodynamic stability. In Horne's model, it has been viewed that the micellar calcium phosphate acts not just as cross links but also as neutralizing agents which bind to negatively charged phosphoserine clusters to reduce the protein charge to the level where the attractive interactions between the hydrophobic regions of the caseins. Once bound to the surface of the protein, it can adopt the more rigid conformational state. The calcium phosphate granules are stable to dissolution provided the milk serum is maintained in a state of super saturation with respect to the crystalline calcium phosphate. They will not undergo further growth if there are no accessible growth sites on the surface of the granules and they are stable to phase transformation if the nucleation of all stable crystalline phases is inhibited (Holt & Davidson, 1991). A schematic representation of the CCP nanocluster is given in Figure (1.4).

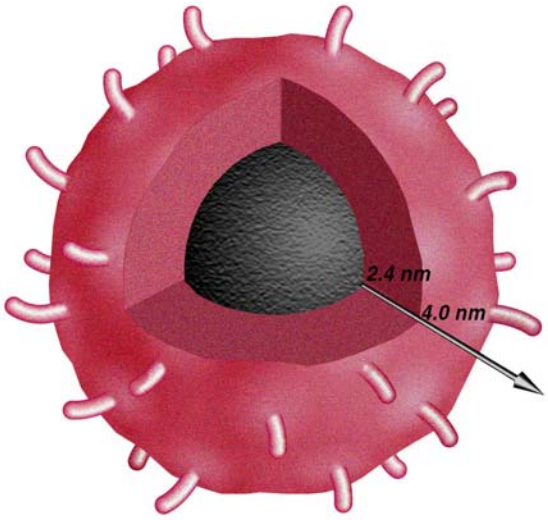

Figure (1.4): Amorphous calcium phosphate core wrapped with phosphopeptides.

(Figure taken from Holt, 2004)

1.6.2 Micelle Stability

The interactions of caseins and their state of association is governed mainly by hydrophobic interactions and through Colloidal Calcium Phosphate (CCP) nano clusters (Horne, 1998) as previously described in Section (1.6.1).

Dalgleish & Law (1989) demonstrated that casein micelles do not fall apart on inducing the removal of colloidal calcium phosphate by decreasing pH, particularly if the temperature was maintained at 30°C or above. The number of positive charges increases due to the protonation of histidine residues, as pH decreases. Removal of calcium phosphate particles by dropping the pH has the effect of increasing the ionic strength, which would decrease the Debye length and hence the size of the interactions between charged residues. This suggests a change in the nature of protein-protein interactions as pH is lowered, or it could indicate the existence of more than one form of binding between proteins and minerals in

the casein micelles. Removing the calcium phosphate takes away one form of stabilization but the other forms of interactions are still present and prevent the micelle from falling apart. This observation clearly shows the importance of hydrophobic interactions in maintaining micellar integrity. On acidification, the micelle was not dissociated by the loss of the CCP. This is due in part to the concurrent partial neutralization of the phosphoserine charge by the acid and to the hydrophobic interactions.

Other types of bonding, where their relative importance have not been identified are hydrogen bonding, electrostatic interactions, van der Waals forces and specific interactions between protein groups along macromolecular chains (Ruettimann & Ladisch, 1987; Horne, 1998).

1.6.3 Colloidal Stability

The colloidal stability of casein micelles is mostly governed by steric stabilization. As two micelles approach to the extent that the hydrophilic regions of the protruding κ-casein overlap, there are increases in the solute concentration in the region between the casein micelles increasing the free energy and thus forcing the casein micelles apart. The stabilization is mainly entropic in nature owing to the large entropy gain when the κ-casein chains are extended freely in to the continuous phase (Horne & Davidson, 1986; Walstra, 1990; Holt & Horne, 1996; Dalgleish, 1998; de Kruif, 1998; Sefcikova *et al.* 1999). The stability of micelles correlates well with voluminosity. A higher voluminosity corresponds to larger particles with a larger radius of curvature. This gives rise to larger overlap of protruding ends and hence stronger steric repulsion. Low voluminosity corresponds with a

compact core with a smaller radius of curvature and lesser overlap of protruding chains. van der Waals attraction is higher (compact core) and steric repulsion is lower (reduced overlaps) resulting in lower stability towards aggregation.

The charges on κ-casein contribute little directly to the colloidal stability of micelles (de Kruif, 1998; 1999) as at high ionic strength electrostatic interactions are very short ranged due to the high screening of charges by counter ions. Darling & Dickson, (1979) attempted to investigate the effect of heat on electrostatic interactions of casein micelles by measuring the zeta potential of cooled milk systems heated to 90°C for 30 min. They found no significant change in the zeta potential of the casein micelles and suggested that the electrostatic interactions are not sufficient to explain the instability of micelles upon heating.

Hydrophobic interactions play a minor role in colloidal stability of casein micelles in natural form. However, when destabilized by external stresses such as acidification, renneting and heating, hydrophobic interactions come in to play. For example, renneting at low temperatures (~12°C) experienced no aggregation of particles. However, as soon as the samples were subjected to high temperatures of around 35°C, particles aggregated readily (Chandrapala, 2005). At high temperatures, more strength in the hydrophobic junctions and less restructuring can be expected in gelled milk systems leading to more compact casein micelles having reduced fractal dimensions. Conversely, low temperatures permit rearrangements in gelled milk systems to a more open structure of casein aggregates characterized by higher fractal dimensions (Vetier *et al.* 2003).

1.7 The Heat Stability of Milk

The ability of milk to withstand high processing temperatures without excessive thickening and coagulation is one of the key factors in the manufacturing of dairy products. The heat stability of milk is a very complex subject**.** Many physical and chemical changes occur in milk proteins during these high temperature processes, which manifest themselves as an increase in viscosity or as coagulation of the milk. Heat stability is affected by a number of compositional factors. The significance of any individual factor is difficult to assess, since variations in one factor influence the others (O'Connell & Fox, 2003). Heat stability can be modified by additives and by varying processing conditions (Fox, 1981). While the precise mechanism of heat coagulation is not yet understood, the influences of a variety of compositional factors and treatment processes on heat stability have been examined.

The heat stability of milk is normally studied in relation to pH. The most commonly used method to study the heat stability of milk is the 'subjective heat stability assay'. This method involves submerging the milk samples in sealed tubes, in a thermostatically controlled oil bath at 140°C (unconcentrated milk) or 120°C (concentrated milk) and measuring the time required to cause coagulation (Singh, 2004). There are two types of Heat Coagulation Time (HCT)-pH profiles; type A and type B. Type A milk exhibits a maximum HCT at pH 6.7 and a minimum HCT at pH 6.9, whereas type B milk HCT increases as a function of pH, as shown in Figure (1.5) (Fox, 1981; Schimdt & Poll, 1986; O'Connell & Fox, 2000, 2001; McSweeney *et al.* 2004; Singh, 2004; Huppertz & Fox, 2006).

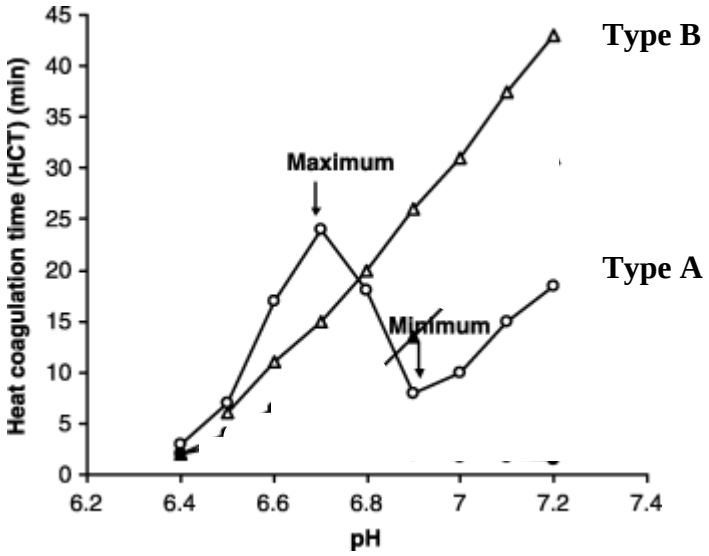

Figure (1.5): The HCT-pH profile of single strength milk heated at 140℃

(*Figure taken from Singh, 2004)*

A more informative method for measuring heat stability is the 'objective heat stability assay'. It measures the percentage of total protein sedimentable by low centrifugal forces as a function of heating time at a constant temperature. The use of this technique is favored if a detailed description of the heat coagulation process is required (Lehmann & Buckin, 2005). Other methods for determining heat stability include the ethanol test, the whitening test and the viscosity determination (Singh, 2004). Most of the heat stability assessment methods correlate poorly with the stability of milk on commercial sterilization (Singh, 2004). Hence, often heat stability was assessed using the objective method, which involve the determination of viscosity after heating under controlled conditions (Augustin & Clark, 1990; Jeurnink & de Kruif, 1992).

Milk is extremely heat stable relative to other food systems due to the presence of caseins, which allows it to be subjected to severe heat treatment with relatively minor changes in comparison to other foods (Fox & McSweeney, 1998; Singh, 2004). Some of the common heat treatments applied in the dairy industry are listed in Table (1.3). The objective of the heat treatment varies with the product being produced.

Table (1.3): **Common heat treatments used in the milk industry**

Type of Heat treatment	Condition
Thermization	65°C for15 sec
Pasteurization - LTLT	65°C for 30 min
Pasteurization- HTST	72°C for 15 sec
Forewarming	90°C for 2-10 min
Sterilization	120°C for 20 sec
UHT	140°C for 3-5 sec
In-container Sterilization	110°C for 10-20 min

(Data taken from Morales *et al.* 2000; O'Connell & Fox, 2003)

(LTLT – Low temperature longer times; HTST- High temperature shorter times)

During the heating process, numerous reactions that influence the nutritional and functional properties of the milk and the subsequent products can occur (Walstra & Jenness, 1984). Some of the changes are listed in Table (1.4).

Table (1.4): **Heat induced changes in milk**

• Denaturation of whey proteins
• Formation of complexes between caseins and whey proteins (eg: κ-casein and β-Lactoglobulin)
• Casein association and solubilization
• Calcium phosphate precipitation onto micelles
• Decrease in pH and calcium activity
• Dephosphorylation of caseins
• Polymerisation of caseins
• Deamidation
• Lysinoalanine formation
• Degradation of lactose
• Maillard reaction

1.7.1 Main Heat Induced Changes

The occurrence and extent of the heat induced reactions are dependent on heating conditions (temperature and duration of heat treatment) as well as on other factors such as milk composition, concentration, and pH. O'Connell & Fox, (1999) found that if the pH was adjusted to pH 5.5 after heating normal milk (pH 6.7) at 140°C for 20 min, it did not coagulate, whereas if normal milk was adjusted to pH 5.5 and heated, it coagulated

instantly. Hence, it was concluded that heat induced reactions affect the inherent heat stability of milk.

In general, the changes induced on heating fall in to two categories. They are tempory changes that are reversed on cooling and permanent changes. The reversible and irreversible reactions are summarized in Figure (1.6) and will be discussed in detail in the remainder of this section.

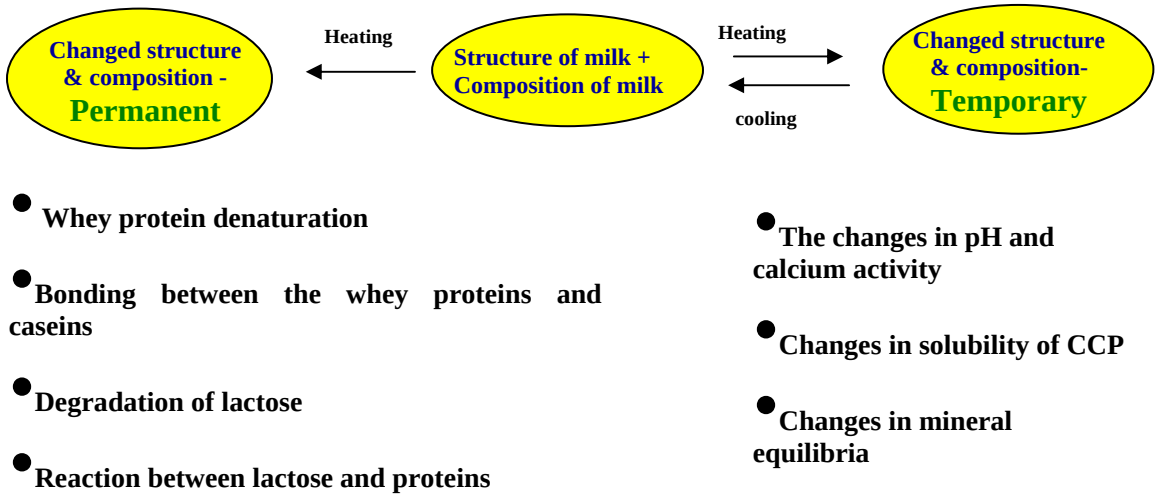

Figure (1.6): The main reversible and irreversible heat induced reactions in milk systems

1.7.1.1. Changes in Minerals

O'Connell & Fox, (2000) pointed out that very small differences in pH can have a very large effect on heat stability of milk. One of the most significant changes during thermal processing is the change in mineral equilibria. Mineral salts affect the partitioning of caseins between the colloidal and serum phases of milk, thus influencing the structure and

stability of proteins. CCP plays an important role in stabilizing the micelle structure (Zhang & Aoki, 1996). There is marked reduction in the solubility of calcium phosphate with increase in temperature, which leads to precipitation of calcium phosphate and thereby a decrease in pH as discussed in Section (1.5).

There is some debate that heating induces a change in the native state of colloidal calcium phosphate or that of the newly precipitated colloidal calcium phosphate. According to Van Dijk, (1990), the calcium phosphate precipitate formed during heating is still able to crosslink casein through phosphoserine residues and thereby causes aggregation of casein micelles. In contrast, Singh & Latham, (1993) and Anema & Klostermeyer, (1997) suggested that the transformed CCP has no ability to cross-link casein molecules leading to the dissociation of caseins from the micelles during heating. However, at the same time the exposure of phosphoserine residues of α_{S-2} and β-casein from the dissociation of κ-casein, which increases the binding sites for CCP and can cause aggregation (Singh & Creamer, 1991). It is unclear how these changes in micellar or soluble calcium phosphate relate to the aggregation and dissociation behaviour of casein micelles during heat treatments (Singh, 1994).

On cooling, there is a reversal of these changes (Augustin & Clark, 1991; Pouliot *et al.* 1989). Zhang & Aoki, (1996) & Zhang *et al.* (2004) stated that approximately 60% of calcium and 40% of phosphate in the serum phase transferred to the colloidal phase on heating to 90°C for 40 min and after recooling to 4°C for 20 hrs, 75–90% of the heat precipitated calcium phosphate was resolubilized. These findings indicate that the

composition of calcium phosphate, its dissolution behavior, and the amount of calcium bound directly to casein remained largely unaffected by heating below 90°C, provided heating was followed by prolonged storage. The slow re-equilibration in the colloidal calcium phosphate composition, which takes place after heating, was attributed by Pouliot *et al.* (1989) to a complex equilibrium. However, when the heating temperature is above 100°C, these changes in micellar calcium phosphate are likely to be irreversible (de la Fuente *et al.* (1998).

The heat stability increases on the addition of calcium chelating agents to the milk (Sweetsur & Muir, 1980; Zadow *et al.* 1983). A possible explanation for the specific effect of added calcium chelating agents on the heat stability of cow's milk is to influence the pH of the milk systems, which is indirectly related to its influence on the nature of the colloidal calcium phosphate. Newstead *et al*. (1975) and Dalgleish *et al.* (1987) have recommended the use of stabilizing salts rather than inorganic acids for pH adjustment of milk. Therefore, it has been proposed when the other factors are kept constant the salt balance of milk becomes a determinant in heat stability (Sommer & Hart, 1922; Webb & Holm, 1932; Kelly *et al*. 1982; Montilla & Calvo, 1997). It was found that an increase in the cation/anion ratio of milk decreased the heat stability. However, the salt balance changes induced by phosphate addition are very small. The effects of pH adjustment on the salt balance of the concentrate are also difficult to interpret since the changes are small and only slight pH adjustments are needed to stabilize the milk (Pouliot & Boulet, 1995). Hence, it is not easy to predict the effect of anion addition on the physico-chemical properties of casein micelles, as it is necessary to consider many factors, which affect the association of anions

with casein micelles (Philippe *et al.* 2005). Some of the factors include the nature and the concentration of added anion and the composition of milk serum.

The buffering capacity of milk products is an important physico-chemical characteristic that corresponds to its ability to be acidified and alkalinized. The buffering capacity of dairy products depends mainly on the composition and distribution of minerals and proteins between aqueous and colloidal phases (Lucey *et al.* 1996; Bikker & Anema, 2003). The natural and induced variations in the composition of milk affect the buffering capacity. Thus, some technological treatments and physico-chemical changes such as heat treatment, membrane separation, high pressure treatment and salt addition, result in a different buffering capacity (Salaun *et al.* 2005). The interactions of cations with acid-base groups of proteins affect their buffering capacities. Binding ions to a protein can also alter its spatial structure and consequently affect protein exchanges with the environment. Depending on the nature of added anion, the milk buffering capacity is affected differently (Salaun *et al.* 2005). The changes are due to the acid-base properties of the anion and also to their interaction with minerals present in milk, especially with calcium. Thus, the addition of the same amount of different anions induces qualitative shifts in the buffering pH range and quantitative increases in buffering capacity. For example, addition of citrate and phosphate to milk induces qualitative and quantitative changes in the buffering capacity. Citrates are calcium chelators and their addition to milk induces solubilization of CCP, where the buffering capacity is shifted towards high pH values. Addition of phosphate increases the soluble phosphate concentration and at the same time the formation of insoluble calcium phosphate is enhanced, which shifts the buffering capacity to lower pH (Salaun *et al.* 2005).

In general, an increase in buffering capacity increases milk stability to heat treatment. The interpretation of qualitative and quantitative changes of buffering capacity is complex because several different biochemical changes occur during heating.

1.7.1.2 Dissociation of Caseins

The micelle has a stable structure as discussed in Section (1.6). The pH, temperature of heating, duration of the heat treatment and the composition of the milk systems play dominant roles in determining the extent of casein solubilization upon heating. The change in charge of individual caseins and/or the change in the state and composition of CCP during heating are suggested to be the primary reasons for the dissociation of casein with change in temperature.

The dissociation of casein increases as the pH and temperature of milk systems increase. At any particular temperature and pH; a higher level of κ-casein than β-casein and a higher level of β-casein than α_S-casein dissociate from the micelles (Anema & Klostermeyer, 1997; Law & Leaver, 1997; Anema, 1998; Ono *et al.* 1999). Low levels of κ-casein were dissociated from the casein micelles on heating at pH 6.6, whereas about 50% were soluble on heat treatment at pH 7.1 at 120°C. This dissociation was partially reversible on readjustment of the pH (Singh & Latham, 1993; Anema & McKenna, 1996). Most casein dissociation processes occur rapidly with the majority occurring during the initial stages of heating, with little further changes on prolonged heating (Anema & Klostermeyer, 1997).

The exact mechanism by which caseins dissociate from the micelles remains unknown. Casein micelle structure is maintained mainly by CCP linkages and hydrophobic interactions and minor interactions such as electrostatic and van der Waals interactions. A temperature and pH induced modification to one of the above mentioned interactions may cause micellar disintegration (Anema & Klostermeyer, 1997). It was suggested that the charge on κ-casein is important in keeping this protein associated with the micelles and that the dissociation of κ-casein at high temperatures (≥ 90°C) and at moderately high pH values is a consequence of the irreversible modification of the protein charge by heat-induced effects (Singh & Creamer, 1991). However, it has been found that there is no significant change to the overall charge of any of the dissociated caseins indicating the impossibility that dissociation is induced through modifications to the protein charge distribution (Anema & Klostermeyer, 1997). It was also hypothesized that the dissociation of casein micelles may occur through changes in the nature of the CCP at elevated pH and temperatures to a form, which is less capable of maintaining the micellar structure. However, the experimental support required for this hypothesis, such as investigations on changes to the composition and structure of the CCP in milk at various temperature and pH combinations is lacking (Anema & Klostermeyer, 1997; Singh, 2004). Horne hypothesized that an increase in pH leads to an increase in conversion of SerP from singly to doubly negatively charged units, which are no longer capable of holding CCP (Horne, 1998). As CCP is involved in maintaining micellar structure, its solubilization is accompanied by the solubilization of the individual caseins (Anema & Klostermeyer, 1997).

κ-casein depleted micelles are supposed to be far less stable with respect to non-depleted ones (Walstra, 1990). Hence, subsequent aggregation of casein micelles occurs as the steric barrier provided by κ-casein is lost (de Jong & van der Linden, 1998; Horne, 2003; O'Connell & Fox, 2003).

1.7.1.3 Denaturation of Whey Proteins and their Subsequent Association with themselves and with Caseins

An important change on heating is the thermal denaturation of the major whey proteins, where the whey proteins retain their native conformation only within relatively limited pH and temperature ranges. Exposing these proteins to extremes of temperature or pH result in protein denaturation (Anema & Klostermeyer, 1996; Corredig & Dalgleish, 1996). The interaction between κ-casein and β-Lg is fundamental to all heat induced modifications of milk product functionality such as the heat stability of concentrated milks (Cho *et al.* 2003).

Protein denaturation and subsequent aggregation reactions are expected to give significantly different values of kinetic and thermodynamic parameters. A denaturation process in which the tertiary structure of the protein is disrupted to give randomly coiled molecules involves rupture of intramolecular bonds, in contrast to an aggregation process in which a few intermolecular bonds are formed (Anema & McKenna, 1996; Lucey *et al.* 1999). The extent and type of the reactions are dependent upon the severity of heating (type and time of heating (indirect or direct heating)) and initial pH (which reflects the levels of soluble calcium & P_{in} and milk concentration) (Corredig & Dalgleish, 1996; Law & Leaver, 1997; Oldfield *et al.* 1998; Anema, 2000; Law & Leaver, 2000; Oldfield *et al.* 2000; Cho *et*

al. 2003). Temperature determines the rate and the extent of denaturation of whey proteins, whereas pH governs that interaction of whey proteins with casein micelles. The degree of denaturation increases with temperature (Jimenez-Guzman *et al.* 2002). The formation of denatured β-Lg may be promoted by prolonged heating times at low temperatures, or by heating at a slower rate to the required temperature.

The unfolded conformation exposes amino acid residue side-chain groups that are normally buried within the native structure, and this causes an increase in the reactivity of some groups (Corredig & Dalgleish, 1999; Oldfield *et al.* 2000; Jimenez-Guzman *et al.* 2002; Bikker & Anema, 2003; Cho *et al.* 2003; Livney *et al.* 2003; Creamer *et al.* 2004). In contrast to the globular whey proteins, the open structured casein proteins are very heat stable. However, the cysteine residues of κ-casein and sometimes $\alpha_{S\text{-}2}$-casein, can be involved in sulfhydryl-disulfide interchange reactions with the denatured whey proteins (Shimada & Cheftel, 1989; Law & Leaver, 1997; Corredig & Dalgleish, 1999; Rasmussen *et al.* 1999; Oldfield *et al.* 2000; Schorsch *et al.* 2001; Jimenez-Guzman *et al.* 2002; Bikker & Anema, 2003; Cho *et al.* 2003; Livney *et al.* 2003; Creamer *et al.* 2004; Considine *et al.* 2007). Non-covalent interactions such as hydrophobic, ionic and van der Waal forces are also involved (Law & Leaver, 1997; Galani & Apenten, 1999; Law & Leaver, 2000; Oldfield *et al.* 2000; Bikker & Anema, 2003; Cho *et al.* 2003; Oldfield *et al.* 2005). Non-covalent complexes form prior to intermolecular disulfide formation (Jang & Swaisgood, 1990; Guyomarc'h *et al.* 2003). Not all the denatured whey proteins complex with the casein micelles. Some remain in the serum where they may form aggregates with other

whey proteins or with serum κ-casein (Anema, 2000; Singh & Waungana, 2001; Guyomarc'h *et al*. 2003; Oldfield *et al.* 2005).

The activation energy of β-Lg denaturation decreases as the pH increases. In contrast, there was no apparent trend for α-La (Oldfield *et al.* 2000). The decrease in activation energy of β-Lg at higher pH values was probably caused by the repulsion of ionized groups within the protein molecule. However, these charged groups, while facilitating protein unfolding (denaturation), hinder intermolecular interactions (aggregation) by their charge repulsion. At the pH of normal milk, β-Lg has a net negative charge and therefore decreasing the pH would reduce the charge and promote aggregation (Law & Leaver, 2000; Oldfield *et al.* 2000; Burova *et al.* 2002). The decrease in activation energy of disulphide-linked aggregation of β-Lg as pH is increased follows that of denaturation, and may reflect the importance protein unfolding has on the development of thiol/disulphide groups capable of forming intermolecular disulphide bonds. In addition, there is a conformational transition at pH 6.9, with an increase in thiol group activity that may promote disulphide-linked aggregation. The net charge on the protein is important in determining the number of favorable collisions between denatured β-Lg that result in aggregation (Oldfield *et al.* 2000). Thus hydrophobic aggregates of β-Lg appeared to form initially along with disulphide-linked aggregates, but as heating progressed the hydrophobic aggregates were able to undergo thiol disulphide interchange reactions. Because hydrophobic interactions between protein molecules are weakened at temperatures above 70°C (Li-Chan, 1983), it is possible that the whey protein aggregates could be broken down in to their unfolded monomeric constituents. Subsequently, the monomeric protein could then interact via

sulphydryl disulphide interchange reactions. Alternatively, the hydrophobic interactions in the aggregate may remain intact and those intermolecular disulphide bonds could form within the aggregate or between β-Lg aggregates. Thus, the aggregates containing disulphide bonds should not be considered exclusive of hydrophobic interactions. Steric effects, thus, seem to be important in dictating disulfide bond formation during heating of whey proteins (Oldfield *et al.* 2000).

Oldfield *et al*. (1998) and (2000) suggested that at least three possible species of denatured β-Lg can associate with micelles; (i) unfolded monomeric β-Lg (ii) self-aggregated β-Lg and (iii) β-Lg/α-La aggregates. Cho *et al.* (2003) showed that the self-aggregation of β-Lg was limited when κ-casein was present. However, at high temperatures and fast heating rates, all whey proteins begin to denature in a relatively short period of time, thus presenting more opportunity for unfolded monomeric β-Lg to self aggregate, which consequently is likely to associate with the casein micelles less efficiently. In contrast, unfolded monomeric β-Lg molecules are expected to penetrate the κ-casein hairy layer with greater ease and have a readily accessible sulphydryl group. The β-Lg aggregates, protrude from the micelles surface, providing a steric barrier against further β-Lg association. In addition, these aggregates may have their reactive sulphydryl group buried within the interior of the aggregate, and therefore unavailable for sulphydryl–disulphide interchange reactions with micellar κ-casein. High ionic strength screens electrostatic repulsion, which promotes aggregation so that proteins approach readily and with more possible orientations and so form more disulfide bonds. Hence, the participation of the different thiol groups in disulphide bonds appeared to depend on their location in the native structure, with surface-

located cysteines more involved than internally located ones. Overall, it may be concluded that despite the unfolding of proteins during the heating process, the position of each cysteine in the native tertiary structure can affect its accessibility and, hence, involvement in disulfide bonding (Corredig & Dalgleish, 1999; Livney *et al.* 2003; Lowe & Anema, 2004).

At pH > 6.9; κ casein/β-Lg complexes dissociate from the micelle during heating whereas at pH < 6.7 complex remains with the micelle, stabilizing the micelle and reducing casein dissociation. The dissociation of *κ*-casein from the casein micelle upon heating has been linked with the formation of the serum type of aggregates, but whether the amount of serum aggregates is a cause or a consequence of the κ-casein dissociation is unclear. These soluble complexes in the serum phase can be separated by size exclusion chromatography. The partition of complexes between soluble and micellar phases has been shown to depend on the pH at which the milk is heated (del Angel & Dalgleish, 2006; Donato & Dalgleish, 2006; Jean *et al.* 2006; Renan *et al.* 2006), as the denatured whey proteins appear to bind more to the casein micelles at low pH, whereas more soluble complexes are formed if the milk is heated at a pH greater than that of the native milk (Vasbinder *et al.* 2004). A model was put forward by Vasbinder & de Kruif, (2003) for the pH dependent association of whey proteins with the casein micelles. It states that heating at pH>6.6 leads to a partial coverage of the casein micelles, whereas heating at pH<6.6 leads to an attachment of almost all of the denatured whey proteins to casein micelles. At pH 6.55 the coverage is rather homogeneous but decreasing/increasing the pH further leads to inhomogeneous coverage of the casein micelles (Vasbinder & de Kruif, 2003). Hence heat treatment of milk results in a

complex mixture of native whey proteins, whey protein aggregates and casein micelles covered with denatured whey proteins. The final composition depends mainly on pH, the temperature of heat treatment (Corredig & Dalgleish, 1996; Anema & Li, 2003; del Angel & Dalgleish, 2006) and the time of exposure to high temperatures (Deeth Pers Com). By considering the Vasbinder & de Kruif, (2003) model, it is evident that very small changes in pH upon heating can make a significant change to the final composition, hence resulting different interactions with particles. As an example, about 75-80% of the denatured whey proteins associated with the micelles at pH 6.5, and this decreased to an association level of only 30% at pH 6.7 (Anema & Li, 2003). At pH 6.55, the casein micelle size increased markedly with a total increase of ~ 25-30 nm. In contrast, at pH 6.7, the casein micelle size increased by only ~5-10nm on heating at 90°C for up to 30 minutes (Anema & Li, 2003). However, it is still not known whether the size changes observed are due mainly to the association of denatured whey proteins with the casein micelle surface or due to the partial aggregation of casein micelles that occurs at the same time (Anema & Li, 2003; Singh, 2004).

1.7.1.4 Other Changes

There are several other reactions, which influences the coagulation of milk proteins to varying degrees during heating. These changes proceed concurrently and possibly influence one another (Singh, 2004). Some of the reactions are, the polymerization of proteins, isomerisation and degradation of lactose, dephosphorylation of caseins, lysinoalanine formation, proteolysis of proteins and most importantly the Maillard reaction. These reactions will be discussed briefly.

Electron microscopic studies showed that high heat treatment (> 120°C) of milk not only increases the size of the casein micelles but also increases the number of protein particles smaller than casein micelles. Extended heating at ultra-high temperatures can also cause changes in the covalent structure of proteins (e.g. loss of phosphate groups from phosphoserine residues) and degradation of proteins in to peptides and amino acids (Singh & Latham, 1993), which results in smaller particles than casein micelles. The increase in particle size at high temperatures could be due to the polymerization of caseins and/or whey proteins as a result of condensation reactions (e.g. Maillard-type and formation of lysinoalanine), where lactose is one of the main components affecting the polymerization of casein on heating (El-Din & Aoki, 1993). Formation of lysinoalanine during heating, also can enhance the polymerization of caseins (Morr, 1985; Ward *et al.* 1996). The formation of lysinoalanine is caused by the β-elimination of phosphoseryl residues (El-Din & Aoki, 1993; Gaucheron *et al.* 2001). Deamidation happens with the liberation of ammonia (van Boekel, 1999; Gaucheron *et al.* 2001).

Lactose isomerisation (through base catalyzed) leading to the formation of lactulose, lactose degradation leading to the formation of epilactose and formic acid and casein dephosphorylation (Castro *et al.* 1986; Andrews & Prasad, 1987; van Boekel, 1998), through the hydrolysis of phosphoserine leading to the formation of phosphates or through β-elimination leading to the formation of dehydroalanine (DHA) and phosphates (El-Din & Aoki, 1993; van Boekel, 1999; Gaucheron *et al.* 2001; Singh, 2004) are partly responsible

for the pH decrease of milk with increase in temperature (> 120°C) for longer periods of time (Castro *et al.* 1986).

Proteolysis of proteins is another change happening during thermal treatment (Morales & Jimenez-Perez, 1998; Gaucheron *et al.* 2001). The lack of secondary structure and the open flexible confirmation of caseins that are rich in proline are responsible for heat induced proteolysis. Proteolysis results in modifications in milk systems such as an increase of polar groups NH_3^+ and COO^- in milk, a decrease in molecular weight of polypeptidic chains and changes in casein association (Gaucheron *et al.* 2001).

Maillard reaction occurs during heating at high temperatures (eg: ≥ 120°C) for considerable time periods (O'Connell & Fox, 1999; Fox, 1981). The early stage of the Maillard reaction is the condensation of lactose with amino groups (lysine) leading to the formation of Schiff's base and this will undergo Amadori rearrangements to form an Amadori product (Lactulosyllysine). The advanced stage breakdown of the Amadori product leads to the formation of various products such as formic acid, lysylpyrraline etc. The final stage is the formation of melanoidins. Consequences of the end product are the loss of nutritional value, formation of flavor compounds, antioxidative and antibacterial compounds, polymerization of milk proteins and the development of brown colour due to melanoidins. Also, the degradation of lysine in the initial stage of the maillard reaction increases the negative charge of the casein micelles (van Boekel, 1998).

1.8 Hypothesis

The primary hypothesis on which this work is based are;

(a) The chemical and structural changes of milk during heat treatment are primarily determined by the pH at the temperature of heating,

(b) The changes in milk concentration and addition of mineral salts, through their effects on the pH at the temperature of heating, influence the chemical and structural changes of milk during heat treatment and hence the heat stability of milk.

1.9 Outline of the Thesis

The object of the present study is to examine the chemical and structural changes of milk systems during heat treatment. The concentrated (12%-21% w/w MSNF) skim milk solutions along with 9% w/w MSNF skim milk solution with or without addition of various amounts calcium chelating agents such as P_{in} and EDTA were used as the main milk systems. The heat treatment used was 90°C for 10 min. The second chapter of the present thesis describes *in situ* physico-chemical changes that occur during heating such as pH, calcium activity and phosphate speciation investigated through ^{31}P NMR. The reversibility of these changes was examined after the heat treatment. The third chapter focuses on the formation and the quantification of the soluble aggregates on heating using HPLC-SEC and SDS-PAGE techniques with respect to *in situ* physico-chemical changes. Chapter four gives the flow properties of the milk systems with respect to the chemical and structural changes of the milk systems. This chapter introduces the use of the Diffusing Wave Spectroscopy (DWS) technique, which is a technique capable of applying Dynamic Light Scattering (DLS) principles to more concentrated systems without dilution. The DWS measurements were done *in situ* conditions to monitor the product of (η^*r) within the milk systems during heating and hence the gel formation. The viscosity was measured to be

compared with the data obtained from DWS. Chapter five concludes the overall chapters and gives the future directions, which can be used to optimize the processing conditions in order to obtain heat stable milk products.

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Chapter 2

Physico-chemical Changes: Effect of pH at heating, milk concentration and added calcium chelating agents

2.1 Introduction

The distribution of salts between serum and micellar phases is affected by the changes in composition that result from the treatment to which the milk is subjected. Small changes in pH and alterations in the distribution of salts can lead to noticeable effects in micellar stability and stability of the milk systems to processing treatments (de la Fuente, 1998).

This chapter primarily considers the changes in mineral composition with temperature, changes in distribution of components between the colloidal and the aqueous phases and changes in P_{in} speciation of skim milk systems.

- The initial focus was on changes in pH in response to;

(a) Changes in temperature from 25°C to 90°C and in some cases to 120°C. The pH changes *in situ* have been examined as a function of initial pH (pH 6.2-7.2), milk concentration (9%-21% w/w MSNF) and the addition of calcium chelating agents (10-30mM P_{in} & EDTA). The pH changes *in situ* as a function of time at high temperatures were also measured in selected samples.

(b) Cooling after heat treatment in order to assess the extent of reversibility of the changes that occur during heating.

(c) Addition of acids and bases in order to measure the buffering capacity at 25°C and at 90°C.

- The second focus was on the changes in calcium activity in response to;

(a) Changes in temperature from 25°C to 60°C. The upper temperature limit was because of the effective operating range of the calcium ion selective electrode.

(b) Cooling after heat treatment in order to assess the extent of reversibility of the changes that occur during heating.

- The third focus was on the changes in P_{in} on heating 9% w/w MSNF milk solutions as determined by ^{31}P NMR. Both the relationship between the chemical shift and the observed changes in pH were compared. These changes have been examined as a function of initial pH and temperature (up to 90°C).
- Fourthly, X-Ray diffraction was used to evaluate the observed phase changes (amorphous/crystalline) in the colloidal calcium phosphate.

2.2 Materials and Methods

2.2.1 Materials

A commercial skim milk powder (SMP) was obtained from Tatura Milk Industries (PO Box 213, Tatura, VIC 3616, Australia). It was a non standardized mid summer (January 2006) milk powder. The preheat treatment given to the milk prior to concentration and drying was 72°C/20s. SMP was stored in sealed 25kg bags at 4˚C. When a bag was opened, SMP was transferred to ~1kg sealed bags in plastic containers at room temperature. All

other chemicals were analytical grade and they were obtained from BDH Chemicals (Kilsyth, VIC 3137, Australia) and Sigma-Aldrich Pty Ltd (Castle Hill, NSW 1765, Australia). Ultra pure water (MilliQ water) was used at all times.

2.2.2 Preparation of Reconstituted Skim Milk Solutions

SMP was reconstituted in MilliQ water at room temperature (~ 25°C ± 3°C) to obtain 180g/kg milk solids non fat (MSNF) solution or 300g/kg MSNF solution. The skim milk solution was continuously stirred for one hour at room temperature (~ 25°C ± 3°C) to ensure complete mixing. MilliQ water was added to dilute the milk solution. The pH adjustment (when required) was carried out by drop wise addition of 0.1M NaOH or 0.1M HCl. Further addition of MilliQ water was made such that at the end of the final pH adjustment, only a small amount of additional MilliQ water was required. The skim milk solution was kept overnight at 4°C. On the next day, the skim milk solution was equilibrated at 25°C for 1 hour. Minor pH adjustments were carried out as necessary and very small amounts (~0.5g) of MilliQ water were added to obtain milk solutions at the final desired concentration (9-21% w/w MSNF) and pH (6.2-7.2).

For the preparation of milk solutions with added salts (P_{in} or EDTA), the salt solutions were added drop wise with continuous stirring. Calculated amounts of MilliQ water was added, before the addition of the respective salt solution such that at the end of the addition and final pH adjustment, only a small amount of MilliQ water was required to obtain the 9% w/w MSNF solution or 18% w/w MSNF solution with the respective amounts of the additive (10-30 mM added P_{in} or EDTA). All amounts were measured by mass. The salt

solutions used were 100 mmol P_{in} (equimolar mixture of Na_2HPO_4 and NaH_2PO_4)/kg and 200 mmol Na_2H_2EDTA/kg. All measurements were performed in replicate on freshly prepared skim milk solutions.

2.2.3 Characterization of the Skim Milk Powder

2.2.3.1 Determination of the Moisture Content

The International Standard FIL-IDF method 26 (1964) was used where 1g of SMP was dried by heating at 102±2°C in an oven to a constant weight such that the difference between consecutive weighings (1 hr intervals) was less than 0.02g. The water content of the powder was taken to be equal to the loss of weight. It was expressed as a percentage of the original weight of the wet powder. Five replicates were done.

2.2.3.2 Determination of the Ash Content

The Australian Standard method 2300.1.5 (1988) was used to determine the ash content. An aliquot (10g) of 9% w/w MSNF solution was dried and the residues charred. Oxidation was completed in a furnace at 500°C. The ash content was expressed as a percentage of the original weight of the wet powder. Five replicates were done.

2.2.3.3 Determination of the Fat Content

The AOAC Official modified Mojonnier ether extraction method 989.05 (1996) was used to determine the fat content. 2g of SMP was mixed with 10ml of concentrated HCl solution and heated to 60°C for 10 minutes. 10ml of 95% ethanol was added to prevent emulsion formation during the extraction. The fat was extracted three times using a mixture of 25ml

of diethyl ether and 25ml of petroleum ether. The organic layer was evaporated to dryness. The fat content was taken to be the mass of the residues. The fat content was expressed as a percentage of the original weight of the wet powder. Five replicates were done.

2.2.3.4 Lactose Determination

Proteins were separated by diluting an aliquot (5g) of 9% w/w MSNF milk solution with 20g of MilliQ water and 25g of 24% w/w trichloroacetic acid (TCA) followed by suction filtration through a Whatman No. 40 filter paper in to a preweighed Buchner flask. The precipitated proteins were washed with 10g of 12% w/w TCA solution. The total mass of the TCA filtrate was then determined.

The Australian Standard method 2300.4.10 (1994) was used to determine the lactose content of the TCA filtrate. An aliquot (10g) of the TCA filtrate was mixed with 22.5g of chloramine T reagent (5.7g/kg) to oxidize the lactose. This was followed by the addition of 7.5g of aqueous potassium iodide (100g/kg). The liberated iodine was determined by titration with ~0.04M pre standardized sodium thiosulphate using starch as the indicator. Chloramine T solution was standardized by carrying out a blank titration. The sodium thiosulphate was standardized against a solution of 0.01mol/L potassium iodate and 100g/L potassium iodide with starch as the indicator. In calculating the mass of lactose present in powder, the molar mass of lactose was taken as 360.32 corresponding to $C_{12}H_{22}O_{11}.H_2O$.

2.2.3.5 Total Phosphorus Determination

The International Standard IDF method 42B (1990) was used to determine the total phosphorus content. Initially, ashing was done as described in Section (2.2.3.2). The ash was dissolved in 2g of 1M HCl and 3g of water and the filtrate was used for analyses after appropriate dilution. Aliquots (2g) of the diluted filtrates were mixed with 2.6g of molybdate-ascorbic acid solution to form a blue coloured complex. The absorbance at 820nm was then measured within 15-30 minutes by UV spectroscopy (CARY 100 BIO UV-Visible spectrometer, Varian Inc., 3120, Hansen Way, Palo Alto, CA 94304-1030, USA). The standard solutions in the range 0–0.6mg of P/g of solution were prepared as described in the method.

2.2.3.6 Ultracentrifugation of Reconstituted Skim Milk Solutions

Reconstituted skim milk solutions were centrifuged at 30,000rpm (78,000g) at 25°C for 90 minutes using a Beckman L8 80M ultracentrifuge with a type 80 rotor (Beckman Instruments Australia (Pty) Ltd, Gladesville, NSW, 2111, Australia). The centrifugation speed was chosen as the minimum required to effectively deposit casein micelles as a firm pellet because at higher speeds some whey proteins and serum casein are deposited with the pellet (Anema & Li, 2003). On ultracentrifugation, skim milk partitions in to three separate layers (Udabage, 1999). These are the clear supernatant layer, the opalescent layer and the firm pellet. The pelleted phase contains the majority of casein micelles whereas the supernatant contains the majority of whey proteins. The opalescent layer is primarily serum but contains a small fraction of the smaller casein micelles. All analyses were carried out on the clear supernatant. However, in calculation of the total amount of a component in the

supernatant, the mass of the opalescent layer was included as part of the total mass of supernatant.

2.2.3.7 Mineral Analyses

Minerals were separated from the proteins as described in Section (2.2.3.4). An aliquot (0.5g of milk or 1.0g of supernatant) was mixed with the suppressant and diluted to a total mass of 50g.

Calcium & Magnesium were determined by atomic absorption spectroscopy (AAS) (Model AA 3110, Atomic Absorption Spectrophotometer; Perkin Elmer, Scoresby, VIC 1270, Australia) using an air acetylene flame and wavelength of 422.7nm and 285.2nm respectively. Commercially available AAS standards for Ca and Mg were used after appropriate dilution (1-4µg/g for Ca and 0.1-0.4µg/g for Mg). All solutions contained 0.2g of $LaCl_3$/g of solution to suppress the ionization of atoms (Udabage, 1999).

Sodium & Potassium were also determined by AAS (Model AA 3110, Atomic Absorption Spectrophotometer, Perkin Elmer, Scoresby, VIC 1270, Australia) using an air acetylene flame and wavelength of 589nm and 766.5nm respectively. Standards were based on freshly prepared 0.4mg/g NaCl and 1mg/g KCl solutions with appropriate dilution (0.15-0.6µg/g for Na and 0.4-2µg/g for K). All solutions contained 0.1g of CsCl/g of solution to suppress the ionization of atoms (IDF 119 A: 1987).

Total inorganic phosphate was determined colorimetrically by the IDF method 42B (1990) using UV spectroscopy. An aliquot (1g) of the TCA filtrate was mixed with ammonium molybdate and potassium antimony tartrate to form phosphomolybdic acid which was then reduced by the addition of ascorbic acid to form a blue complex. The absorbance of the colour is proportional to the concentration of the inorganic phosphate content. The absorbance was measured at 820nm within 15-30 minutes after mixing by UV spectroscopy (CARY 100 BIO UV-Visible spectrometer, Varian Inc., 3120, Hansen Way, Palo Alto, CA 94304-1030, USA). The standard solutions in the range 0–0.6mg of P/g of solution were prepared as described in the method. The values obtained from this method showed a much lesser amount. Hence, the samples were commercially analyzed by using PHOS 03 08.93 (Dairy Technical Services Ltd, Kensington, VIC 3031, Australia) and those values obtained were used in the present study.

Citrate was determined by a colorimetric method based on Marier & Boulet (1958). An aliquot (1g) of the TCA filtrate was mixed with 1.3g of pyridine and 5.7g of acetic anhydride to form a yellow coloured complex. The sample was placed in a 32°C water bath for 30 min and the absorbance was measured at 428nm (CARY 100 BIO UV-Visible spectrometer, Varian Inc., 3120, Hansen Way, Palo Alto, CA 94304-1030, USA). The standards were made from tri sodium citrate (0-0.15mg/g).

2.2.3.8 Nitrogen Analysis

The Kjeldahl method based on the Standards Association of Australia (1991) was used to determine the total nitrogen (TN), non casein nitrogen (NCN) and non protein nitrogen

(NPN). Casein nitrogen (CN) content was calculated by subtracting the amount of nitrogen from the total nitrogen content of the skim milk solutions. The N of the denatured whey proteins is included as casein nitrogen. These were used for estimation of protein in the skim milk solutions. The factor of 6.38 was used to convert the protein nitrogen to calculate the protein content of each group of protein (IDF 20B: 1993).

For the determination of NCN, 20g of milk was diluted with 20g of water and was warmed to 35°C. Caseins were separated by the addition of 2ml of 10% w/w acetic acid followed after 10 min with 2ml of 1M sodium acetate. Water was added once a clear supernatant formed to obtain a final weight of 50g. Caseins were removed by filtering through a Whatman No. 40 filter paper. The filtrate was analyzed.

For the determination of NPN, Total Protein (TP) was separated by acidification of 20g of milk with 80g of 15% w/w TCA followed by filtering through a Whatman No. 40 filter paper. The filtrate was analyzed.

2.2.3.8.1 The Kjeldahl Method

The particular filtrate or skim milk solution was digested with 2 kjeltabs Cu/3.5 as the catalyst, 10ml of concentrated sulphuric acid and 4ml of 30% hydrogen peroxide (added 2ml at a time) and 2-3 drops of Dow Corning DB-110A antifoam. The digestion was carried out in a Tecator 2006 digestion block at 420°C until a clear solution obtained. After digestion, the solutions were allowed to cool. After cooling, the solution was transferred to a Kjeldahl distillation unit (Kjeltec system 1026 distilling unit) containing approximately

110ml of water and 70ml of 40% sodium hydroxide. The distillate was collected in a flask containing 20ml of boric acid solution (4g/L boric acid containing bromocresol green and methyl red as indicators). The liberated ammonia was determined as the difference in the amounts of 0.1M HCl required causing a colour change from green to red in boric acid solution with ammonia and the blank.

2.2.3.9 The Extent of Denaturation of Whey Proteins

The caseins and denatured whey proteins were precipitated from skim milk by adjusting the pH to 4.6 with 1M HCl. The samples were held 30 min and then were centrifuged for 30 min at 1,000g at 25°C using a Beckman J2-MC ultracentrifuge (Beckman Instruments Australia (Pty) Ltd, Gladesville, NSW, 2111, Australia).

The supernatant containing the undenatured whey proteins was analyzed by SDS-PAGE under reducing conditions along with the total milk (Parris & Baginski, 1991). The supernatant and the skim milk portion were each dissolved in MilliQ water to a total protein content of 2.5mg/mL. The samples (25µL) were mixed with 20µL of NuPage LDS sample buffer at pH 8.4 and 5µL of 1mM dithiothreitol. All samples were heated at 70°C for 10 min in order to ensure complete reduction. Aliquots (10µl) of the heated samples were applied to the gels and run at 125V for 10 min then at 180V for further 50 min. The running buffer used was NuPage MES (Morpholino Ethane Sulphonic acid) SDS buffer with the composition of 50mM MES, 50mM tris base, 0.1% SDS, 1mM EDTA at pH 7.3. Standard solutions of sodium caseinate and whey protein isolate were run to identify and quantify the bands. The gels were stained using Invitrogen Coomassie Blue staining kit following the

manufacturer's instructions. The stained gels were then photographed and the photographs analyzed using Scion Image software (Scion Co-operation, 82 Worman's Mill Court, Maryland 21701, USA). The integrated intensities of each band were measured and used to determine the relative proportions of the whey proteins present in the supernatant and skim milk samples as percentage of total proteins.

2.2.3.10 X-Ray Diffraction Measurements

X-Ray Diffraction (XRD) of skim milk solutions were obtained on a Phillips 1140/90 powder diffractometer using a Cu tube operated at 40kV and 20mA fitted with a carbon monochromator. The measurements were made over the range of scattering angles $2 \leq 2\theta \geq 60°$.

2.2.4 Heat Treatment of Reconstituted Skim Milk Solutions

2.2.4.1 At 90℃

Aliquots (50g) of milk were transferred to stainless steel tubes (Figure 2.2) and heated in a water bath at 90°C. Figure (2.1) shows the temperature profile during heating. The time to reach 90°C was 8 min. The skim milk solutions were held for a further 10 min at 90°C. After the heat treatment, the samples were cooled by immersion in a water bath held at 25°C for 5-10 min. The cooled solution was transferred to refrigerator for overnight storage at 4°C. The next day, the skim milk solutions were removed from the refrigerator and placed in a water bath at 25°C for a minimum of 1 hr prior to measurements.

2.2.4.2 At 120℃

Aliquots (50g) of milk were transferred to stainless steel tubes and heated in a glycerol bath at 120°C. Figure (2.1) shows the temperature profile during heating. The time to reach 120°C was 8 min. The skim milk solutions were held for a further 10 min at 120°C.

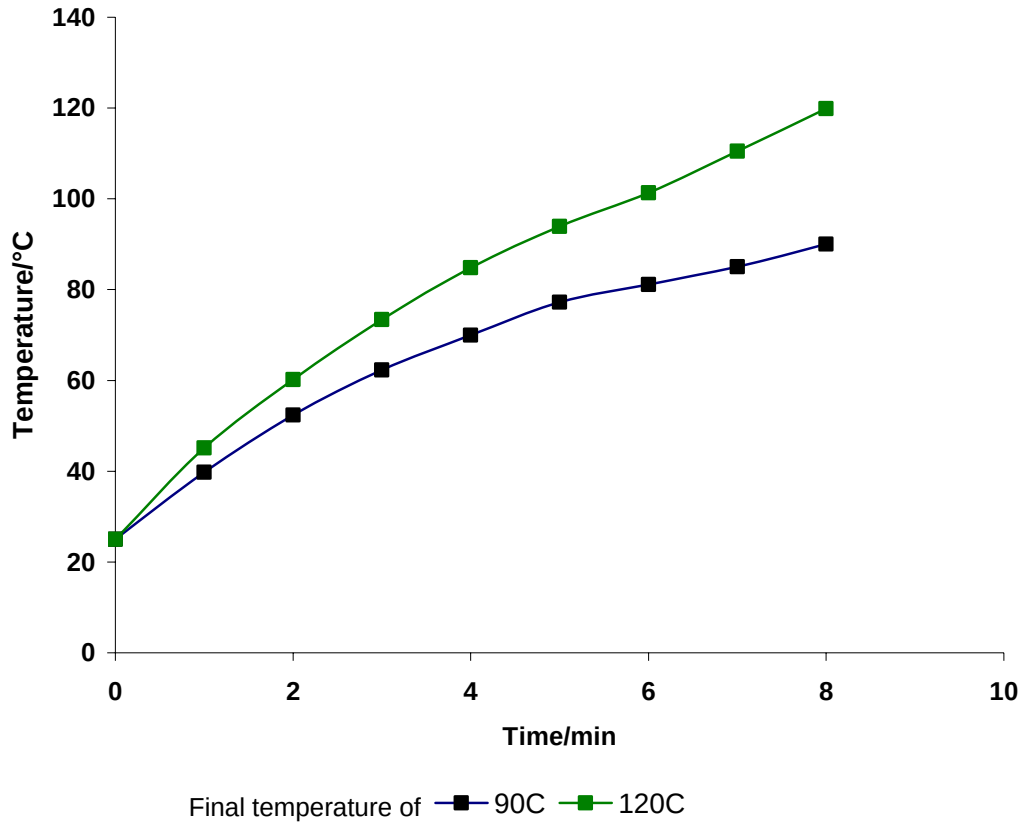


Figure (2.1): The temperature profile of skim milk solutions reaching 90℃ and 120℃ using the high temperature pH probe

2.2.5 pH Measurements

2.2.5.1 pH at 25℃

The pH of the skim milk solutions at 25°C was measured by an InPro 2000 liquid electrolyte pH electrode with an integrated temperature sensor (Mettler Toledo, Australia) connected to a Metrohm pH meter (Metrohm AG, Oberdorfatrasse 68, 9101 Herisau, Switzerland). The pH probe was calibrated at 25°C using phosphate buffer at pH 6.86 and phthalate buffer at pH 4.01 (CRC Hand book of Chemistry and Physics).

2.2.5.2 pH at High Temperatures

The pH of the skim milk solutions at 90°C/120°C was measured by an InPro 2000 liquid electrolyte pH electrode with an integrated temperature sensor (Mettler Toledo, Australia) connected to a Metrohm pH meter (Metrohm AG, Oberdorfatrasse 68, 9101 Herisau, Switzerland). The temperature compensation of the pH electrode was checked against the buffer systems used in the present study. The pH of the phosphate buffer system at 90°C was calculated with the use of equilibrium constants using following equations and the pH of the phthalate buffer system at 90°C was used as 4.205 (CRC Hand book of Chemistry and Physics). The actual pH measured by the meter was compared with the calculated values and the compensation of the pH meter was accurate within pH unit ± 0.018 for the buffer solutions in the pH range of 4 to 8.

$$\ln K_{90} = \frac{\Delta H_r^0}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] + \ln K_{25} \qquad \textit{Equation 1}$$

$$pH = pK + \log \frac{a_{HPO_4^{2-}}}{a_{H_2PO_4^-}} \qquad \textit{Equation 2}$$

$$a = \gamma c$$

where, a = activity of a particular substance,

γ = activity coefficient,

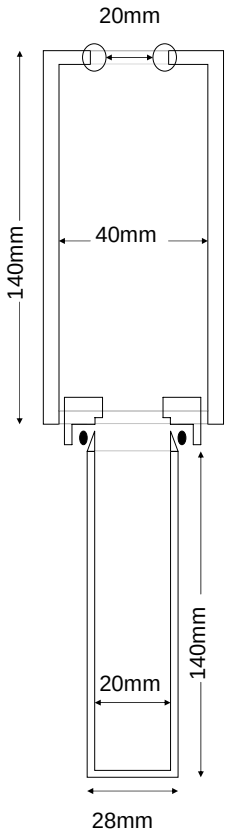
c = concentration

The values used for the calculation were;

K_{25} = 6.31*E-08

P Species	ΔH^0_f /(kJ/mol)	γ at 25°C	γ at 90°C
HPO_4^{2-}	-310.4	0.38	0.33
$H_2PO_4^-$	-311.3	0.79	0.76

(The values were taken from Robinson & Stokes, 1954)



The pH probe was designed for temperatures up to 140°C and pressures up to 6 bar. For milks, at temperatures above 90°C, a sealed cell was designed (Figure 2.2).

Figure (2.2): A schematic representation of the sealed cell

2.2.6 Measurement of Calcium Activity

Ca^{2+} activity was measured at 25°C using a Ca Ion Selective electrode connected to a pH meter (Metrohm, AG CH-9101, Hensau, Switzerland) fitted with a reference Ag/AgCl electrode. Calibrations were carried out with $CaCl_2$ solutions in the range of 0.0003M – 0.025M, with an ionic strength of 0.08M, adjusted with KCl. Standard curve of potential vs $\ln a_{Ca^{2+}}$ was used. The Ca^{2+} activity ($a_{Ca^{2+}}$) of these standard solutions was calculated as:

$$a_{Ca^{2+}} = \frac{c_{Ca^{2+}}}{c^0}\gamma_{Ca^{2+}} \qquad \textit{Equation 3}$$

where, c^0 = 1mmol/L and $\gamma_{Ca^{2+}}$ = 0.425 is the activity coefficient of the Ca^{2+} as given by Debye-Huckel approximation (MacInnes, 1961). Regular calibrations were performed during analysis of samples.

Ca^{2+} activity was measured at 60°C using the same electrode. The standards were calibrated at 60°C when measuring at 60°C. The $\gamma_{Ca^{2+}}$ of 0.403 was used at 60°C, which was calculated using Debye-Huckel approximation. Although the manufacturers stated that the upper limit for the electrode was 80°C, it was found that the practical upper limit was 60°C.

2.2.7 High Resolution ^{31}P NMR Spectroscopy

^{31}P NMR spectra of skim milk solutions were obtained at each different temperature in a 300MHz BRUKER spectrometer (Bruker BioSpin GmbH, Silberstreifen 4, 76287 Rheinstetten, Germany) at a frequency of 121.51MHz. 6.75μs was used as the pulse width. The spectral width was 10,000 Hz and the line broadening was 2Hz. The acquisition time

was 0.73s. Chemical shifts were given in ppm relative to 85% phosphoric acid. 500 transients were acquired. 5 mm sample NMR tubes were used.

2.3 Results and Discussion

The composition of the skim milk powder used throughout the experiments is given in Table (2.1). These values are in good agreement with the literature (Walstra & Jenness, 1984).

Table (2.1): The composition of the skim milk powder used in the experiments

Component	**Experimental results (g/100g powder)**
Moisture	3.50 ± 0.17
Ash	8.80 ± 0.02
Fat	0.53 ± 0.01
Lactose	48.45 ± 1.92
Total Crude Protein	36.50 ± 0.30
Mineral component	**Concentration (g/100g powder)**
Calcium	1.31 ± 0.10
Magnesium	0.10 ± 0.02
Sodium	0.40 ± 0.02
Potassium	1.64 ± 0.03
Citrate	1.20 ± 0.17
Total P	1.05 ± 0.04
P_{in}	0.61 ± 0.01
Nitrogen component	**Concentration (g/100g of powder)**
Casein	28.50 ± 0.20
Whey Proteins	7.00 ± 0.13
Non Protein Nitrogen	0.144 ± 0.007

Mean values and s.d. of ($n \geq 3$) analyses.

SDS-PAGE was used to evaluate the amount of denatured whey proteins (Table 2.2). About 5% of the total whey proteins were denatured in the low heat skim milk powder used. This low level of denatured whey proteins is consistent with the low heat treatment received during powder manufacture.

Table (2.2): The extent of whey protein denaturation of the SMP analyzed through SDS-PAGE

Sample	Whey Proteins (as % of total protein)
9% w/w MSNF milk solution (Total)	19.81 ± 0.21
Supernatant obtained from ultracentrifugation (Undenatured)	18.74 ± 0.16
*The difference (Denatured)	1.07 ± 0.04

Mean values and s.d. of (n = 2) analyses.
*The value for % whey protein obtained from 9% w/w MSNF milk solution is that of the total whey proteins and the % whey protein obtained from the relevant supernatant gives only the undenatured whey proteins. The denatured whey proteins were calculated as the difference.

Supernatants were obtained by ultracentrifugation of 9% w/w MSNF milk solutions as described in Section (2.2.3.6). The Ca, P_{in} and citrate contents of the supernatant are given in Table (2.3). About 28% of calcium, 85% of citrate and 49% of P_{in} were in the supernatant, which was in agreement with the literature values (Walstra & Jenness, 1984).

Table (2.3): The serum calcium, citrate and P_{in} content of reconstituted 9% w/w MSNF milk solution

Component	Total concentration/ (mmol/kg of milk)	Serum concentration/ (mmol/kg of milk)
Calcium	32.75 ± 2.70	9.10 ± 0.05
Citrate	6.20 ± 0.87	5.20 ± 0.04
P_{in}	18.97 ± 0.30	9.29 ± 0.02

Mean values and s.d. of (n = 3) analyses.

2.3.1 Skim Milk Solutions of 9 - 21% w/w MSNF

2.3.1.1 The Change of pH with Temperature

The pH of a series of skim milk solutions with concentrations ranging from 9%-21% w/w MSNF with initial values of pH adjusted to 6.2 to 7.2 at 90°C and at 25°C (after heat treatment (90°C/10min), cooling at 4°C overnight and re-equilibrate at 25°C for 1 hour) are listed in Table (2.4). The unadjusted pH values of 9%-21% w/w MSNF milk solutions are listed in the table in bold italic. As demonstrated in the table, the pH at 25°C decreased with increasing milk concentration as observed by others (Nieuwenhuijse *et al.* 1988). The pH decreased when heated to 90°C. This decrease in pH was also observed when heated to 120°C. However, a larger decrease in pH heated at 120°C for 10 min was observed in comparison with the pH decrease heated at 90°C for 10 min as can be seen from Table (D.1) in Appendix D.

Table (2.4): The changes in pH with temperature and its reversibility upon cooling as described in (Section 2.2.4.1)

Sample	Initial pH at 25°C	pH at 90°C	Δ pH (pH at 25°C–pH at 90°C)	pH at 25°C after overnight equilibration
9%	6.25 ± 0.00	5.86±0.01	0.39 ± 0.01	6.26±0.01
	6.41± 0.02	5.98±0.01	0.43 ± 0.01	6.42±0.02
w/w	***6.65± 0.02***	***6.18±0.00***	***0.48 ± 0.02***	***6.63±0.01***
	6.83± 0.02	6.31±0.01	0.51 ± 0.01	6.77±0.01
MSNF	7.02± 0.02	6.46±0.01	0.56 ± 0.03	6.95±0.01
	7.21± 0.02	6.60±0.01	0.61 ± 0.03	7.07±0.02
12%	6.22±0.03	5.77±0.03	0.45 ± 0.01	6.23±0.01
	6.44±0.01	5.99±0.01	0.45 ± 0.01	6.44±0.02
w/w	***6.55±0.03***	***6.09±0.01***	***0.46 ± 0.03***	***6.57±0.02***
	6.84±0.00	6.30±0.01	0.54 ± 0.01	6.78±0.01
MSNF	7.03±0.02	6.42±0.03	0.61 ± 0.02	6.90±0.01
	7.23±0.01	6.56±0.01	0.67 ± 0.00	7.08±0.03
15%	6.23±0.00	5.78±0.00	0.45 ± 0.00	6.24±0.01
	6.42±0.01	5.94±0.02	0.48 ± 0.01	6.44±0.02
w/w	***6.50±0.00***	***6.02±0.00***	***0.48 ± 0.00***	***6.51±0.01***
	6.84±0.00	6.27±0.03	0.57 ± 0.03	6.85±0.01
MSNF	7.03±0.02	6.37±0.00	0.66 ± 0.03	7.02±0.03
	7.24±0.00	6.51±0.03	0.73 ± 0.03	7.16±0.02
18%	6.23±0.03	5.80±0.03	0.43 ± 0.02	6.24±0.01
	6.46±0.00	***5.98±0.01***	***0.48 ± 0.01***	***6.47±0.02***
w/w	6.61±0.03	6.08±0.03	0.53 ± 0.01	6.63±0.02
	6.81±0.02	6.24±0.02	0.57 ± 0.01	6.82±0.01
MSNF	7.04±0.02	6.38±0.02	0.65 ± 0.02	7.01±0.01
	7.21±0.02	6.50±0.03	0.72 ± 0.02	7.17±0.01
21%	6.23±0.01	5.76±0.01	0.47 ± 0.02	6.24±0.01
	6.43±0.01	***5.91±0.02***	***0.51 ± 0.02***	***6.43±0.02***
w/w	6.63±0.02	6.07±0.03	0.56 ± 0.03	6.63±0.02
	6.80±0.01	6.19±0.03	0.62 ± 0.03	6.81±0.02
MSNF	7.01±0.02	6.33±0.03	0.68 ± 0.03	7.00±0.01
	7.21±0.02	6.45±0.02	0.76 ± 0.01	7.18±0.01

Mean values and s.d. of (n = 3) analyses.
The skim milk solutions at their unadjusted pH values are given in bold italic in the table.

The pH decreased with increasing temperature as observed by others (Chaplin & Lyster, 1988; Ma & Barbano, 2003). Early works were limited to temperatures up to 80°C, while this study was extended to higher temperatures of up to 120°C. The pH at the temperature of heating as a function of temperature (25°C to 120°C) of 9% w/w MSNF milk solutions with different values of initial pH at 25°C (pH 6.2, 6.65 & 7.2) are represented in Figure (2.3). The graphs are approximately linear over this temperature range. The linear coefficients obtained in this study were -0.0070, -0.0079 and -0.0091 at pH values of 6.2, 6.65 and 7.2 respectively. These values are consistent with the coefficients of -0.0073 reported by Chaplin & Lyster, (1988) for pH 6.65 and -0.0078 pH unit/°C reported by Ma & Barbano, (2003) for pH 6.58 milk solutions. The negative slopes increase with the increase of the initial pH of the skim milk solution.

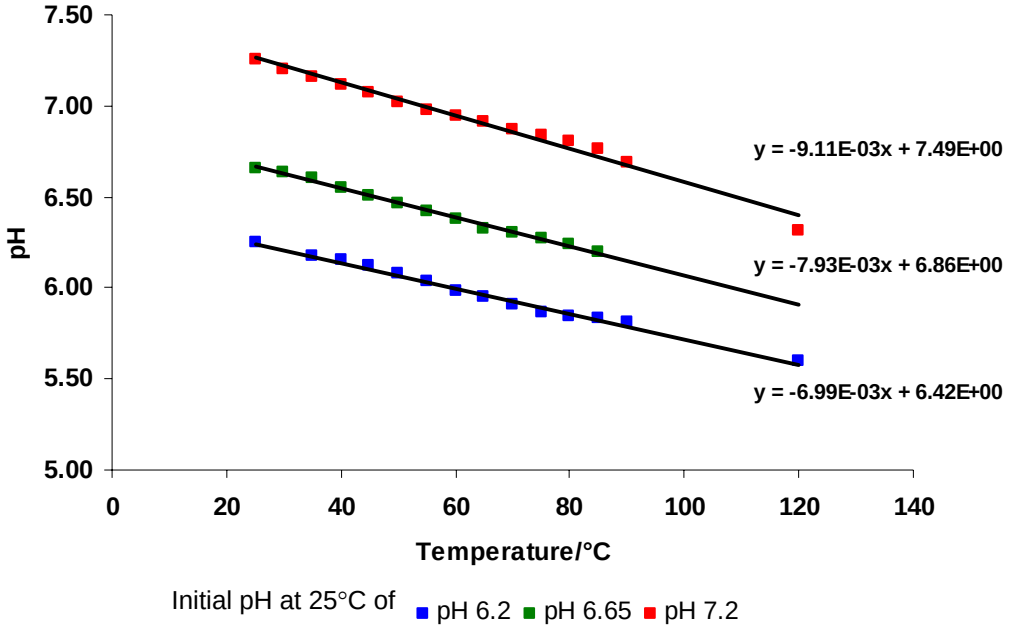


Figure (2.3): The pH of the 9% w/w MSNF solutions at initial pH values of 6.2, 6.65 and 7.2 as a function of temperature. The errors in slopes for pH 6.2, 6.65 and 7.2 milks are 0.0007, 0.0003 and 0.0005 respectively.

Figure (2.4) reports the decrease in pH on heating from 25°C to 90°C with respect to milk concentration (9% to 21% w/w MSNF) and with respect to initial pH at 25°C before heating from pH 6.2 to 7.2. The decrease in pH on heating from 25°C to 90°C ($\Delta pH_{(25°C - 90°C)}$) was defined as the difference between the pH at 25°C before heating and pH at 90°C (measured at 90°C).

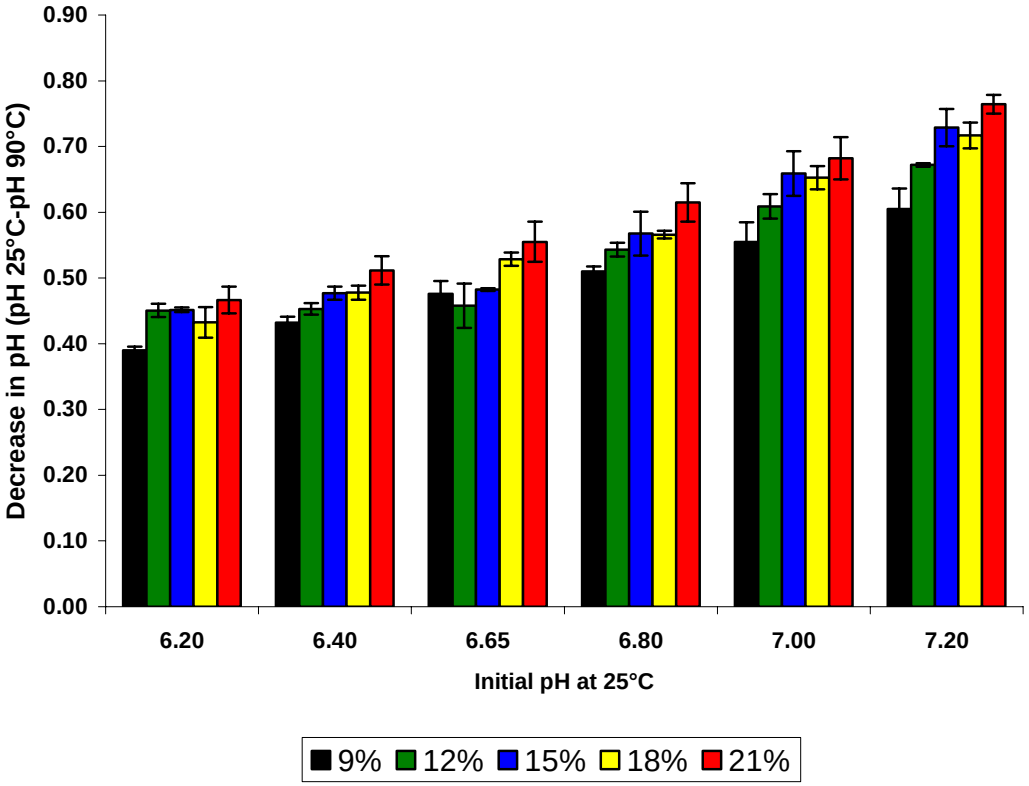


Figure (2.4): The decrease in pH on heating from 25°C to 90°C as a function of initial pH and milk concentration (9% - 21% w/w MSNF)

The magnitude of the decrease in pH at 90°C increased both as a function of increasing initial pH at 25°C and increasing milk concentration from 9%-21% w/w MSNF. The pH

measurements at 120°C followed the same trends as at 90°C (Table D.1 in Appendix D). As illustrated in Chapter 1, Section (1.7.1.1), the changes in pH during heat treatment is primarily due to the changes in minerals in the serum phase. It was found that the reconstituted (in water) casein micelle dispersions (CMD) heated at 95°C in the absence of serum showed no change in pH highlighting the minor contribution from the components of the micellar phase to the change in pH upon heating (Le Ray *et al.* 1998). Van Boekel *et al.* (1989) also observed that the whey proteins present in the aqueous phase did not contribute to the change in pH during heating. The decrease in pH during heating is qualitatively consistent with the changes that occur to calcium and phosphate (Chaplin & Lyster, 1988; Pouliot *et al.* 1989*a*; Singh, 2004). The solubility of calcium phosphate in milks decreases with increasing temperature leading to the precipitation of calcium phosphate (Eq 1). This process is in accord with the observation of progressive transfer of Ca and P_{in} from the aqueous phase to the colloidal phase during thermal processing (Hardy *et al.* 1984; Nieuwenhuijse *et al*. 1988; Pouliot *et al.* 1989*a*; Zhang & Aoki, 1996; De la Fuente *et al.* 2002; Singh, 2004). During heating, precipitation of calcium phosphate results a re-equilibration between the HPO_4^{2-} and $H_2PO_4^-$ in the serum phase (Eq 2), which produces H^+ ions and results a decrease in pH.

$$Ca^{2+}{}_{(aq)} + 0.7\,HPO_4^{2-}{}_{(aq)} + 0.2PO_4^{3-}{}_{(aq)} \leftrightarrow Ca\left(HPO_4^{2-}\right)_{0.7}\left(PO_4^{3-}\right)_{0.2(s)} \qquad \text{Eq 1}$$

$$H_2PO_4^-{}_{(aq)} \leftrightarrow HPO_4^{2-}{}_{(aq)} + H^+{}_{(aq)} \qquad \text{Eq 2}$$

$$HPO_4^{2-}{}_{(aq)} \leftrightarrow H^+{}_{(aq)} + PO_4^{3-}{}_{(aq)} \qquad \text{Eq 3}$$

Our results showed an increased magnitude of the decrease in pH during heating as a function of milk concentration; the higher the milk concentration, higher the decrease in pH on heating from 25°C to 90°C. The changes in free Pin and serum calcium when the milk concentration is increased from 9% to 18% w/w MSNF are described in Appendix E. The amounts of free phosphate ions present in the aqueous phase were greater with increase in milk concentration at a given pH. This will lead to a greater re-equilibration of phosphate ions in the aqueous phase during heating and hence a greater release of H^+ ions, which will result in an increased pH change from 25°C to 90°C.

Chaplin & Lyster, (1988) stated that the dissolved phosphates are present partly as $H_2PO_4^-$ & HPO_4^{2-}, but the solid material formed during heating will contain only PO_4^{3-} or possibly HPO_4^{2-}. However as described in Chapter 1-Section (1.7.1.1), it is not yet fully elucidated whether this newly insolubilised calcium phosphate is similar to the native form or may be attributed to another form of calcium phosphate. The composition of calcium phosphate, its dissolution and the amount of calcium bound directly to casein remained largely unaffected by heating below 90°C provided heating was followed by sufficiently prolonged cooling (Pouliot *et al.* 1989*b*; de la Fuente *et al.* 2002). Our XRD profiles shown in Figure (2.5) resulted in no sharp peaks, which demonstrate that the form of the calcium phosphate formed during heating was amorphous and not crystalline. This is in accord with other studies (Lyster *et al*. 1984; Holt *et al.* 1996). However, the ability to detect non-crystallinity of a solid in a mixture of crystalline and amorphous materials depends on the experimental techniques used. If a higher proportion of non-crystalline material is present in comparison

to crystalline material, the techniques would have not distinguished between each other, except to give an indication of the bulk phase (Lyster *et al.* 1984).

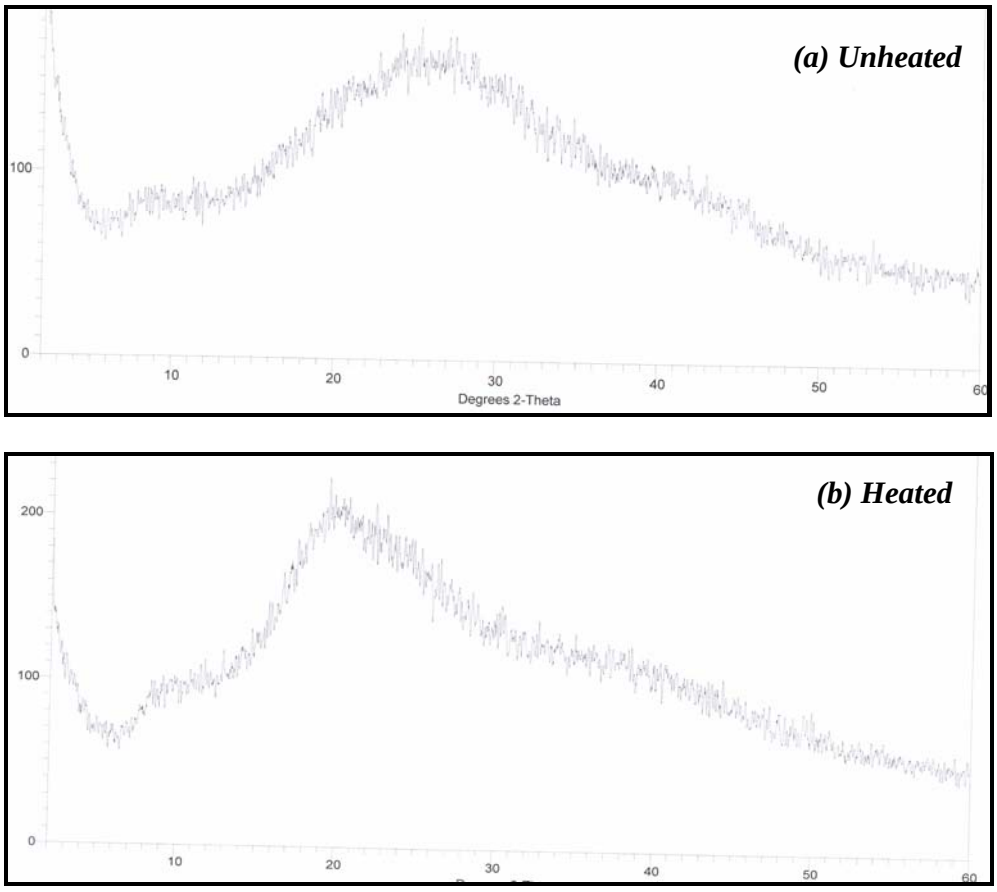


Figure (2.5): XRD patterns of (a) unheated (b) heated 9% w/w MSNF milk solutions at pH 7.2

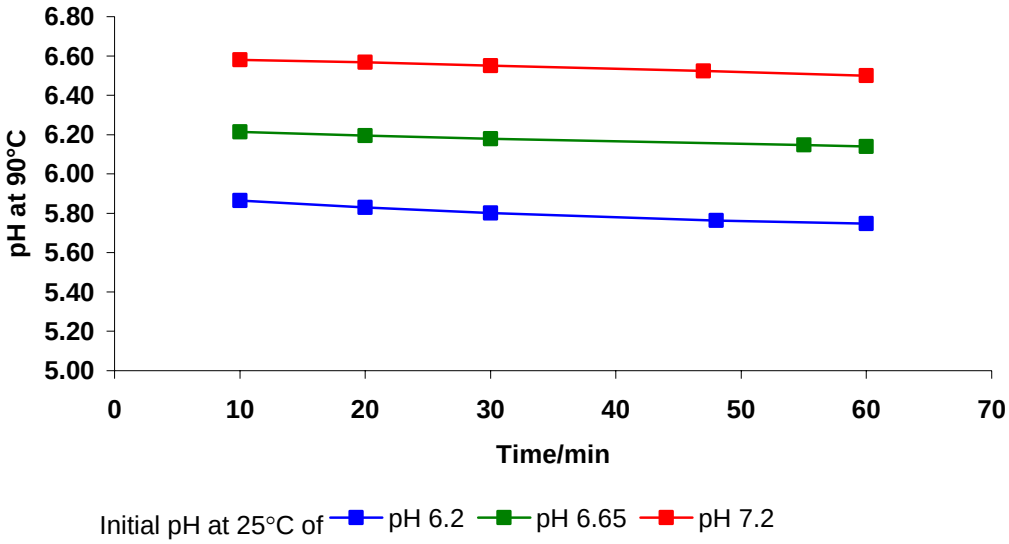
Some changes during heating are reversible and others are irreversible (Chapter 1-Section 1.7.1) and can lead to a reduction of the nutritional value and of the technological properties of milk (Le Ray *et al.* 1998). In the present study, the pH changes were largely restored after overnight equilibration at 4°C for skim milk solutions studied. Table (2.4) demonstrates, that at low initial pH values the change in pH was completely reversible

irrespective of milk concentration. However, at higher values of pH the change in pH was not completely reversible. But the reversibility increased with increase in milk concentration (Table 2.4). So whatever the changes that takes place at high pH values for concentrated milks did not affect the reversibility. The results of 9% w/w MSNF solutions in the present study are in accordance with Pouliot *et al.* (1989*b*) where they showed a 90-95% recovery of changes in partitioning of Ca and P_{in} of single strength milks heated to 85°C for 40 min on subsequent cooling at 4°C for 24 hrs.

Although the decrease in pH on heating has been known, the actual changes were unknown. The decrease in pH on heating had been previously reported as the difference in pH between the initial pH at 25°C before heating and pH at 25°C after the heat treatment (Pouliot *et al.* 1989*a*; Van Boekel *et al.*1989). Hence, the magnitude of the decrease in pH on heating from 25°C to 90°C in the present study was greater than reported previously. In the present study, the pH measured at 90°C was used to evaluate the magnitude of the pH decrease, in contrast to other studies, where the immediate reversibility of the changes was monitored. Due to the reversibility of the pH changes, measuring the pH after the heat treatment might have established a new equilibrium and therefore does not represent the actual system during heating. Our study represents a more realistic representation of the magnitude of the pH changes during heating. Thus allow to control and maintainence of the desired pH values during processing in order to avoid instability.

The pH measurements at 90°C and at 120°C as a function of time for up to 60 and 45 min respectively for 9% w/w MSNF milk solutions with initial pH values of 6.2, 6.65 and 7.2 are presented in Figure (2.6).

(a)



(b)

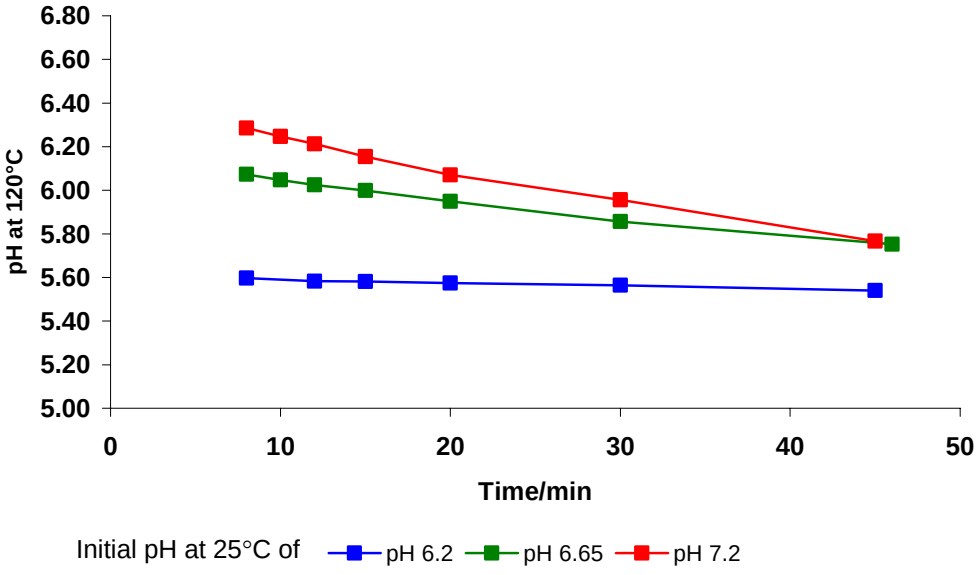
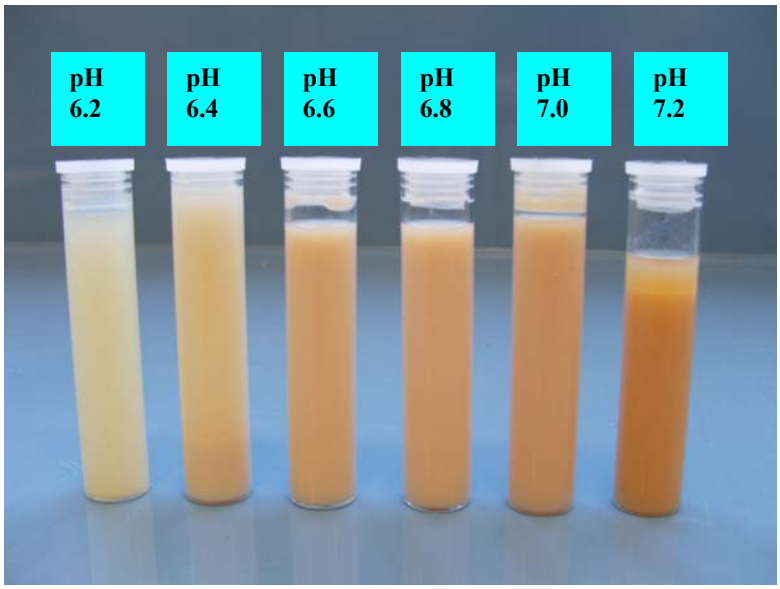


Figure (2.6): The pH (a) at 90℃ (b) at 120℃ as a function of time for 9% w/w MSNF solutions at initial pH values of 6.2, 6.65 and 7.2

The change in pH on reaching 90°C was instantaneous with minimal changes on prolonged heating (Figure 2.6*a*). Once 90°C had been reached, further heating at 90°C to a time period of 60 min resulted in no further change in pH (Figure 2.6*a*). However, at 120°C and pH ≥ 6.65, there was a gradual decrease during prolonged heating at 120°C after the initial decrease of pH while approaching 120°C (Figure 2.6*b*). Van Boekel *et al.* (1989) obtained pH at 25°C after the heat treatment at 130°C for 10 min, and attributed the initial decrease in pH to calcium phosphate precipitation and the further decrease due to the formation of the acids. The present data clearly show an occurrence of two-processes, where the first process is fast and independent on time whereas the second process is dependent of time. Pouliot *et al. (*1989*a*) measured the calcium and phosphate contents in ultrafiltration permeate obtained from milk solutions during heating. They found that, there was a steep decrease in concentration of both calcium and phosphate while heating to 90°C followed by a slower and a smaller decrease with time held at 90°C. This is in accordance with the pH changes in the present study. Chaplin & Lyster, (1988) pointed out that on prolonged heating at temperatures above 85°C, the formation of less soluble calcium phosphates can be expected. However, Visser *et al.* (1986) interpreted the new form of calcium phosphate formed during heating (15 min at 100°C) as a change from an amorphous to a crystalline form at higher temperatures. But our XRD data (Figure 2.5) showed that no obvious phase change was observable upon heating to 90°C for 10 min.

The increase in intensity of browning in 9% w/w MSNF milk solutions heated at 120°C as a function of initial pH can be clearly seen in picture (2.1). As the initial pH increases, the intensity of browning of the milks increased (Picture 2.1). The gradual decrease observed at

120°C for milk solutions at pH ≥ 6.65 on prolonged heating may be partly due to the occurrence of the Maillard reaction as the rate of the Maillard reaction increases with increase in initial pH (Van Boekel *et al.* 1989). Hence, the increased browning with increase in pH confirms the increased occurrence of the Maillard reaction at high pH. However, the other changes such as dephosphorylation of caseins, which increases with increase in pH (Dalgleish *et al.* 1987), lactose degradation and lactulose formation (Castro *et al.* 1986) cannot be ruled out completely for the gradual decrease in pH on prolonged heating (Figure 2.6*b*).



Picture (2.1): The browning due to Maillard reaction as a function of initial pH for 9% w/w MSNF milk solutions heated at 120℃ for 45 minutes

2.3.1.2 The Change of Calcium Activity with Temperature

Calcium activity is as important as pH in determining mineral equilibria during heating. However, previous studies have been limited to measuring calcium activity at 25°C after cooling the heated milks. In contrast, in the present study the calcium activity was measured at a higher temperature (60°C) without cooling.

The results of calcium activity at 25°C prior to heating, at 60°C and the magnitude of the decrease in calcium activity on heating from 25°C to 60°C for 9% - 21% w/w MSNF milk solutions as a function of initial pH of 6.2-7.2 at 25°C are given in Table (2.6). The calcium activities of 9%-21% w/w MSNF milk solutions at unadjusted pH values are given in bold italic in the table. For unheated milks, the calcium activity decreased with increasing milk concentration and with increasing initial pH at 25°C. These results are in accordance with others (Nieuwenhuijse *et al.* 1988; Van Boekel *et al.* 1989; Augustin & Clark, 1990, 1991; De la Fuente, 1998; Lin *et al.* 2006), and can be explained by the complex equilibrium as described in Sections (1.5; 1.7.1.1; 2.3.1.1). As pH decreases, the dissolution of calcium phosphate from the colloidal phase to the aqueous phase occurs (Van Hooydonk *et al.* 1986; Dalgleish & Law, 1989; Goddard & Augustin, 1995). This leads to an increase in the amount of Ca^{2+} ions in the serum, which results in an overall increase in calcium activity.

Table (2.5): The changes in calcium activity as a function of pH at 25°C for 9%-21% MSNF milk solutions

Sample	Initial pH at 25°C	Ca activity at 25°C (Before heating)	Ca activity at 60°C	Decrease in Ca activity ($aCa^{2+}_{25°C} - aCa^{2+}_{60°C}$)
9%	6.25 ± 0.00	1.136	0.584	0.552
	6.41± 0.02	0.833	0.311	0.522
w/w	***6.65± 0.02***	***0.636***	***0.180***	***0.456***
	6.83± 0.02	0.516	0.110	0.406
MSNF	7.02± 0.02	0.422	0.072	0.350
	7.21± 0.02	0.360	0.045	0.315
12%	6.22±0.03	0.948	0.436	0.512
	6.44±0.01	0.717	0.264	0.453
w/w	***6.55±0.03***	***0.635***	***0.242***	***0.393***
	6.84±0.00	0.435	0.086	0.349
MSNF	7.03±0.02	0.367	0.045	0.322
	7.23±0.01	0.286	0.030	0.256
15%	6.23±0.00	0.787	0.281	0.506
	6.42±0.01	0.581	0.159	0.422
w/w	***6.50±0.00***	***0.462***	***0.099***	***0.363***
	6.84±0.00	0.398	0.068	0.330
MSNF	7.03±0.02	0.322	0.039	0.283
	7.24±0.00	0.269	0.025	0.244
18%	6.23±0.03	0.710	0.254	0.456
	6.46±0.00	***0.531***	***0.140***	***0.391***
w/w	6.61±0.03	0.406	0.082	0.324
	6.81±0.02	0.335	0.042	0.293
MSNF	7.04±0.02	0.259	0.030	0.229
	7.21±0.02	0.214	0.020	0.194
21%	6.23±0.01	0.601	0.169	0.432
	6.43±0.01	***0.511***	***0.135***	***0.376***
w/w	6.63±0.02	0.371	0.056	0.315
	6.80±0.01	0.335	0.049	0.286
MSNF	7.01±0.02	0.254	0.024	0.230
	7.21±0.02	0.197	0.017	0.180

Mean values of (n = 3) analyses
Pooled s.d. = 0.024
The skim milk solutions at their unadjusted pH values are given in bold italic in the table.

The calcium activity decreased on heating which is in line with other authors (Geertz *et al.* 1983; Nieuwenhuijse *et al.* 1988; Vanboekel *et al.* 1989; Augustin & Clark, 1990; De la Fuente *et al.* 2002; Singh, 2004; Lin *et al.* 2006). The Ca^{2+} ions in the serum phase at 25°C will be transferred to the colloidal phase with the formation of calcium phosphate precipitate as discussed in Sections (1.5; 1.7.1.1; 2.3.1.1). This will result in a decrease in calcium activity during heating.

Figure (2.7) reports the decrease in calcium activity on heating from 25°C to 60°C with respect to milk concentration (9% to 21% w/w MSNF) and with respect to initial pH at 25°C before heating from pH 6.2 to 7.2. The decrease in calcium activity on heating from 25°C to 60°C (Δcalcium activity $_{(25°C - 60°C)}$) was defined as the difference between the calcium activity at 25°C before heating and calcium activity at 60°C (measured at 60°C). The magnitude of the decrease in calcium activity upon heating from 25°C to 60°C, was lower with increase in milk concentration and increase in initial pH at 25°C (Figure 2.7). This is the opposite behavior to the decrease in pH during heating where a larger decrease with increasing milk concentration and increasing initial pH was observed. However at equivalent pH, there are lesser amounts of calcium ions present in the aqueous phase of more concentrated systems (ref: Table E.2), which results in less transfer of calcium ions from the serum phase to the colloidal phase during heating and hence results in a lower decrease in calcium activity as milk concentration increases as well as when the initial pH of the milk solution increases.

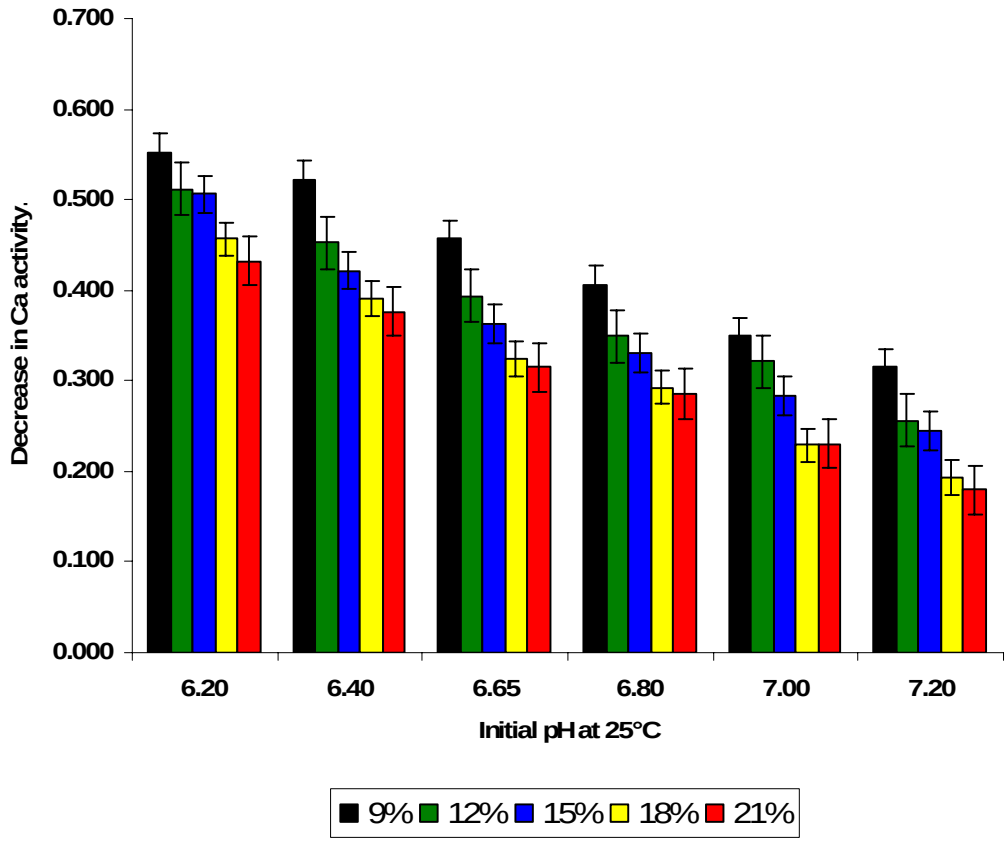


Figure (2.7): The decrease in calcium activity upon heating from 25 ℃ to 60 ℃ as a function of initial pH at 25 ℃ and milk concentration

The heat induced (90°C/10min) calcium activity changes were largely reversible when enough time was given for equilibration. Augustin & Clark, (1991) found that heat induced changes in calcium activity are reversible upon cold storage of milk that has been heated at temperatures up to 85°C provided enough time was allowed for equilibration. Thus, the present study (Table 2.6) is in line with those observed by Augustin & Clark, (1991).

Table (2.6): The reversibility of the changes in calcium activity as a function of pH at 25°C for 9%-21% MSNF milk solutions

Sample	Initial pH at 25°C	Ca activity at 25°C (Before heating)	Ca activity at 25°C (after heating)
9%	6.16	0.990	0.981
	6.43	0.803	0.795
w/w	6.66	0.610	0.607
	6.82	0.489	0.524
MSNF	7.01	0.432	0.433
	7.20	0.377	0.379
12%	6.23	0.890	0.895
	6.44	0.701	0.699
w/w	6.61	0.543	0.555
	6.80	0.424	0.419
MSNF	7.02	0.352	0.367
	7.24	0.276	0.270
15%	6.25	0.710	0.699
	6.41	0.580	0.591
w/w	6.56	0.458	0.469
	6.80	0.361	0.369
MSNF	6.99	0.299	0.288
	7.22	0.251	0.256
18%	6.26	0.682	0.690
	6.49	0.482	0.487
w/w	6.60	0.382	0.386
	6.81	0.317	0.316
MSNF	6.99	0.282	0.269
	7.20	0.239	0.236
21%	6.23	0.591	0.601
	6.44	0.483	0.482
w/w	6.65	0.389	0.390
	6.85	0.311	0.315
MSNF	7.01	0.269	0.277
	7.25	0.231	0.235

Mean values of (n = 3) analyses
Pooled s.d. for calcium activity = 0.019
Pooled s.d. for pH = ± 0.02

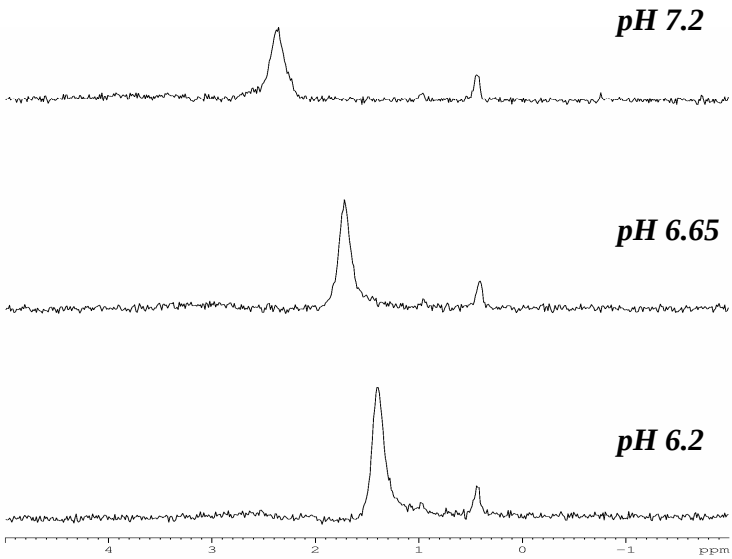
2.3.2 ^{31}P NMR Measurements at High Temperatures

^{31}P NMR provides a means of detecting and separating the signals from the different phosphorylated compounds present in milk since each attached phosphate displays a characteristic chemical shift according to the chemical nature and structure of the compound it belongs to (Belloque *et al.* 2000). It investigates the chemical nature of phosphorylated molecules based on the interpretation of three main spectral features. They are the chemical shift which gives information of local environments of the phosphorus nuclei, the nuclear spin-spin coupling patterns which gives an identity of neighboring groups and the line widths which reveals the effect of dynamic processes. The intra and inter molecular exchange between non equivalent sites and the chemical exchange processes which lead to an averaging of the chemical shifts of the various forms are responsible for the line broadening of the peaks (Ho *et al.* 1969). Phosphorus in milk is present in a variety of forms including P_{in}, which may be associated with calcium both in the serum and micellar phase and SerP which are associated with caseins and sugar phosphates (Belloque *et al.* 2000).

Owing to the low sensitivity and slow relaxation of the phosphorus nuclei, acquiring quantitative NMR spectra is a lengthy procedure. However, a quantitative analysis of various phosphorus components at high temperatures by ^{31}P NMR had not yet been developed but information on changes to the speciation of the phosphorus component is possible.

The ^{31}P NMR spectra of 9% w/w MSNF skim milk at 30°C as a function of initial pH of pH 6.2, 6.65 and 7.2 and 9% w/w MSNF skim milk at pH 6.65 as a function of temperature of measurement are depicted in Figure (2.8*a*) & Figure (2.8*b*) respectively. The ^{31}P NMR spectra of a phosphate buffer solution at an initial pH value of 6.65 at 25°C as a function of temperature are presented towards the side of Figure (2.8*b*). There were mainly four peaks observed in the spectra at 25°C as reported by early literature (Belton *et al.* 1985; Wahlgren *et al.* 1986; Belloque *et al.* 2000; Ishii *et al.* 2001; Belloque & Ramos, 2002; Hubbard *et al.* 2002; de la Fuente *et al.* 2004). Andreotti *et al.* (2006) also found these peaks in buffalo milk and they concluded that these milks are similar to bovine milks with respect to low molecular weight molecules containing phosphorous. However, the exact chemical shifts were different. This is in part because of the variation in pH of the milks studied in different works. Within the present study, the repeatability of the spectra was good.

(a)



(b)

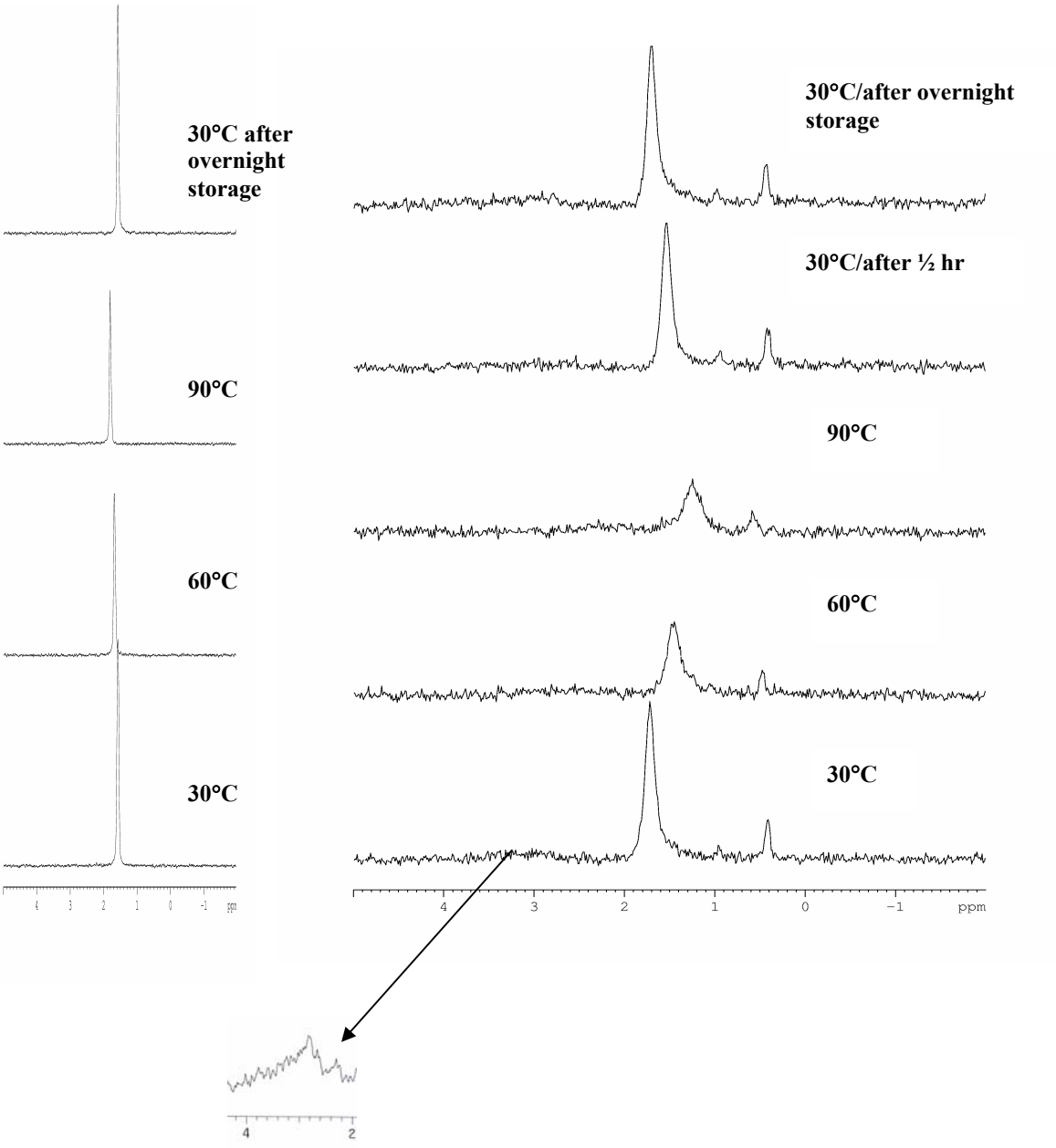


Figure (2.8): (a) Overlayed ^{31}P NMR spectra of 9% w/w MSNF milk solution at 30℃ as a function of pH from 6.2 to 7.2 (b) Overlayed ^{31}P NMR spectra of 9% w/w MSNF milk solution at initial pH of 6.65 at different temperatures. The sharp signals were assigned as follows; P_{in} (1.72ppm), Glycerophosphorylethanolamine (GPA) (0.97ppm), Glycerophosphorylcholine (GPC) (0.43ppm). The SerP appeared as a broad signal between (2-4 ppm) (* The same milk sample was heated and cooled)

LHS – Overlayed ^{31}P NMR spectra of a phosphate buffer solution at pH 6.65 as a function of temperature

The sharp peak at 1.72 ppm was assigned as $P_{in.}$ This peak was dependent both on temperature and pH (Figure (2.8*a*) & Figure (2.8*b*)). Increasing pH at lower temperatures shifted the resonance downfield (Figure (2.8*a*)) whereas increasing temperature shifted the resonance upfield and caused the peaks to broaden (Figure (2.8*b*)). Our results supported the reports by others, which showed the pH dependency of the chemical shift in the ^{31}P NMR spectrum (Belton *et al.* 1985; Mariette *et al.* 1993; Ishii *et al.* 2003). They explained that the chemical shift depends on the state of ionization of the P forms. Changing pH results in changes in the equilibria between various phosphate species (Calcium phosphate nanoclusters, PO_4^{3-}, $H_2PO_4^{2-}$, HPO_4^{-}) (Philippe *et al.* 2005). At high pH, the more basic species predominates and this is consistent with the downfield shift in resonance peaks of the ^{31}P NMR spectrum of milk samples (Sleigh *et al.* 1983). The upfield shift on increasing the temperature is related to the decrease in pH on heating and the increased proportion of acidic phosphate species.

Downfield shift – ***as pH increases***

$$H^+_{(aq)} + HPO_4^{2-}{}_{(aq)} \rightleftharpoons H_2PO_4^-{}_{(aq)}$$

Upfield shift – ***as temperature increases***

The comparison of the P_{in} peaks as temperature increases showed that the line width for P_{in} was much narrower at lower temperatures whereas it broadens with temperature. In general, there are two reasons for the broadening of a NMR signal. They are the molecular exchange between non-equivalent sites, which lead to an averaging of the chemical shifts due to different species forms and/or the formation of high molecular weight complexes with less mobility (Ho *et al.* 1969). Wahlgren *et al.* (1990) observed a line broadening of the ^{43}Ca

signal in heated milks. Belton *et al.* (1985) found a significantly greater P_{in} width with milk solutions containing caseins and concluded that it is probably because of the binding or chemical exchange with the casein micelles. In the present study, it was found that the width of the P_{in} peak in milk was greater than in buffer systems indicating the chemical exchange with other components present in milk.

In the present study, broadening has been attributed either to formation of more than one species, which are in dynamic equilibrium with each other or to the formation of calcium phosphate attached to a phosphoserine residue in a protein molecule. The line broadening could arise from the exchange rates for such complexes being in the intermediate regime, being too slow to give a sharp signal and too fast to be resolved into two distinct peaks. The signal intensities and line widths varied with the environment and were affected by the redistribution of calcium and phosphate during heating. The width of the P_{in} peak in buffer systems did not change as temperature was increased. In this study, even though CCP is formed on heating, the conformational freedom of the P_{in} seems to be retained during heating. Holt & Hukins, (1991) suggested that the calcium phosphate in milk comprises small clusters of Ca^{2+} & HPO_4^{2-} in a fairly disordered state. These clusters are linked through SerP in caseins (Aoki *et al.* 1987; Aoki, 1989). Multinuclear NMR spectroscopy of solutions containing calcium phosphate nanoclusters and phosphopeptides showed line broadening of the P_{in} due to the dynamic exchange of the nanoclusters with the peptides and small ions in the solutions (Holt *et al.* 1996). It was further stated that the calcium phosphate is linked to the proteins through its sequence of phosphorylated residues but these phosphopeptides with calcium phosphate nano clusters bound retain the conformational freedom as was in their unbound state (Holt *et al.* 1996). Hence, it can be

suggested that the calcium phosphates formed during heating in milk solutions may be of some sort of a form that are attached to a single protein apart from casein micelles that is small enough to be detectable with NMR spectroscopy, even though there is no direct evidence found in this present study.

There was a broad peak around 2-4 ppm, which was assigned to SerP that are in a mobile state. This consists of multiple peaks averaged within the range. Using magic angle spinning and cross polarization of native casein micelles, Thomsen *et al.* (1995) showed that most of the SerP residues are immobilized and most likely by interactions with calcium phosphates. They suggested that the SerP are directly involved in colloid formation and that the inorganic constituents of CCP may be ascribed to hydroxyl apatite and an immobile component leading to a somewhat broader resonance. The SerP residues in the same casein molecule but in different locations give rise to different resonances in the spectrum. That is mainly because of the differences in pK_a's of different phosphoserines (Baumy *et al.* 1989). The SerP peaks also behave the same as the P_{in} peak with respect to temperature and pH. Belloque & Ramos (2002) used ^{31}P NMR to analyze the casein content of milk by quantification of the area under the resonances corresponding to SerP of caseins. They observed that there was no alteration of the casein content by heating to 95°C for 15 min. They further observed that the content of proteins precipitatable at pH 4.6 (i.e. casein and denatured whey proteins) determined by the Kjeldahl method was higher than the protein content obtained through ^{31}P NMR. This was because of the fact that most whey proteins associating with caseins on heating are included in the Kjeldahl method when pH 4.6 precipitation is used. Hence, it was concluded that bound whey proteins did not interfere with the ^{31}P NMR measurements (Belloque & Ramos, 2002).

The two upfield peaks at 0.97 ppm and at 0.43 ppm originate from glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC) respectively as assigned in early literature. The GPC peak was reported to be independent of pH in the pH range of 5.8 to 7.3 (Belton *et al.* 1985). However, our results showed a slight variation in chemical shift with increasing temperature. The change in chemical shift of GPA peak as a function of temperature is hard to distinguish due to the overlapping of the P_{in} peak as temperature increases.

All the resonance changes of milk solutions in the present study were largely reversible upon cooling if enough time was given. Almost 90% were recovered within 30 min after cooling. The NMR spectra showed a partial reversibility with 9% skim milk solutions at higher pH values at 25°C, in comparison to full reversibility at lower pH values at 25°C. This is in accordance with the pH measurements described in Section (2.3.1.1).

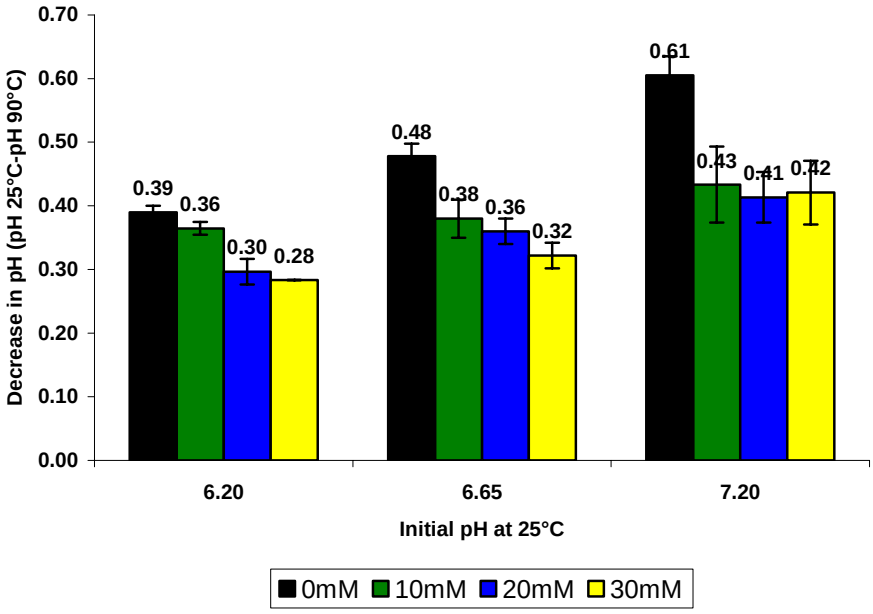
2.3.3 Skim Milk Solutions with Added Calcium Chelating Agents (P_{in} & EDTA)

2.3.3.1 The Change of pH with Temperature

Previous work in the same laboratory (McKinnon *et al.* 2008) examined the effect of added calcium (10mM). It was found that the addition of calcium increased the change in pH on heating. In this work, different amounts (10-30mM) of calcium chelating agents such as P_{in} and EDTA were added to milk solutions.

The decrease in pH upon heating of 9% w/w MSNF milk solutions with or without addition of (a) P_{in} or (b) EDTA from 25°C to 90°C as a function of initial pH at 25°C with respect to different amounts of addition of calcium chelating agents are shown in Figure (2.9). The decrease in pH upon heating from 25°C to 90°C was less for skim milk solutions with the addition of calcium chelating agents (P_{in} and EDTA) in comparison to skim milk solutions without the additives at a particular pH. For example, the decrease in pH was 0.43 and 0.45 for milk solutions with added 10mM Pin and EDTA respectively at pH 7.2, whereas the decrease in pH was 0.61 for milk solution without the addition at that particular pH. Chaplin & Lyster, (1988) also showed a smaller decrease in pH on heating to 80°C with EDTA added milk systems compared to that of without the addition of EDTA. Addition of calcium chelating agents, reduces the level of calcium ions available for calcium phosphate precipitation during heating. This leads to a lower pH decrease during heating, as less calcium ions are available for precipitation. Also, the increased buffering capacity due to the additives will result in a lower decrease in pH during heating. In milk solutions containing additional phosphate, the distribution of ions between serum and micellar phases was qualitatively and quantitatively modified showing that the milk systems acquire a thermodynamically more favourable state. These mineral transfers were quantitatively dependent on pH and are related to the state of ionization of the phosphate ions (Gaucher *et al.* 2007). Hence, the influence of the addition of phosphate to the newly established thermodynamical state of the milk systems results in different magnitudes of pH decrease during heating.

(a)



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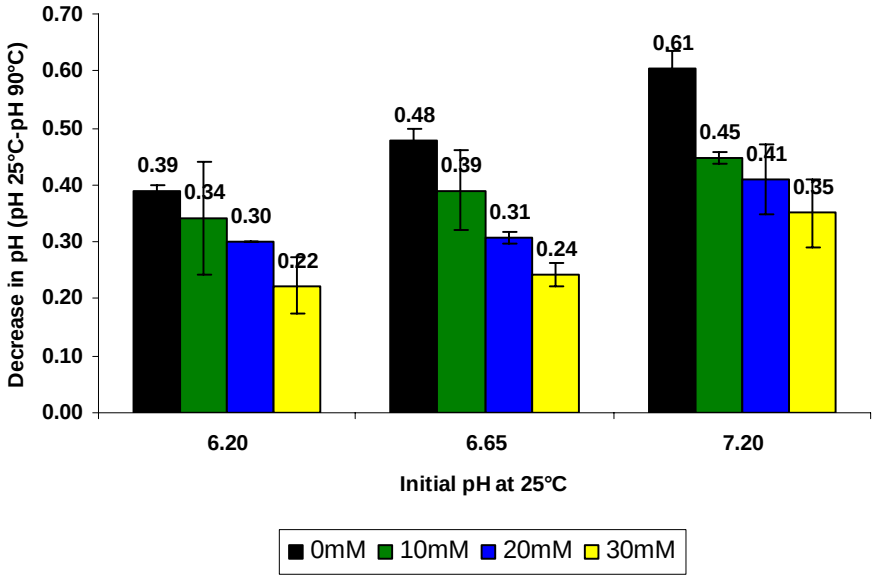


Figure (2.9): The decrease in pH on heating from 25℃ to 90℃ as a function of initial pH at 25℃ with different amounts of (a) P_{in} (b) EDTA added 9% w/w MSNF milk solutions

Two levels of added calcium chelating agents presenting different effects were identified; each indicating different effects. The decrease in pH upon heating from 25°C to 90°C was not markedly different up to 20 mM additions of P_{in} and EDTA for a given pH value at 25°C (Figure 2.9). However, further increase in concentration of added EDTA (>20mM), resulted in a lower decrease in pH upon heating from 25°C to 90°C, than the same amount of added P_{in} to skim milk solution for a given pH at 25°C. EDTA is a much stronger chelating agent than P_{in}. Hence, the calcium availability for the precipitation of calcium phosphate is less in milk solution with added EDTA, thereby resulting in a lower pH decrease on heating compared with milk solution with added P_{in}.

It was found that the pH values were largely restored back when enough time was given for equilibration on cooling as observed in Section (2.3.1.1). In the presence of chelating agents, the reversibility was lower for higher pH value samples than low pH value samples, as was also observed in the absence of calcium chelating agents. Hence, it can be concluded that this lower reversibility happens for higher pH skim milk solutions irrespective of the addition of calcium chelating agents. Udabage, (1999) found that the casein micelles did not reform after addition of ≥20mM of EDTA. But even though the micelles were not restored, the pH was largely reversible indicating the independence of the proteins towards the reversibility of the pH measurements. This favours the hypothesis by Pouliet *et al*. (1989*b*) who stated that the release of salts from the micellar phase during cooling takes place by a different mechanism than the uptake of salts during heating.

The following Figure (2.10) describes the behavior of pH at 90°C as a function of time for 10mM P_{in} added to 9% w/w MSNF skim milk solution at different pH values at 25°C of 6.2, 6.65 and 7.2. The pH change on prolonged heating at 90°C of skim milk solutions with added calcium chelating agents behaved similar to milk solutions without additives (Figure 2.10). Hence, it is important to note that the calcium chelating agents do not influence the mineral equilibria further on prolonged heating.

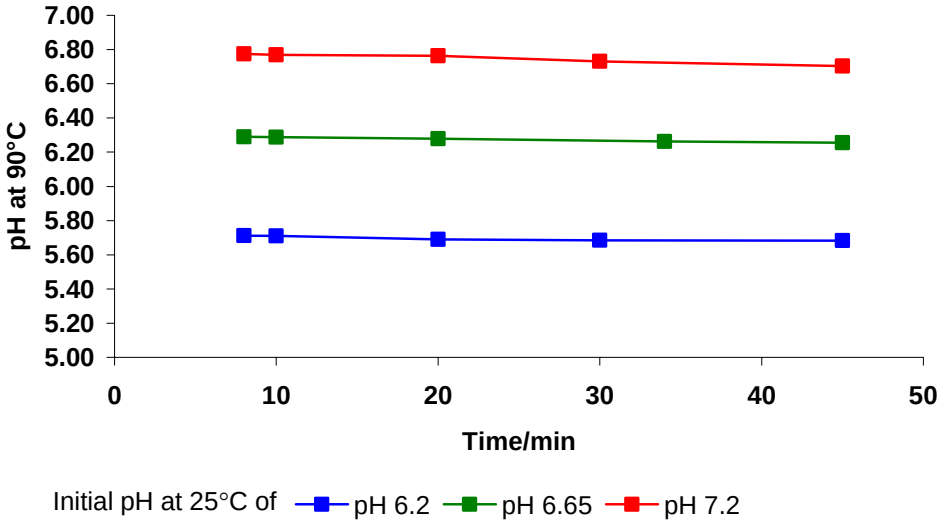


Figure (2.10): The pH at 90℃ for 10mM P_{in} added to 9% w/w MSNF milk solutions as a function of time at pH values of 6.2, 6.65 and 7.2

For a given pH, the amount of added calcium chelating agents did not affect the pH on prolonged heating (Figure 2.11). There were no markedly different trends observed on prolonged heating with the addition of different amounts of calcium chelating agents at a particular pH at 25°C, although there was the dependency of the amount of added calcium chelating agent on the initial decrease in pH upon heating from 25°C to 90°C. The

following Figure (2.11) represents the pH at 90°C as a function of time for 9% w/w MSNF milk solution with different amounts of added P_{in} at pH 6.2.

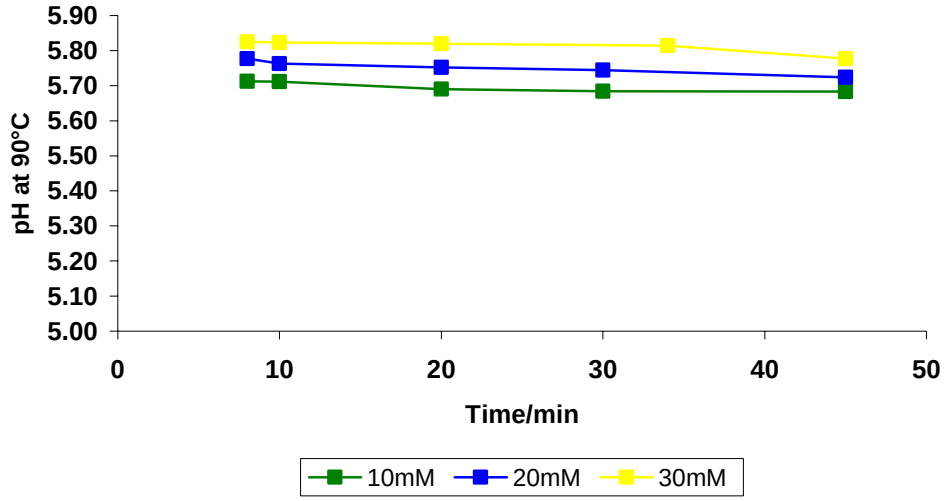


Figure (2.11): The pH at 90℃ for 10mM, 20mM and 30mM P_{in} added 9 % w/w MSNF milk solutions as a function of time at pH value of 6.2

Milk solutions with added EDTA behaved similar to milk solutions with added P_{in} on prolonged heating, where there was no change in pH after the temperature of heating was attained.

2.3.3.2 The Change in Calcium Activity with Temperature

The results of calcium activity at 25°C prior to heating, at 60°C and the magnitude of the decrease in calcium activity on heating from 25°C to 60°C for 9% w/w MSNF milk solutions with/without addition of P_{in} and EDTA as a function of initial pH of 6.2-7.2 at 25°C are given in Table (2.7).

Table (2.7): The changes in calcium activity as a function of pH at 25°C for 9% w/w MSNF milk solutions with/without addition of P_{in} & EDTA

Additions to milk (mmol/kg of milk)	Initial pH at 25°C	Ca activity at 25°C (Before heating)	Ca activity at 60°C	Decrease in Ca activity ($aCa^{2+}_{25°C} - aCa^{2+}_{60°C}$)
None(control)	6.25 ± 0.00	1.136	0.584	0.552
	6.65 ± 0.02	0.636	0.180	0.456
	7.21 ± 0.02	0.360	0.045	0.315
P_{in}				
10	6.23 ± 0.02	1.074	0.541	0.533
20	6.20 ± 0.01	0.575	0.226	0.349
30	6.25 ± 0.01	0.263	0.091	0.172
10	6.68 ± 0.02	0.504	0.071	0.433
20	6.63 ± 0.02	0.354	0.033	0.321
30	6.65 ± 0.01	0.140	0.017	0.123
10	7.20 ± 0.02	0.326	0.023	0.303
20	7.21 ± 0.01	0.255	0.008	0.247
30	7.19 ± 0.02	0.098	0.003	0.095
EDTA				
10	6.24 ± 0.01	0.828	0.453	0.375
20	6.20 ± 0.00	0.453	0.169	0.284
30	6.19 ± 0.02	0.033	0.002	0.031
10	6.65 ± 0.02	0.425	0.119	0.306
20	6.65 ± 0.00	0.325	0.074	0.251
30	6.63 ± 0.02	0.028	0.002	0.026
10	7.18 ± 0.01	0.205	0.019	0.186
20	7.18 ± 0.02	0.180	0.011	0.169
30	7.20 ± 0.01	0.019	0.001	0.018

Mean values of (n = 3) analyses. Pooled s.d. = ± 0.019

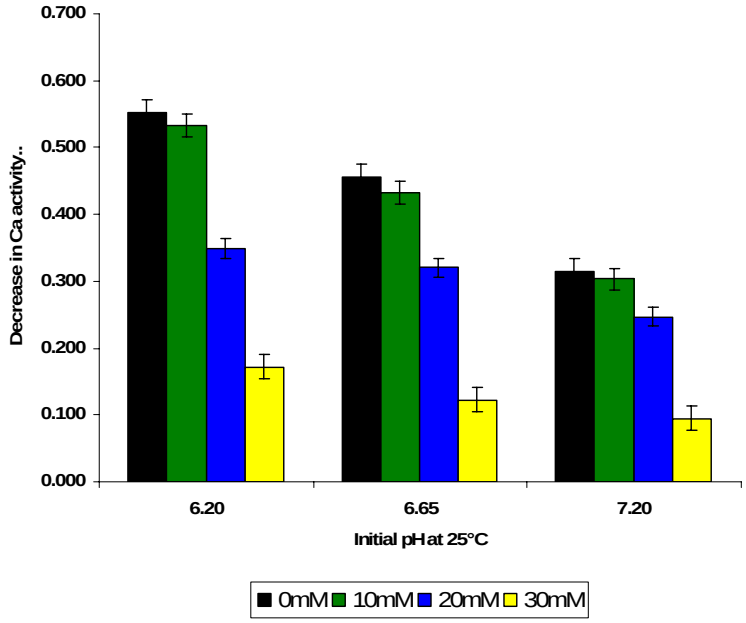
In this study, it was observed that the addition of calcium chelating agents led to a reduction in calcium activity as observed by others due to the complexation of the added calcium chelating agents with calcium (Van Hooydonk *et al.* 1986; Niewenhuijse *et al.* 1988; Udabage *et al.* 2000). Most of the added P_{in} and EDTA remained in the serum fraction (Udabage, 1999; Udabage *et al.* 2000; McKinnon & Chandrapala, 2006). The complexation of the calcium ions with the added phosphates or added EDTA resulted in a decrease in calcium activity compared to milk solutions without additives. This is in line with others (Hardy *et al.* 1984).

The decrease in calcium activity of 9% MSNF milk solutions with different amounts of added of P_{in} and EDTA upon heating from 25°C to 60°C as a function of pH at 25°C is shown in Figure (2.12). The calcium activity decreased with increasing temperature (Table 2.7). However, this decrease in calcium activity upon heating from 25°C to 60°C was lower for systems with added calcium chelating agents in comparison to systems without the added calcium chelating agents for a given pH value. Most of the calcium ions are already complexed with the added agents, which results in less available calcium ions to be precipitated at high temperatures.

Table (2.8): The reversibility of changes in Ca activity for salt added 9% w/w MSNF milk systems

Additions to milk (mmol/kg of milk)	Initial pH at 25°C	Ca activity at 25°C (Before heating)	Ca activity at 25°C (after heating and cooled overnight)
P_{in} 10	6.23 ± 0.02	1.07	1.05
P_{in} 20	6.20 ± 0.01	0.57	0.56
P_{in} 30	6.25 ± 0.01	0.26	0.25
P_{in} 10	6.68 ± 0.02	0.50	0.48
P_{in} 20	6.63 ± 0.02	0.35	0.34
P_{in} 30	6.65 ± 0.01	0.14	0.14
P_{in} 10	7.20 ± 0.02	0.33	0.31
P_{in} 20	7.21 ± 0.01	0.25	0.24
P_{in} 30	7.19 ± 0.02	0.10	0.10
EDTA 10	6.24 ± 0.01	0.83	0.80
EDTA 20	6.20 ± 0.00	0.45	0.44
EDTA 30	6.19 ± 0.02	0.03	0.03
EDTA 10	6.65 ± 0.02	0.42	0.41
EDTA 20	6.65 ± 0.00	0.32	0.30
EDTA 30	6.63 ± 0.02	0.03	0.03
EDTA 10	7.18 ± 0.01	0.20	0.19
EDTA 20	7.18 ± 0.02	0.18	0.18
EDTA 30	7.20 ± 0.01	0.02	0.02

(a)



(b)

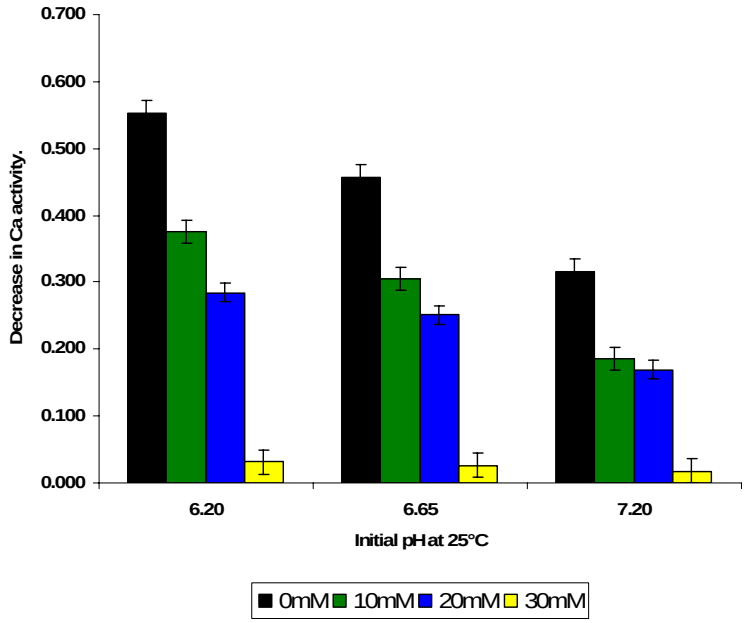


Figure (2.12): The decrease in calcium activity heating from 25 ℃ to 60 ℃ as a function of initial pH for different amounts of (a) P_{in} (b) EDTA added 9% w/w MSNF milk solutions

The decrease in calcium activity upon heating from 25°C to 60°C was lesser for systems with EDTA addition than P_{in} addition at a particular pH. EDTA is a much higher chelator of calcium ions than P_{in}, which will lead to more complexation of calcium ions in the serum. Increasing amounts of added calcium chelating agents resulted in a lesser decrease in calcium activity. Increasing amounts resulted in less calcium ions available for precipitation of calcium phosphate. However, increased amounts of EDTA addition led to a markedly lower drop in calcium activity, which is in accord with the increased disruption of the micelles (Lin *et al.* 1972). In the presence of low concentrations of phosphate, it is evident that phosphate does not displace micellar calcium phosphate because the affinity of calcium is higher for the micellar phosphate (Gaucher *et al*. 2007).

2.4 Conclusion

The pH of the skim milk solutions decreases as temperature increases. The pH decrease on heating from 25°C to 90°C was greater with increase in initial pH (pH at 25°C before heating) and increase in milk concentration. These changes were largely reversible if enough time is given for equilibration.

The decrease in pH on heating from 25°C to 90°C in skim milk solutions with added calcium chelating agents was less than the control skim milk solutions without the addition of chelators for a given pH at 25°C. The decrease in pH on heating from 25°C to 90°C was independent of the type of calcium chelating agent for given concentration of added salts of up to 20mM. However, the decrease in pH on heating from 25°C to 90°C was lower with

the addition of higher levels of >20mM of EDTA than P_{in} at the same pH at 25°C. This highlights the dependence of the type of calcium chelating agent at higher concentrations of addition on the decrease in pH on heating. The decrease in pH on heating from 25°C to 90°C was less when there is a disruption of the casein micelles. The changes in pH on heating from 25°C to 90°C for calcium chelating agents added skim milk solutions were also largely reversible.

The results highlighted the influence of variations in the milk composition on the decrease in pH on heating from 25°C to 90°C. The decrease in pH during heating was very much predictable from the pH at 25°C before heating as long as the milk composition is kept constant. If the mineral composition of the milk varies, the initial pH at 25°C before heating can not be used to predict the decrease in pH during heating.

The pH at 90°C hardly changed on prolonged heating for skim milk solutions at different pH values of pH 6.2, 6.65 and 7.2. The type of calcium chelating agents added and their respective amounts did not affect the pH at 90°C on prolonged heating. Although, the pH at 120°C did not change on prolonged heating at lower initial pHs, at higher initial pH values, there was a gradual decrease in pH on prolonged heating. This indicated the influence of other reactions especially, the Maillard reaction.

The calcium activity of the skim milk systems decreased as temperature increased. This is associated with the formation of calcium phosphate described by the simple chemical equilibria. The calcium activity decrease on heating from 25°C to 60°C was less with

increase in initial pH (pH at 25°C before heating) and increase in milk concentration, in contrast to the greater decrease in pH on heating. These changes were largely reversible if enough time is given for equilibration.

The decrease in calcium activity upon heating from 25°C to 60°C in skim milk solutions with added calcium chelating agents was less than without the addition at the same pH at 25°C. The decrease in calcium activity on heating was lesser with the addition of EDTA than the addition of P_{in} for all the calcium chelating agent concentrations studied. These calcium activity changes were largely reversible.

There was an up field shift with increase in temperature in the ^{31}P NMR spectrum of skim milk solutions, which correlates to the change in pH on heating from 25°C to 90°C. The P_{in} peak showed a much broader and a shorter peak as temperature increases. This is possibly due to the formation of different species of calcium phosphate, which are in dynamic exchange between each other and/or to the formation of calcium phosphate with a single protein attached. These changes in chemical shifts were largely reversible on cooling.

The results suggested that the decrease in pH and mineral equilibria on heating from 25°C to 90°C is a function of the milk composition. Hence, it is interesting to examine how these changes in milk composition with respect to pH and mineral equilibria affects the behavior of the proteins resulting in aggregation leading to heat stability of milks.

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Chapter 3

The Formation and Quantification of Soluble Aggregates: Effect of pH at heating, milk concentration and added calcium chelating agents

3.1 Introduction

Extensive studies have been done focusing on the formation of soluble aggregates with respect to changes in different properties of milk solutions such as pH (Singh & Latham, 1993; Menard *et al.* 2005; del Angel & Dalgleish, 2006; Donato & Dalgleish, 2006; Jean *et al.* 2006; Renan *et al.* 2006), casein micellar structure (del Angel & Dalgleish, 2006; Renan *et al.* 2007), different genetic variants (Donato *et al.* 2007) and protein composition (Dalgleish *et al.* 1997; Donato *et al.* 2007; Guyomarc'h *et al.* 2003). Soluble aggregates are defined as the aggregates present in the supernatant of milk solutions after mild centrifugation conditions. It has been shown that the pH is one of the predominant factors, which governs the formation of these soluble aggregates (Singh & Latham, 1993; Menard *et al.* 2005; del Angel & Dalgleish, 2006; Donato & Dalgleish, 2006; Jean *et al.* 2006; Renan *et al.* 2006). However, there has been no detailed study of the effect of pH *in situ*, milk concentration and added calcium chelating agents on the formation and the composition of the soluble aggregates.

In the present study, the effect of the pH at 90°C on the formation and the composition of soluble aggregates in reconstituted single strength (9% w/w MSNF) and concentrated (12% - 21% w/w MSNF) milk solutions was examined. In addition, the effect of added calcium

chelating agents (P_{in} & EDTA) on the formation and the composition of soluble aggregates on heating 9% w/w MSNF milk solutions for 90°C/10min was investigated. A combination of Size Exclusion Chromatography (SEC) and SDS-PAGE analysis was performed. Emphasis was placed on skim milk solutions at initial pH of 6.20 to 8.00 at 25°C, as this is the region, where the greatest change in association of whey proteins with the casein micelles occurred.

3.2 Materials and Methods

3.2.1 Preparation of Reconstituted Skim Milk Solutions

Reconstituted skim milk solutions with and/or without added calcium chelating agents (P_{in} & EDTA) were prepared and equilibrated as described in Section (2.2.2).

3.2.2 Heat Treatment of Reconstituted Skim Milk Solutions

The heat treatment at 90°C was done as described in Section (2.2.4.1).

3.2.3 pH measurements

The pH measurements at 25°C and at 90°C were done as described in Section (2.2.5).

3.2.4 Ultracentrifugation of Skim Milk Solutions and Measurement of Nitrogen Content

The heated and unheated skim milk solutions were ultracentrifuged at 33,000 g for 60 min at 25°C using a Beckman LK 90 M ultracentrifuge with a type 55.2Ti rotor (Beckman

Instruments Australia (Pty) Ltd, Gladesville, NSW 2111, Australia). At approximately 30 min prior to ultracentrifugation, the concentrated milk solutions (12-21% w/w MSNF) were diluted to 9% by weight with MilliQ water. In some samples (i.e. 21% w/w MSNF, pH at 25°C < 6.4) where soft gels were formed on heating, these were broken by vigorous shaking prior to dilution by using the method adapted by Anema, (2000) to ensure redispersion. The clear supernatant (excluding the opalescent layer) was carefully removed and filtered using a 0.45 μm filter and stored at 4°C until further analysis. These were generally used within 1-2 days after separation. Separate experiments showed that the amount and size of the aggregates did not change over this period. A supernatant sample obtained from 21% w/w MSNF undiluted skim milk solution was used to determine if there was an effect of dilution prior to centrifugation. The results suggested that dilution did not alter the amount and size of the soluble aggregates.

The total nitrogen content of each reconstituted skim milk solution and the corresponding supernatant before and after heat treatment was determined using a LECO FP-2000 Nitrogen Analyser (LECO Australia Pty Ltd, Castle Hill, NSW, Australia). The factor of 6.38 was used to convert nitrogen to protein concentration (IDF 20B: 1993). The protein content was calculated by subtracting the contribution from non proteins of the total milk.

3.2.5 Size Exclusion Chromatography

For comparison of the elution profiles of the supernatants from different skim milk solutions, HPLC-SEC was conducted using a Shimadzu LC 20HPLC system equipped with a UV-VIS detector. Samples (20 μL) of the filtered supernatants were injected in to a

Phenomenex TSK GEL 17 u G5000PW 1000A (Phenomenex Australia Pty LTD, PO box 4084, Lane Cove, NSW 2066, Australia) column and run at 0.5 ml/min in 100mM ammonium bicarbonate buffer at pH 7.0. The elution profiles were monitored at 280 nm. The total run time was 35 mins.

Size Exclusion Chromatography (SEC) was carried out on a larger scale to enable collection of sufficient sample for further analysis of the composition of the aggregates. For this purpose, a 40 ml aliquot of each supernatant was introduced to the column (5 cm diameter and 90 cm long packed with Sephacryl S500HR, Amersham Bioscience Baulkham Hills, NSW, Australia) with a nominal fractionation range of 40-100,000 kDa. Ammonium bicarbonate (100 mM, pH 7) at a flow rate of 3mL/min was used as the running buffer. The column was run on a Pharmacia FPLC system using a LKB 2152 HPLC controller, LKB 2150 HPLC pump and a LKB 2151 variable wavelength monitor at 280 nm set at 150mV full scale. The total elution time for each sample was 1000 min. Fractions were collected between 270-470 min using a LKB Superac 2211 Fraction Collector. Fractions were pooled in groups of three and freeze dried before further analysis using SDS polyacrylamide gel electrophoresis.

The elution profiles obtained using the small and large scales were similar. The results are average of at least duplicate analysis.

3.2.6 SDS Polyacrylamide Gel Electrophoresis

Mono-dimensional SDS-PAGE was used to determine the composition of the soluble aggregates. Precast 4-12% acrylamide gels (NuPage Novex Bis Tris gels, Invitrogen Australia Pty Ltd, Mt.Waverley, Victoria, Australia) were used and electrophoresis was performed under reducing and non-reducing conditions.

Fractions and the relevant supernatants were dissolved in ammonium bicarbonate buffer (100 mM, pH 7) and in MilliQ water respectively to a crude total protein content of 2.5 mg/mL. For reduced gels, samples (25µL) were mixed with 20µL of NuPage LDS sample buffer at pH 8.4 and 5µL of 1mM dithiothreitol. For non-reduced gels, 5µL MilliQ water was used in place of the reducing agent. Samples of sodium caseinate and Whey Protein Isolate (WPI) were run as standards. All samples were heated at 70°C for 10 min in order to ensure complete reduction. Aliquots (10µl) of the heated samples were applied to the gels and run at 125 V for 10 min then at 180 V for further 50 min. The running buffer used was NuPage MES (Morpholino Ethane Sulphonic acid) SDS buffer with the composition of 50 mM MES, 50 mM tris base, 0.1% SDS, 1mM EDTA at pH 7.3. The gels were stained using Invitrogen Coomassie Blue staining kit following the manufacturer's instructions. The stained gels were then photographed and the photographs analyzed using Scion Image software. For reduced gels, the integrated intensities of each band were analyzed to determine the relative proportions of the major proteins in the soluble aggregate fraction.

3.3 Results and Discussion

3.3.1 Total Protein Content in Supernatants

3.3.1.1 Skim Milk Solutions of 9 - 21% w/w MSNF

Table (3.1) shows the total protein content in supernatants obtained from unheated 9% w/w MSNF milk solutions as a function of initial pH at 25°C. At pH 6.65, the protein content in supernatant from unheated milk was 20.98 ± 0.26%. As the pH of the unheated milk was increased, the protein content in the supernatant increased linearly with pH up to 27.34 ± 0.29% at pH 7.26. These data are in agreement with Donato & Dalgleish, (2006). Other authors also found an increase in total protein content with increase in pH (Dalgleish & Law, 1989; Anema, 2007). It might be due to the increase in dissociation of all types of caseins. However, the dissociation of κ-casein was found to be dominant with increase in pH, which can result in an increase in the total protein content in the supernatants (Anema & Klostermeyer, 1997*a*,*b*; Anema, 1998; Manard *et al.* 2005; Anema, 2007). Anema & Klostermeyer (1997*b*) had a possible explanation for this dissociation of caseins. They hypothesized that it is due to a pH dependent conversion of the native Colloidal Calcium Phosphate (CCP) to an alternative form of calcium phosphate, which is less capable of maintaining the micellar integrity, particularly at higher pH values where the charges of the proteins are greater.

The exact values were quite different from other works, as most of the other works used very high or low centrifugal forces to study the protein distributions. In this study 33,000g was used. According to Anema, (2007), centrifugal forces around 25,000g are sufficient to

deposit the casein micelle in skim milk. The milder centrifugation conditions (i.e. 33,000g) may be more selective in separating serum phase aggregates from the colloidal phase when compared to the markedly higher forces (> 50,000g) used in earlier studies.

Table (3.1): The total protein content in supernatants obtained from unheated 9% w/w MSNF solutions as a function of initial pH at 25°C

Initial pH at 25°C	Total Protein Content in Supernatant (%)
6.65	20.98 ± 0.26
6.82	22.32 ± 0.32
7.26	27.34 ± 0.29
7.39	28.10 ± 0.21
8.02	32.88 ± 0.45
8.51	37.87 ± 0.42

Mean values & s.d. of (n=4) analyses
Pooled s.d. for pH = ± 0.02

Representative reduced SDS gels for the protein content in supernatants from unheated 9% w/w MSNF milk solutions as a function of initial pH at 25°C from ~6.7 to 7.4 are shown in Figure (3.1). Donato & Dalgleish, (2006) had found that the total protein content in the serums from unheated milk solutions at pH < 6.7 did not show any marked differences with milk solutions at pH 6.7. Hence, the minimum pH of ~6.7 was chosen to determine the total protein content in the serum from unheated milk solutions in the present study. The intensity of the bands of caseins increased as pH increases indicating the increase in

amounts. This is due to the dissociation of caseins, mainly κ-casein, from the casein micelles as described above. The levels of β-Lg and α-La were essentially constant at all pH values in unheated milks (Figure 3.1). This indicates that there was no transfer of whey proteins between micellar and serum phases as pH increased. Higher molecular weight (MW) whey proteins behaved the same as β-Lg and α-La, where they were constant at all pH values in unheated milks (Figure 3.1).

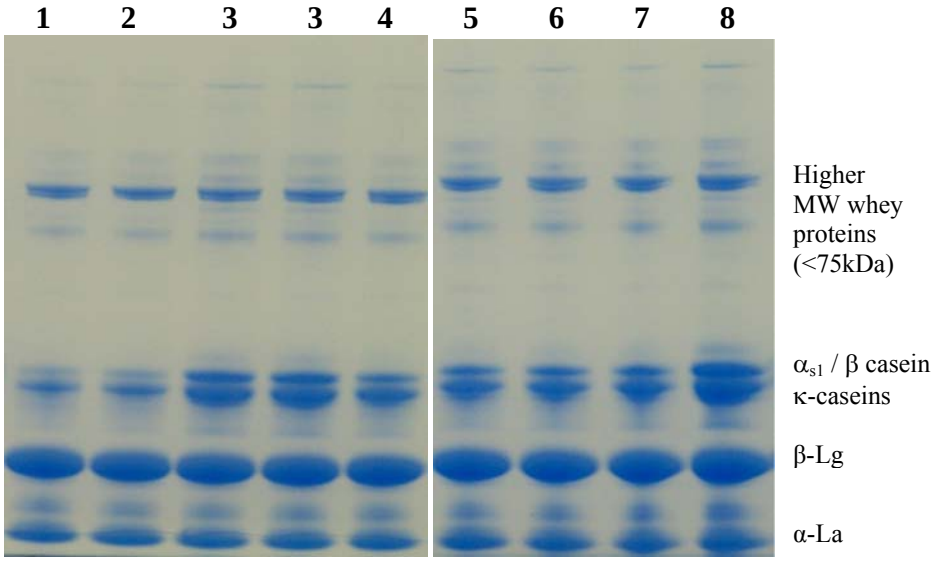


Figure (3.1): Reduced SDS-PAGE patterns of supernatants obtained from unheated 9% w/w MSNF solutions as a function of initial pH at 25℃; Lane 1 – pH 6.75, Lane 2 – pH 6.79, Lane 3 – pH 7.05, Lane 4 – pH 7.26, Lane 5 – pH 6.85, Lane 6 – pH 6.94, Lane 7 – pH 7.14, Lane 8 – pH 7.39

Table (3.1b): The amount of caseins present in the supernatants of unheated 9% w/w MSNF milks as function of initial pH at 25°C

pH	Concentration/mg/ml of supernatant
6.75	1.08
6.79	1.18
6.85	1.42
6.94	1.77
7.04	2.32
7.14	2.79
7.26	3.40
7.39	3.82

The total protein content in supernatants as a function of initial pH at 25°C for heated and unheated 9% w/w MSNF milk solutions are shown in Figure (3.2). At pH 6.65, the protein content in supernatant from unheated milk was 20.98 ± 0.26%. As the pH of the unheated milk was increased to pH 8.51, the protein content in the supernatant increased linearly with pH up to 37.87 ± 0.42% (Figure 3.2).

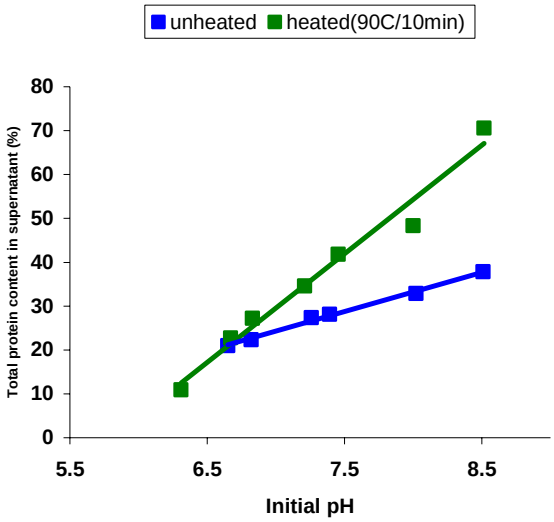


Figure (3.2): The total protein content in supernatants obtained from heated and unheated 9% w/w MSNF milk solutions as a function of initial pH at 25℃

The increase in the total protein content in the heated supernatants between pH 6.3 and 8.5 appeared to be approximately linear with pH at 25°C (Figure 3.2). The increase in the protein concentration with increase in pH is due to the increased dissociation of caseins particularly κ- casein, which is in accordance with others (Nieuwenhuijse *et al.* 1991; Singh, 1994; Anema & Klostermeyer, 1996; Anema & Klostermeyer, 1997*a,b*; Anema, 1998; Menard *et al.* 2005; Donato & Dalgleish, 2006; Anema, 2007; Renan *et al.* 2007). After heating at pH around 6.65, there was no significant change in the total protein content in the supernatant compared to that of unheated milk (Figure 3.2). After heating at lower pH values than pH 6.65, the total protein content in the supernatant decreased. In contrast, the total protein content in the supernatant of heated milk increased with increase in pH above 6.65 (Figure 3.2). In a very recent study by Donato & Dalgleish (2006), the effect of pH on the distribution of protein components between the serum and micellar phases in heated milks was examined. They found that there was no significant change in the total protein concentration in the serum compared to that of unheated milk at pH 6.7, whereas at lower pH values, the protein concentration in supernatant decreased and at higher pH values the protein concentration increased. Our results are in accordance with their observations.

Heat treatment of milk causes denaturation of whey proteins leading to a mixture of whey proteins coated casein micelles and whey protein-whey protein aggregates as discussed in Section (1.7.1.3 in Chapter 1). This casein-whey protein association is highly dependent on pH. This was clearly illustrated by Vasbinder & de Kruif (2003). With respect to association of whey proteins to caseins, there are two possibilities that exists; whey protein forms a complex with micellar κ-casein which dissociates from the micelles, or whey

protein remains in the serum and forms a complex with the dissociated κ-caseins. However, the question of whether this casein-whey protein interaction proceeds or follows the dissociation of κ-casein is still debated. According to Anema, (2007) & van Boekel *et al.* (1989), at pH < 6.65 most of the κ-casein is in the micellar phase and therefore the predominant interaction of the denatured whey proteins is with the κ-casein in the micellar phase. As the pH increased progressively more κ-casein was found in the serum phase and hence there appears to be a preferential reaction between the denatured whey proteins and the serum phase κ-caseins. This may be a consequence of easier access of the disulfide bonds of the serum phase κ-casein than the micellar phase κ-casein for thiol reactions with the denatured whey proteins. However, the interactions will also be diffusion limited and the interactions between denatured whey proteins and the serum phase κ-casein will probably be more rapid than the interactions between denatured whey proteins and the micellar phase κ-casein. As a consequence, when there is a segregation of κ-casein between phases the ratio of whey protein to κ-casein is higher in the serum phase than in the micellar phase (Anema, 2007). Anema & Li, (2003) and Anema *et al.* (2004*a* & 2004*b*) suggested that as the pH is increased, the micelle surface charge will increase, which will cause the hairs of the casein micelles to extend further from the micelle surface. This may reduce the association of denatured whey proteins with casein micelles when the pH is increased and therefore increase the tendency for the serum phase reactions. In contrast, Donato *et al*. (2007) suggested that the reaction between κ-casein and whey proteins in milk heated at 90°C takes place preferentially on the surfaces of the casein micelles rather than in the serum phase. The observation of the preferential reaction between whey proteins

and κ-caseins in the serum phase in the present study are highlighted and discussed in Section (3.3.3) with the results obtained in heated milks with added EDTA.

Figure (3.3) represents the reduced SDS-PAGE patterns of supernatants of heated 9%-18% w/w MSNF milk solutions at their unadjusted pH values. For comparison purpose, unheated 9% w/w MSNF milk solutions at pH 6.65 at 25°C are shown in Figure (3.3).

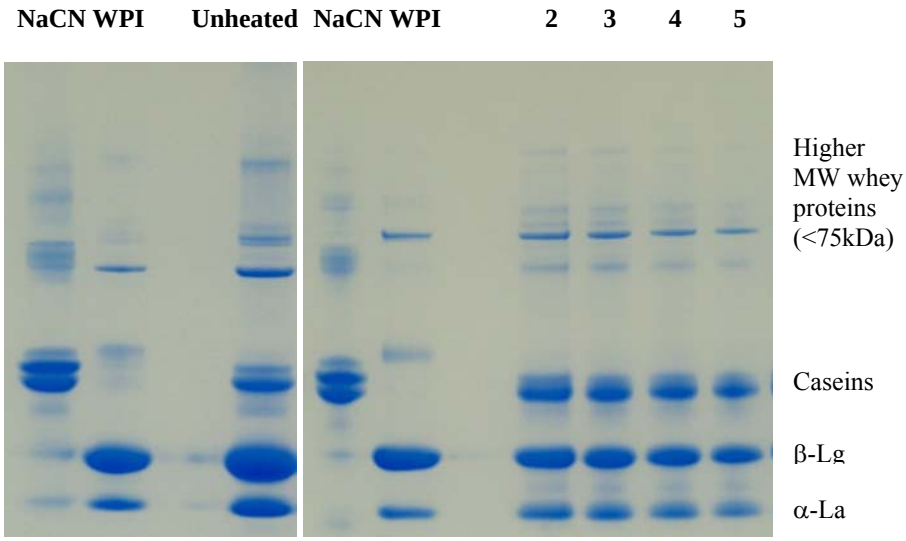


Figure (3.3): Reduced SDS-PAGE patterns of supernatants obtained from heated 9%-18% w/w milk solutions; Lane 2- 9% w/w MSNF pH 6.65, Lane 3- 12% w/w MSNF pH 6.55, Lane 4- 15% w/w MSNF pH 6.50, Lane 5- 18% w/w MSNF pH 6.46. (Lane 1 represents the supernatant obtained from unheated 9% w/w MSNF milk solution at pH 6.65)

As expected, for the heated samples, the levels of β-Lg and α-La were low indicating the transfer to the micellar phase during heating as discussed earlier. However, the levels of whey proteins in supernatants appeared much lower at lower pH values in the present study

(Figure 3.3), which is in accordance with the model put forward by Vasbinder & de Kruif, (2003). It stated that heating at a pH > 6.6 leads to a partial coverage of the casein micelles and the formation of separate whey protein aggregates. Heating at a pH < 6.6 leads to an attachment of increasing amounts of whey proteins to the casein micelles as the pH is decreased. A schematic representation of the model is depicted in Figure (3.4). The high molecular weight species such as BSA, Ig and Lactoferrin followed the same trends as other whey proteins having lower contents in heated milks. This is in accordance with Donato & Dalgleish, (2006).

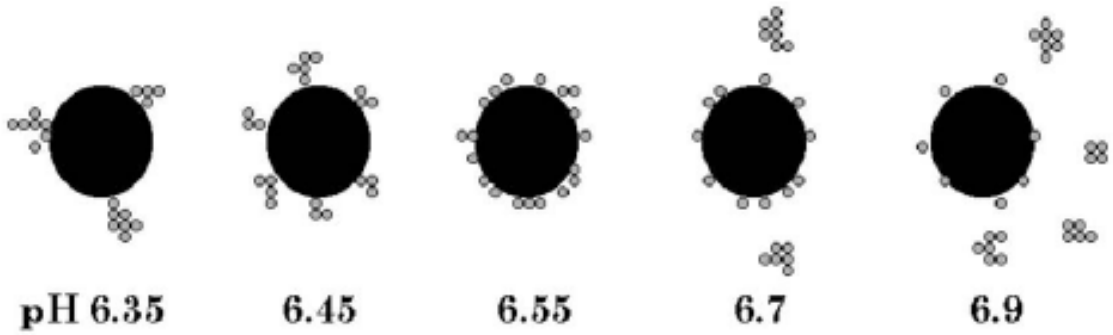


Figure (3.4): A schematic representation of the interaction between casein micelles and whey proteins occurring in milk during heat treatment for 10 min at 80℃ at pH values ranging from 6.35 to 6.9 *(Figure taken from Vasbinder & de Kruif, 2003)*

Table (3.2) shows the total protein content in supernatants obtained from heated 9%-21% w/w MSNF milk solutions at different pH values at 25°C and at 90°C. The data allows a comparison of systems having known pH values at 90°C, as opposed to knowledge of pH prior to heating. To the best of the knowledge, this is the first time that such data is

available that combines knowledge of pH *in situ* at 90°C and protein content in the supernatant.

The 9%-21% w/w MSNF milk solutions at their natural pH values at 25°C, showed a gradual decrease in the protein content in supernatants with increase in milk concentration. This decrease can be more attributed to the decrease in pH at 25°C with increase in milk concentration. On the other hand, heating of skim milk solutions of each milk concentration, resulted in a linear increase in the total protein content in the supernatant with increase in pH. For example, 9% w/w MSNF milk solutions showed an increase in total protein content in supernatants from 10.3-48.3% in the pH range of 6.3 to 8.0 (Table 3.2), whereas 21% w/w MSNF milk solutions showed an increase from 10.4-44.6% in the same pH range (Table 3.2). These results clearly suggest that it is the pH that has the primary influence on the total protein content in supernatants in the range of pH 6.3 to 8.0 at 25°C, which represents pH 5.9 to 7.2 at 90°C. The milk concentration plays a very minor role. However, at higher pH values (pH > 8.0 at 25°C) milk concentration appears to play a role in determining the protein content in the supernatants.

Table (3.2): The total protein content in the supernatants obtained from heated 9%-21% w/w MSNF milk solutions as a function of pH

Sample	Initial pH at 25°C	pH at 90°C	Total Protein Content in Supernatant (%)
9%	6.30	5.90	10.34 ± 0.65
	6.31	5.91	10.91 ± 0.67
w/w	***6.67***	***6.19***	***22.75 ± 0.42***
	6.83	6.31	27.18 ± 0.74
MSNF	7.21	6.61	34.58 ± 0.22

	7.45	6.8	41.78 ± 0.78
	8.00	7.22	48.35 ± 0.59
	8.52	7.61	70.55 ± 0.47
12%	6.30	5.87	10.42 ± 0.32
	6.34	5.90	10.16 ± 0.68
w/w	***6.55***	***6.09***	***21.62 ± 0.67***
	6.65	6.14	22.28 ± 0.67
MSNF	6.86	6.30	28.03 ± 0.47
	7.21	6.56	38.29 ± 0.45
	7.55	6.82	38.93 ± 0.62
	8.01	7.17	46.93 ± 0.57
	8.50	7.55	68.31 ± 0.52
15%	6.30	5.85	10.47 ± 0.26
	6.37	5.90	10.64 ± 0.75
w/w	***6.50***	***6.02***	***18.43 ± 1.27***
	6.66	6.12	19.15 ± 0.68
MSNF	6.96	6.33	29.84 ± 0.67
	7.20	6.50	38.19 ± 0.52
	7.61	6.79	40.79 ± 0.67
	7.99	7.07	46.70 ± 0.63
	8.50	7.43	67.03 ± 0.48
18%	6.30	5.86	11.40 ± 0.67
	6.37	5.91	10.40 ± 0.77
w/w	***6.46***	***5.98***	***18.10 ± 0.30***
	6.65	6.11	19.04 ± 0.37
MSNF	7.00	6.35	31.34 ± 0.32
	7.21	6.50	38.83 ± 0.48
	7.64	6.81	40.68 ± 0.83
	8.03	7.07	44.97 ± 0.54
	8.51	7.42	60.84 ± 0.49
21%	6.30	5.83	10.44 ± 0.72
	6.41	5.90	11.54 ± 0.72
w/w	***6.43***	***5.92***	***13.66 ± 0.68***
	6.66	6.08	18.25 ± 0.57
MSNF	7.03	6.33	31.04 ± 0.38
	7.20	6.45	40.27 ± 0.57
	7.68	6.80	41.43 ± 0.75
	8.01	7.01	44.63 ± 0.61
	8.51	7.36	59.75 ± 0.58

Mean values & s.d. of (n=2) analyses
Pooled s.d. for pH = ± 0.02
The skim milk solutions at their unadjusted pH values are given in bold italic in the table.

3.3.1.2 9% w/w MSNF milk solutions with added Calcium Chelating Agents (P_{in} & EDTA)

Table (3.3) represents the total protein content in supernatants obtained from unheated and heated 9% w/w MSNF milk solutions with/without added different amounts of calcium chelating agents (P_{in} & EDTA) at different pH values at 25°C and at 90°C.

Table (3.3): The total protein content in supernatants of unheated and heated 9% w/w MSNF milk solutions with/without added P_{in} & EDTA as a function of pH

Additions to milk (mmol/kg of milk)	Initial pH at 25°C	pH at 90°C	Total Protein Content in Unheated Supernatant (%)	Total Protein Content in Heated Supernatant (%)
None(control)	6.30	5.90	Nd	10.34 ± 0.65
	6.67	6.19	20.98 ± 0.26	22.75 ± 0.42
	7.26	6.61	27.34 ± 0.29	34.58 ± 0.22
	7.45	6.80	28.10 ± 0.21	41.78 ± 0.78
P_{in}				
10	6.23	5.92	25.37 ± 0.47	9.51 ± 0.34
20	6.23	5.94	24.55 ± 0.51	9.84 ± 0.25
30	6.23	5.96	23.28 ± 0.61	9.68 ± 0.27
10	6.65	6.23	24.10 ± 0.87	24.14 ± 0.30
20	6.58	6.22	23.92 ± 0.13	24.94 ± 0.56
30	6.55	6.23	23.25 ± 0.33	26.76 ± 0.58
10	7.23	6.82	31.24 ± 0.46	44.79 ± 0.68
20	7.23	6.84	31.33 ± 0.62	52.59 ± 0.64
30	7.22	6.82	34.50 ± 0.21	54.06 ± 0.59
EDTA				
10	6.67	6.24	36.49 ± 0.24	28.65 ± 0.63
20	6.54	6.20	46.25 ± 0.68	30.54 ± 0.19
20	6.66	6.37	Nd	34.35 ± 0.25

10 **20**	7.24 7.11	6.83 6.83	Nd 95.80 ± 0.86	58.72 ± 0.69 57.45 ± 0.72

Mean values & s.d. of (n=2) analyses
Pooled s.d for pH= ± 0.02
N.d. = Not determined

The total protein content in supernatants of unheated milk solutions with added P_{in} was higher at all pH values when compared to milk solutions at similar pH at 25°C without added P_{in} (Table 3.3). The higher the pH at 25°C, the more pronounced the effect was. Increasing the amount of added P_{in} to a milk solution from 10 to 30mM at a given pH < 6.65 at 25°C did not change the total protein content in unheated supernatant markedly (Table 3.3). However, the total protein content in the supernatant obtained from the unheated 9% w/w MSNF milk solution with 30mM added P_{in} in milk with initial pH at 25°C of pH 7.2 showed an increase in comparison with the supernatants obtained from the 9% w/w MSNF milk solutions with added 10-20mM P_{in}. This is in accordance with data obtained by Gaucher *et al*. (2007). At the same pH at 25°C, the addition of EDTA showed marked increases in the total protein content in the supernatants of unheated milk solutions compared to unheated milk solutions with added P_{in} (Table 3.3). The total protein content in the supernatants obtained from unheated milk solutions increased with increase in pH at 25°C and increased amounts of added EDTA. This suggests the strong chelating power of EDTA, which results in an increased dissociation of caseins from the casein micelles.

At low pH values at 25°C (pH<6.65), heated skim milk solutions with added P_{in} showed similar behavior as milk solutions without P_{in} addition. Both skim milk solutions with or without addition of P_{in} at pH 6.2 – 6.3 at 25°C showed a decrease in the total protein

content in supernatants obtained from heated milk solutions compared to unheated milk solutions, whereas no marked change was observed at around pH 6.65 at 25°C (Table 3.3). However, increasing pH at 25°C increased the total protein content in the supernatant of heated milk solutions with added P_{in} compared to supernatants of heated milk solutions without added P_{in}. In contrast, heated skim milk solutions with added EDTA showed a lower total protein content in the supernatants than unheated milk solutions with added EDTA at all equivalent pH values at 25°C. This suggests a possible re-association of the dissociated caseins to the casein micelles in the heated skim milk solutions with added EDTA.

Unlike the relationship between pH at 25°C and the total protein content in supernatants obtained from heated 9%-21% w/w MSNF milk solutions, the total protein content in the supernatants obtained from heated 9% w/w MSNF milk solutions with added calcium chelating agents having the same initial pH at 25°C was not constant, although there was a trend of increasing protein concentration with increasing pH at 25°C. This suggests that the changes in the composition of milk as a result of the addition of mineral salts (including the consequent differences in speciation of the components of milk) affect the aggregation of the proteins during heating.

3.3.2 The Formation of Soluble Aggregates

3.3.2.1 Supernatants of heated and unheated 9% w/w MSNF milk solution at their natural pH of 6.65

To elucidate the nature of the particles produced by the proteins on heating, the HPLC-SEC chromatograms for supernatants corresponding to heated and unheated 9% w/w MSNF milk solutions at their natural pH of 6.65 was obtained (Figure 3.5).

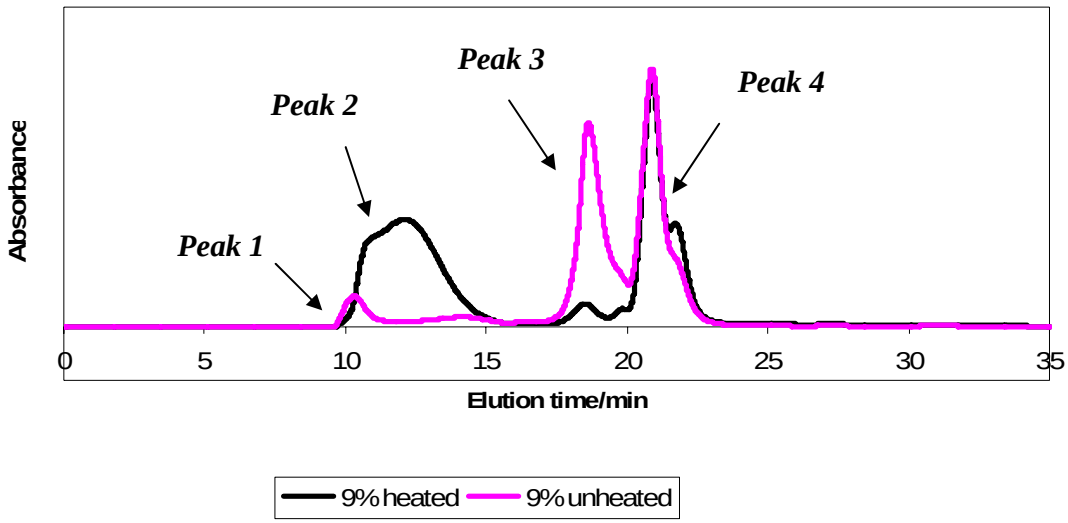


Figure (3.5): HPLC-SEC profiles of supernatants obtained from heated and unheated 9% w/w MSNF milk solutions at pH 6.65. The peaks were assigned as follows: Peak 1 – dead volume of the column; Peak 2- soluble aggregate peak; Peak 3 – native whey protein and monomeric caseins; Peak 4 – orotic acid and other dialyzable solutes

**The total protein content in supernatants for unheated and heated milk solutions were 20.98% and 22.75% respectively.*

The profiles obtained from supernatants of 9% w/w MSNF milk solutions at pH 6.65 prior to heating and after the heat treatment at 90°C/10 min were typical of those obtained previously (Guyomarc'h *et al.* 2003; Menard *et al.* 2005; del Angel & Dalgleish, 2006;

Donato & Dalgleish, 2006; Donato *et al.* 2007; Renan *et al.* 2006; Renan *et al.* 2007). The HPLC-SEC profiles of the supernatant from unheated milk showed four contributions (Figure 3.5). The first peak eluting at a time of around 10 min, defines the dead volume of the column. This region does not contain proteinaceous material and probably contain very small fat globules that redisperse after centrifugation and filtration (Donato & Dalgleish, 2006). Peak 2 which represents the soluble aggregates is missing in the unheated profile. Peak 3 consists of small quantities of caseins and significant amounts of native whey proteins. Guyomarc'h *et al.* (2003) suggested that these amounts of caseins present were small relative to the total amount of casein in milk and showed that they represented less than 10% of the total casein in milk. Peak 4, which elutes at a time around 20 min, consists of orotic acid and other dialyzable solutes. Orotic acid is very stable to heat and to chemical changes. It also gives a well resolved peak on separation by Sephacryl and was therefore a good material to use as the internal standard to compare the profiles of the milk solutions (Guyomarc'h *et al.* 2003).

Upon heating, it can be seen by the decrease in Peak 3 area that the amounts of native whey proteins and monomeric caseins decreased considerably, while a large symmetrical peak corresponding to soluble aggregates appeared (Peak 2). The decrease in Peak 3 area on heating is due to the involvement of the whey proteins and monomeric caseins in the formation of the soluble aggregates as described in Section (1.7.1.3 & 3.3.1.1). Hence, it can be concluded that heat treatment has a large effect on the formation of soluble aggregates. However, Guyomarc'h *et al.* (2003) pointed out that heat induced dissociation of κ-casein was implicated in the formation of the soluble aggregates and indicated that a

significant amount of κ-casein was left unreacted after heating. Heat treatment did not affect the area of Peak 4 (Figure 3.5).

It has previously been found that after milk is heated at 95°C for 10 min and cooled and re-heated again for further 10 min, the profiles were superimposable. This indicates that the denaturation of whey proteins and aggregation reactions are completed by heating to 95°C for 10 min (Guyomarc'h *et al.* 2003; Anema *et al.* 2004*b*). Hence, the heating conditions (90°C/10min) used in the present study represent the overall denaturation and the association pathways of whey proteins and caseins.

3.3.2.2 Supernatants of unheated 9% w/w MSNF milk solutions at high pH values

Figure (3.6) shows the HPLC-SEC profiles of supernatants obtained from unheated 9% w/w MSNF milk solutions at high pH values of 6.8, 8.0 & 8.5. As the pH of the skim milk solutions increased, there was a more noticeable peak eluting at ~13-14 min (denoted as Peak 5 in Figure 3.6). SDS-PAGE analysis of this region had shown that this peak contained small quantities of caseins, significant amount of β-Lg and traces of α-La (Donato & Dalgleish, 2006). Hence, it was suggested that some of the whey proteins identified in Peak 3 may be somewhat aggregated and therefore elute at a shorter elution time than the main native whey protein peak. The whey protein self-aggregation was found to be less pronounced when κ-casein is present in the medium (Cho *et al.* 2003). However, as the pH increases, the whey proteins tend to self-aggregate more efficiently. At alkaline pH, the intramolecular disulfide bonds that help to maintain the secondary structure of whey proteins are easily broken and repulsive electrostatic forces are increased (Rattray &

Jelen, 1997). Also, it has been suggested that the increase in ionic strength by adding acids or bases to adjust pH of milk may help to promote the self-aggregation of the pH induced denatured whey proteins. In the present study, the presence of whey protein aggregates is reflected in the HPLC-SEC profiles (Figure 3.6) by the decrease in the native whey protein peak (Peak 3) and an increase in peak 5.

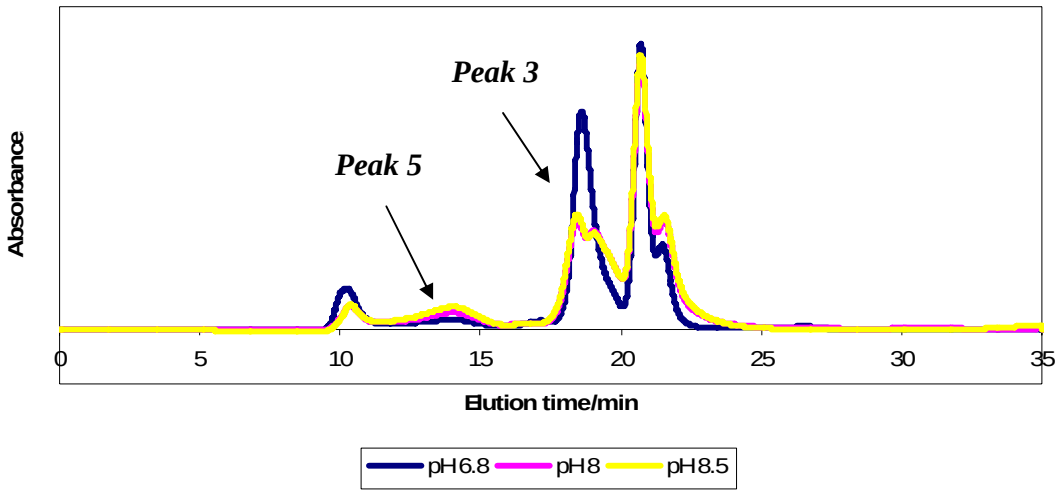


Figure (3.6): HPLC-SEC profiles of unheated supernatants obtained from 9% w/w MSNF milk solutions at pH values of 6.8, 8.0 and 8.5 at 25℃

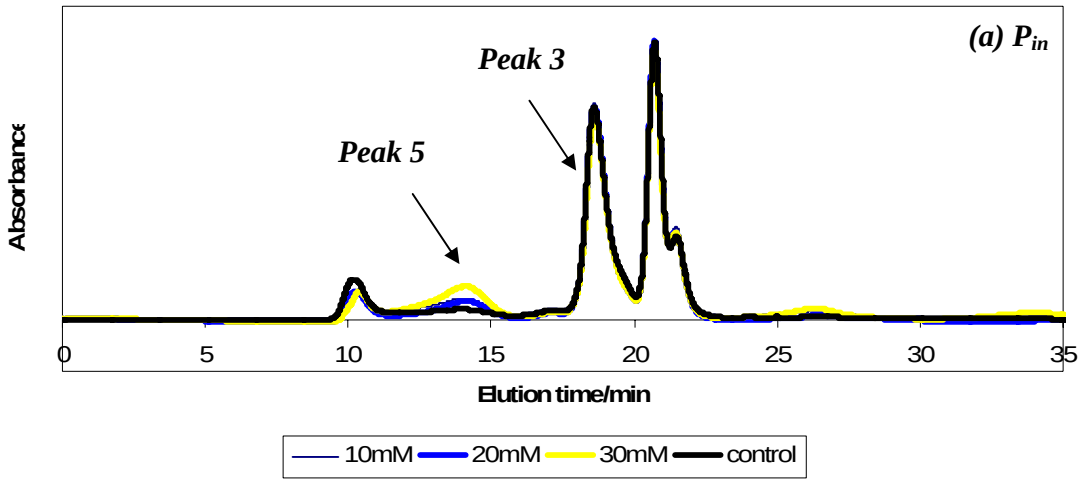
**The concentration of the protein content in supernatants were 22.32%, 32.88% & 37.87% respectively.*

3.3.2.3 Supernatants of unheated 9% w/w MSNF milk solutions with added calcium chelating agents (P_{in} & EDTA)

Figure (3.7) shows the HPLC-SEC profiles of supernatants obtained from unheated 9% w/w MSNF milk solutions with/without addition of P_{in} and EDTA. The elution times of the peaks obtained after the addition of calcium chelating agents were similar to the elution

times observed without the additions. However, the area of Peak 5 increased with both the additions of P_{in} and EDTA, having a more pronounced effect with increase in the amounts of added calcium chelating agents (Figure 3.7). The increase in the area of Peak 5 was more pronounced with the addition of EDTA, which confirms the extensive disintegration of the micelles with EDTA by being a more powerful chelating agent than P_{in} (Figure 3.7*b*). Interestingly, the monomeric protein peak, which comprises mainly native whey proteins and a small amount of monomeric caseins (Peak 3), was not affected by the added calcium chelating agents. An explanation for this is that the whey proteins are not disturbed by the additions of calcium chelating agents.

With the addition of EDTA, there was an appearance of another peak (Peak 6 in Figure 3.7 *b*). It should be some kind of very small molecules and does not appear with the addition of P_{in}.



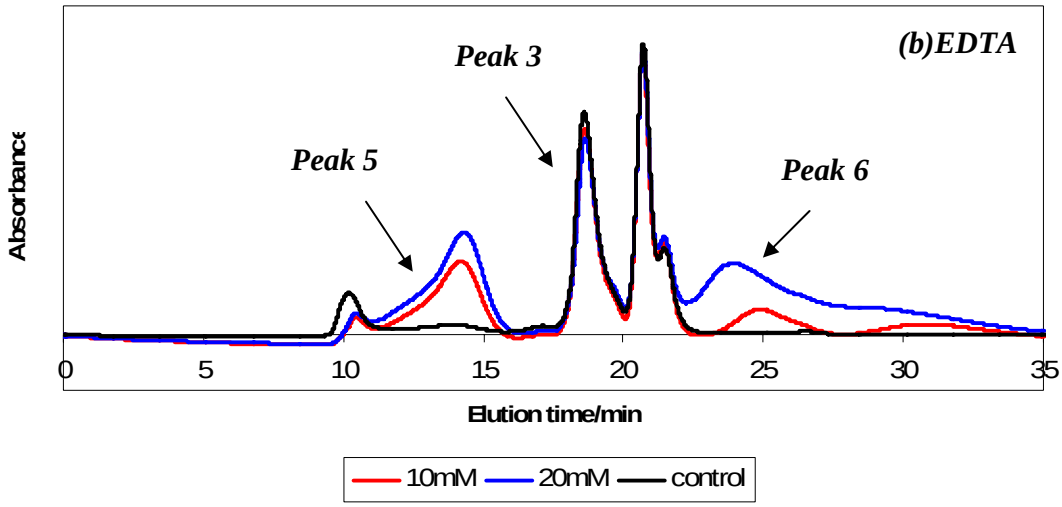


Figure (3.7): HPLC-SEC profiles of unheated supernatants obtained from 9% w/w MSNF milk solutions with/without added (a) P_{in} (b) EDTA with pH values of 7.2 at 25℃

3.3.2.4 Supernatants of heated 9% to 21% w/w MSNF milk solutions at their unadjusted pH values

The HPLC-SEC elution profiles of supernatants of heated milk solutions (9-21% w/w MSNF) at their unadjusted pH values are given in Figure (3.8). It has been demonstrated previously that the amounts of soluble aggregates in the serum phase depend on the pH of the milk solution (Singh & Latham, 1993; Menard *et al.* 2005; del Angel & Dalgleish, 2006; Donato & Dalgleish, 2006; Jean *et al.* 2006; Renan *et al.* 2007). Increasing milk concentration, which decreases their natural pH, resulted in a decrease in the formation of soluble aggregates as reflected by the reduced area of the soluble aggregate peak (Figure 3.8). Less soluble aggregates were formed as the pH of the solution decreased; lowest

amounts of soluble aggregates were observed with 21% w/w MSNF milk solution having a pH of 6.4. These data are in line with others who obtained a decreased amount of soluble aggregates as pH decreased (Menard *et al.* 2005; del Angel & Dalgleish, 2006; Donato & Dalgleish, 2006; Renan *et al.* 2006). These results shows a clear correlation between a lower pH and a higher proportion of micelle bound denatured whey protein aggregates as discussed in Sections (1.7.1.3 & 3.3.1.1), with the use of Vasbinder & de Kruif, (2003) model.

With increase in milk concentration from 9-12% w/w MSNF, there was an increase in peak area of the soluble aggregate peak and a shift to higher MW soluble aggregates as shown by appearance of the peak at ~11 min, indicating the formation of larger aggregates in milk with the higher concentration. However further increases in milk concentration beyond 12% w/w MSNF (i.e. 12-21% w/w MSNF), the area of the soluble aggregate peak decreased with increase in milk concentration but its position did not change markedly. This implies that the amount of soluble aggregates varied, whereas their sizes did not change markedly in the pH range of 6.55 to 6.40 that was studied. These results are in accordance with the results obtained by del Angel & Dalgleish, (2006).

These patterns were superimposed in repeated measurements, indicating the consistency of the formation of soluble aggregates. The formation of lower amounts of soluble aggregates could either be due to a concentration effect and/or to a pH effect according to the observations in the current data set studied. Hence, it is hard to distinguish between these two factors within the data given in Figure (3.8) alone.

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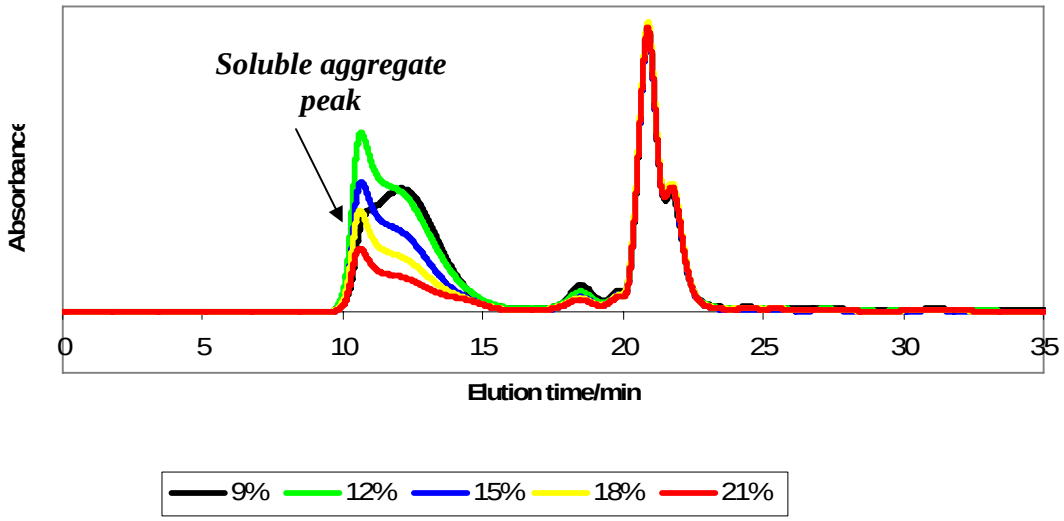


Figure (3.8): HPLC-SEC profiles of heated supernatants obtained from 9% to 21% w/w MSNF milk solutions at their natural initial pH values at 25℃; 9%- pH 6.65, 12%- pH 6.55, 15%- pH 6.50, 18%- pH 6.46, 21%- pH 6.43

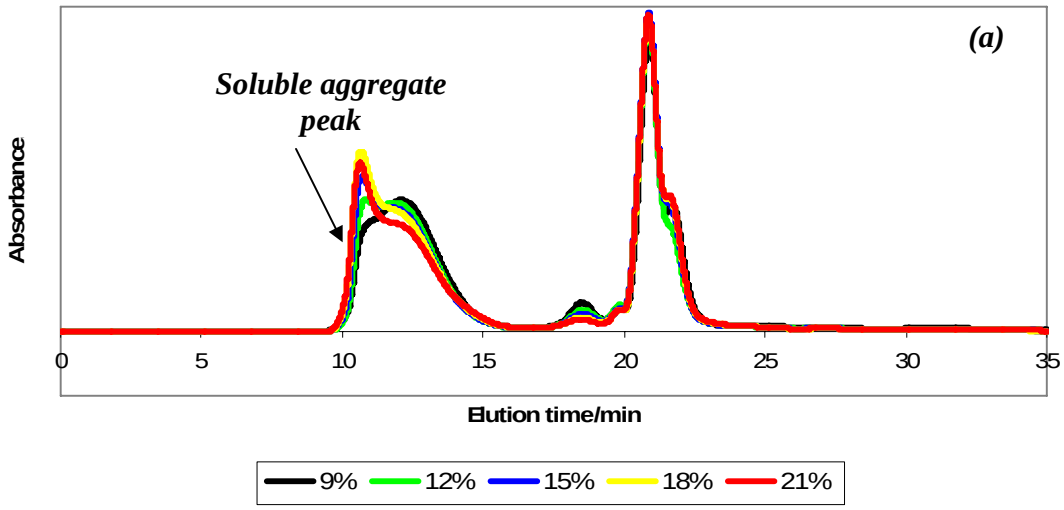
**The concentration of the protein content in supernatants were 22.75%, 21.62%, 18.43%, 18.10%, 13.66% respectively.*

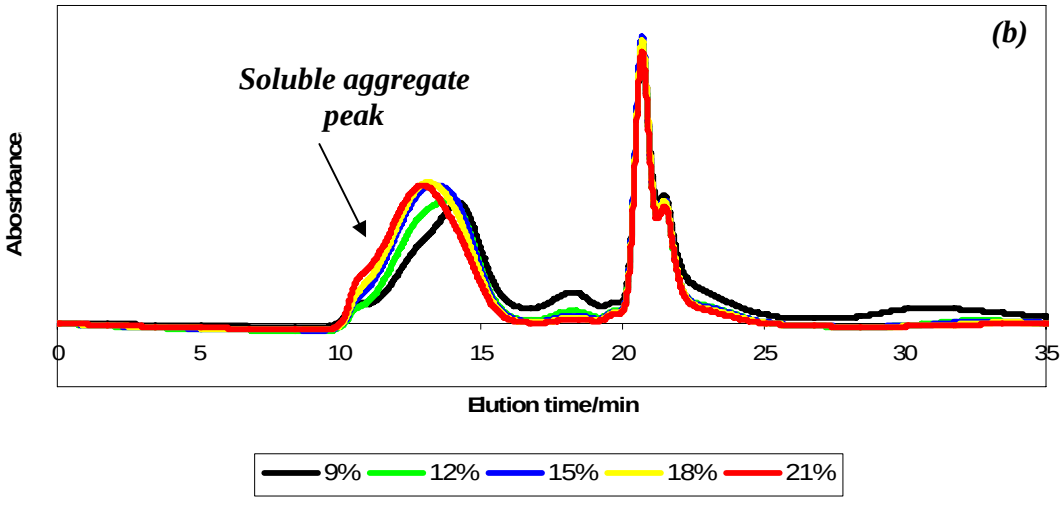
3.3.2.5 Supernatants of heated 9% to 21% w/w MSNF milk solutions at constant pH values at 25℃ before heating

The question that arose in the previous Section (3.3.2.4) was how to distinguish between the effect of pH and the effect of milk concentration on the formation of soluble aggregates. Hence, the formation of soluble aggregates in skim milk solutions having the same pH values at 25°C was studied as a function of milk concentration (9%-21% w/w MSNF).

Figure (3.9) shows the HPLC-SEC profiles of supernatants obtained from heated 9%-21% w/w MSNF milk solutions having constant pH values at 25°C.

At pH 6.65, there were minor differences in the area of the soluble aggregate peaks, with varying milk concentration, although for 18% & 21% w/w MSNF milk solutions, there were increased amounts of soluble aggregates formed during heating. At the same time, there were slight shifts towards shorter elution times as milk concentration increased, indicating the formation of somewhat larger aggregates. The formation of larger aggregates with increase in milk concentration becomes much more prominent with increase in pH as indicated by the decrease in elution times of the soluble aggregate peak in Figure (3.9*b* & 3.9*c*). The shift in the soluble aggregate peak of single strength milk solutions with increasing pH is in line with del Angel & Dalgleish, (2006), who found that the area of the peak changed along with a slight shift in elution time, implying a small change in size with increase in pH up to pH 7.2. They further suggested that the effect of pH not only alters the amounts of soluble aggregates but also changes their size. Generally, it was found that the aggregates were almost spherical and have the hydrodynamic radius of about 50-70nm as measured by dynamic light scattering (del Angel *et al.* 2006; Jean *et al*. 2006).


(a)
Soluble aggregate peak
Absorbance
0
5
10
15
20
25
30
35
Elution time/min
9%
12%
15%
18%
21%


(b)
Soluble aggregate peak
Abosrbance
0
5
10
15
20
25
30
35
Elution time/min
9%
12%
15%
18%
21%

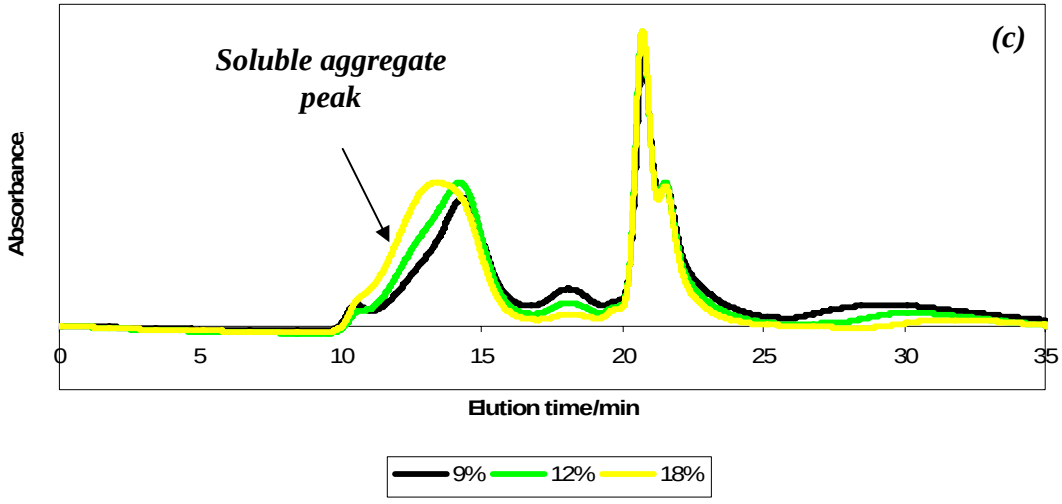


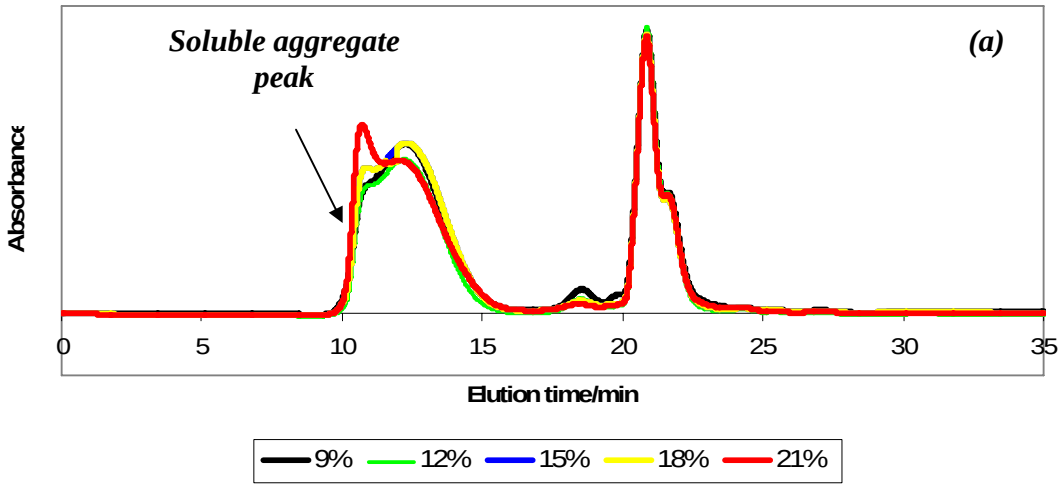
Figure (3.9): HPLC-SEC profiles of heated supernatants obtained from 9% to 21% w/w MSNF milk solutions at constant pH values of (a) pH 6.65 (b) pH 8.00 (c) pH 8.50 at 25℃ before heating

The present set of data suggests the dependence of pH on the quantity and the size of the soluble aggregates formed during heat treatment than the effect of milk concentration.

3.3.2.6 Supernatants of heated 9% to 21% w/w MSNF milk solutions at constant pH values at 90℃

Figure (3.10) shows HPLC-SEC profiles of supernatants obtained from heated 9% to 21% w/w MSNF milk solutions having constant pH values of (a) pH 6.30 (b) 6.80 at 90°C. The area of the soluble aggregate peaks with increase in milk concentration (9%-18% w/w MSNF) showed no marked differences, although 21% w/w MSNF milk solutions showed

some differences in the soluble aggregate peak. It is indicated by an appearance of a sharp shoulder in the soluble aggregate peak at around 10 min (Figure 3.10*a*). This is less pronounced at higher pH values (Figure 3.10*b*). There were no significant changes in the elution times indicating not much size change of the soluble aggregates with increase in milk concentration having a constant pH of 6.3 at 90°C. However, at pH 6.8, there was a more prominent shift towards shorter elution times of the soluble aggregate peaks with increase in milk concentration as compared to that obtained at pH 6.3 at 90°C. These results also clearly confirm the importance of pH to the quantity and size of the soluble aggregates being formed during heat treatment.



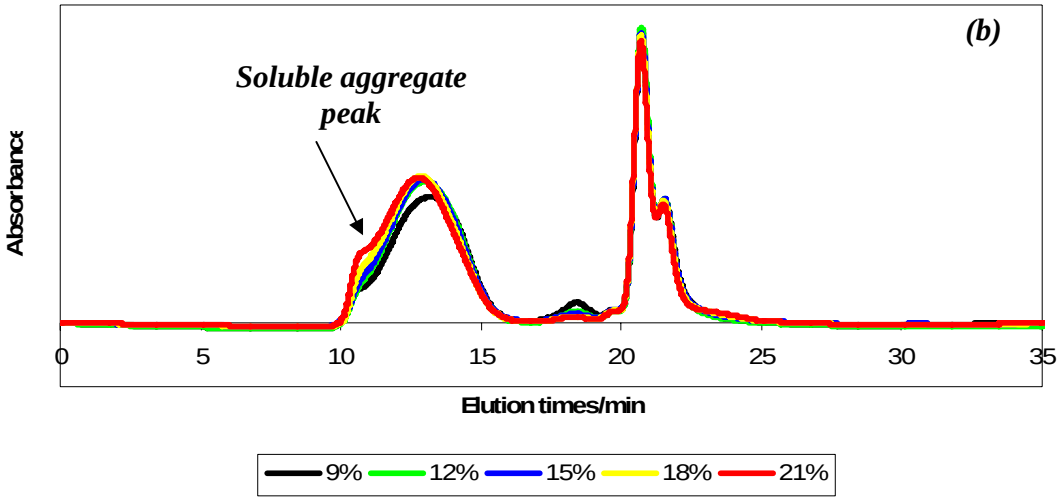


Figure (3.10): HPLC-SEC profiles of supernatants obtained from heated 9% to 21% w/w MSNF milk solutions at constant pH values of (a) pH 6.3 (b) 6.8 at 90℃

3.3.2.7 Supernatants of heated 9% w/w MSNF milk solutions with added Calcium Chelating Agents (P_{in} & EDTA)

Figure (3.11) represents the HPLC-SEC profiles of supernatants obtained from 9% w/w MSNF milk solutions with various amounts of added P_{in} (10-30mM) and EDTA (10-20mM) at constant pH of 6.8 at 90°C. The addition of P_{in} resulted in a slight change in the shape of the soluble aggregate peak compared to that of without addition, even though the pH at 90°C was kept constant. Based on the elution profiles, there were a slightly higher proportion of smaller aggregates with increasing amounts of added P_{in} as can be seen by the (*) in Figure (3.11*a*).

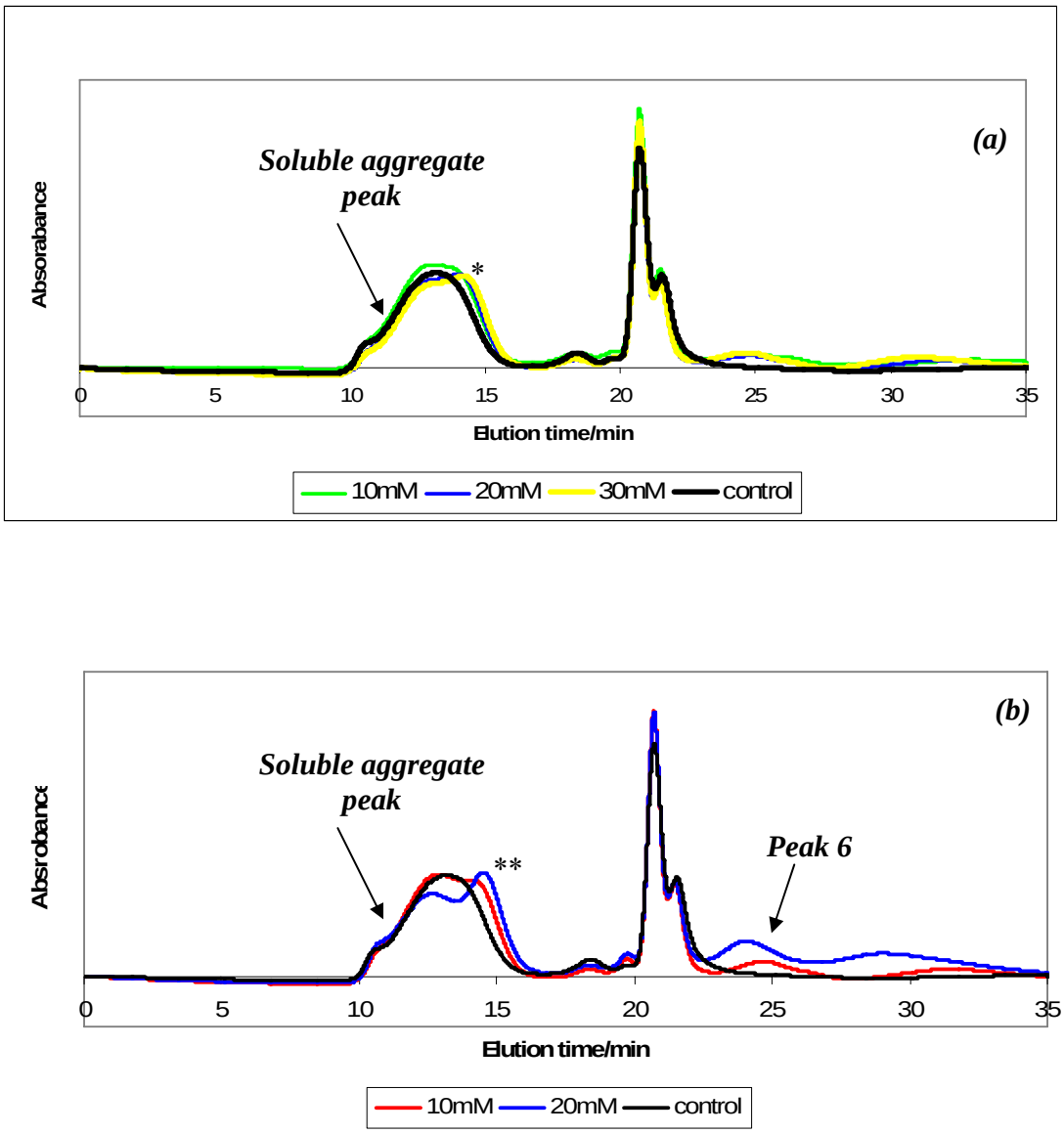


Figure (3.11): HPLC-SEC profiles of heated supernatants obtained from 9% w/w milk solutions with added (a) P_{in} (b) EDTA at constant pH of 6.8 at 90℃

The addition of EDTA resulted in a similar but much more pronounced change in the shape of the soluble aggregate peak than with the addition of P_{in} (Figure 3.11*b*). There is a tendency of large amounts of small soluble aggregates, which is shown by (**) in Figure (3.11*b*). In order, to distinguish the compositional characterization of the two types of soluble aggregates (small and large), quantification through SDS-PAGE was done for the fractions obtained from each elution times. The results which will be discussed in Section (3.3.3) suggests that increased tendency of the dissociated caseins to be involved in the formation of soluble aggregates, that was also observed with the data obtained in Section (3.3.1.1).

The Peak 6 also can be identified in heated profiles, although the area of that peak showed a reduction. This peak was only prominent with the addition of EDTA. It concludes that the protein materials eluting as Peak 6 involves in some kind of a reaction during heating. However, in the present study it was not identified and characterized to that extent of what those protein materials are and how those protein materials participate during heating.

3.3.3 The Characterisation of the Soluble Aggregates

It is extremely difficult to determine the direct interactions between various species in heated milk and within the soluble aggregates. In particular it is difficult conclusively to determine the direct interactions between the denatured whey proteins interact with κ-casein and the predominant bonding involved in the interactions (Anema, 2007). To elucidate the bonding within the soluble aggregates, the soluble aggregate fractions were analyzed through SDS-PAGE under reducing and non-reducing conditions. The proteins

resolved on non reducing gels were assumed to contain protein aggregated by non covalent bonds (Oldfield *et al.* 2005).

The SDS-PAGE electrophoragrams done under non-reducing conditions showed no protein bands (Figure 3.12*a*), whereas protein bands were observed under reducing conditions (Figure 3.12*b,c*). Under non reducing conditions, the proteins were in an aggregated state where they were stuck on top of the gel without migrating through the gel, in contrast under reducing conditions the aggregated proteins were reduced to individual proteins and migrated through the gel. This result suggested that the primary bonding within the soluble aggregates is through disulfide bonding, where under reducing conditions that most of the disulfide cross linked aggregates were broken and hence results in monomeric proteins which will migrate along the gels. This is in line with other works (Dalgleish, 1990; Anema, 2000; Anema, 2001; Henry *et al.* 2002; Guyomarc'h, *et al.* 2003; Lowe *et al.* 2004; Oldfield *et al.* 2005; Havea, 2006; Jean *et al.* 2006; Patel *et al.* 2006; Renan *et al.* 2006; Anema, 2007; Jovanovic *et al.* 2007; Renan *et al.* 2007). However, other interactions such as hydrophobic and hydrogen bonding are also likely to be involved in forming and maintaining the structure (Menard *et al.* 2005; Havea, 2006; Jovanovic *et al.* 2007).

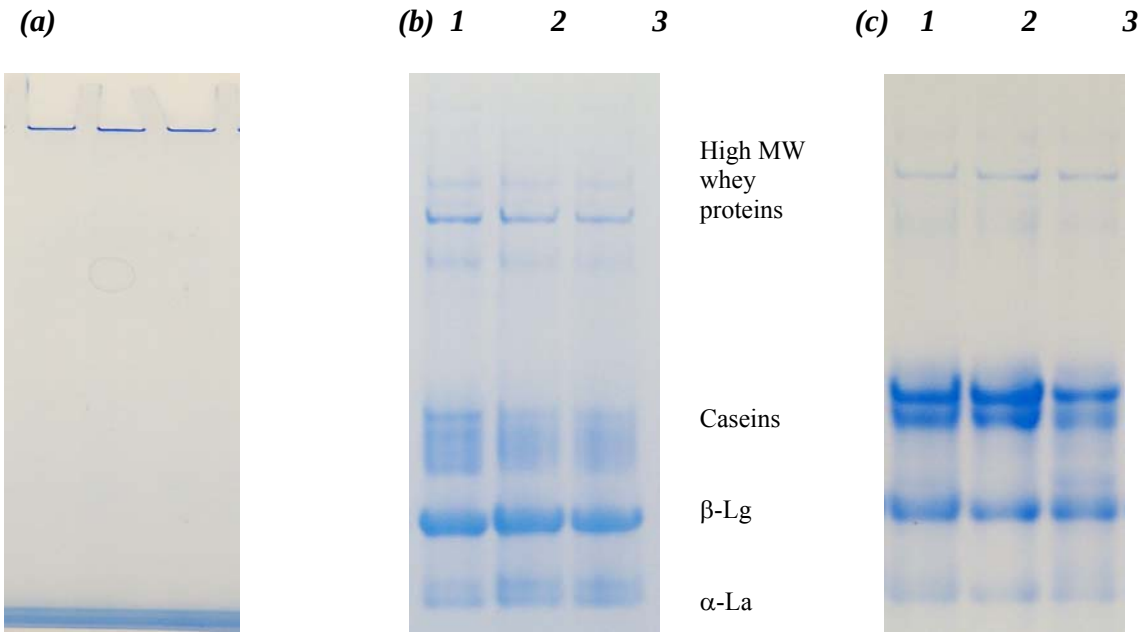


Figure (3.12): Non- reduced (a) Reduced (b & c) SDS-PAGE patterns of soluble aggregate fractions (Peak 2) obtained from heated (b) Lane 1 – 9% w/w MSNF; Lane 2 – 15% w/w MSNF; Lane 3 – 18% w/w MSNF milk solutions at a constant pH of 6.8 at 90℃ (c) 9% w/w milk solutions with added P_{in}; Lane 1 – 10mM; Lane 2 – 20mM; Lane 3 – 30mM at a constant pH of 6.8 at 90℃

The amounts of caseins associated with the soluble aggregates of selected heated (90°C/10min) skim milk solutions are shown in Table (3.4). The 9% - 21% w/w MSNF milk solutions at their natural pH values were chosen as these milk solutions were used as controls (denoted in Italic in the table). Then 9%-21% w/w MSNF milk solutions (without added salts) having a constant initial pH at 25°C (denoted in blue) and pH at 90°C (denoted in green and maroon) were chosen in order to find out the importance of the initial pH at

25°C and pH *in situ* on the nature of the aggregates. Milk solutions (9% w/w MSNF) with added P_{in} having a constant pH at 25°C of 6.2 (denoted in pink) and with added P_{in}/EDTA having a constant pH at 90°C of 6.2 and 6.8 (denoted in orange and maroon respectively) were chosen to find out the importance of milk composition on the nature of the aggregates. The different sets of milk solutions are colour coded in Table (3.4) as described earlier for easy recognition.

Table (3.4): The amounts of caseins present in the soluble aggregate fractions of 9%-21% w/w MSNF milk solutions with/without added EDTA/P_{in} as a function of pH

Sample	Additions to milk (mmol/kg of milk)	pH at 25°C	pH at 90°C	Casein present in soluble aggregates (%)
9% w/w MSNF	No addition	*6.67*	*6.19*	*20.24 ± 1.36*
		6.83	6.31	22.88 ± 2.52
		7.45	6.80	31.39 ± 0.70
12% w/w MSNF	No addition	*6.55*	*6.09*	*12.98 ± 1.28*
		6.65	6.14	11.21 ± 1.13
		6.86	6.30	22.12 ± 1.78
15% w/w MSNF	No addition	*6.50*	*6.02*	*7.25 ± 0.69*
		6.66	6.11	9.58 ± 0.97
		6.96	6.33	21.71 ± 1.21
		7.61	6.79	29.44 ± 1.22
18% w/w MSNF	No addition	*6.46*	*5.98*	*9.73 ± 0.89*
		6.65	6.11	9.82 ± 0.77
		7.00	6.35	22.98 ± 1.82
		7.64	6.81	30.19 ± 2.02
21% w/w MSNF	No addition	*6.43*	*5.92*	*3.51 ± 0.42*
		6.66	6.08	10.64 ± 1.00
		7.03	6.33	20.28 ± 1.57

9% w/w MSNF	P_{in} 10	6.23	5.77	13.07 ± 0.95
	20	6.23	5.85	15.67 ± 1.05
	30	6.23	5.90	19.59 ± 1.24
	10	6.66	6.23	26.93 ± 1.35
	20	6.58	6.22	21.84 ± 1.29
	30	6.52	6.20	22.11 ± 1.04
	10	7.23	6.82	59.94 ± 1.89
	20	7.23	6.84	66.47 ± 1.93
	30	7.22	6.82	67.51 ± 1.75
9% w/w MSNF	EDTA 10	7.25	6.83	56.72 ± 1.69
	20	7.11	6.83	59.21 ± 1.46

Mean values & s.d. of (n=3) analyses
Pooled s.d. for pH = ± 0.02

The amount of caseins present in soluble aggregates obtained from 9%-21% w/w MSNF milk solutions at their natural pH values decreased with increase in milk concentration (Table 3.4). This is mostly due to the decrease in pH with increase in milk concentration rather than the increased concentration of the components as described in Section (3.3.2.5. & 3.3.2.6). del Angel & Dalgleish, (2006) concluded that the effect of pH is not simply to alter the amounts of soluble aggregates but also to change their size and possibly their composition. Our results clearly showed that there was definitely a change in composition of the soluble aggregates with respect to changing the pH of the milk systems. However, keeping pH values constant at 90°C showed less variations in the amount of caseins present in the soluble aggregates with increase in milk concentration. For example, the amount of caseins present in soluble aggregates for 9% w/w MSNF milk solutions were 31.29 ± 0.70% whereas, for 21% w/w MSNF milk solutions were 30.19 ± 2.02% for a given pH of 6.8 at 90°C. In contrast, the amount of caseins present in the soluble aggregates at a

constant pH value at 25°C showed much more variation in comparison with the amount of caseins present in the soluble aggregates at a constant pH value at 90°C. For example, the amount of caseins present in soluble aggregates for 9% w/w MSNF milk solutions were 20.24 ± 1.36% whereas, for 21% w/w MSNF milk solutions were 10.64 ± 1.00% for a given pH of 6.65 at 25°C. In this case, the pH at 90°C was different even though the pH at 25°C was kept constant; the pH at 90°C ranged from 6.19-6.08 with increase in milk concentration keeping pH values of 6.65 ± 0.01 at 25°C constant. Hence, these results suggest the importance of pH at the temperature of heating (at 90°C in the present study) on the composition of the soluble aggregates. It also highlights the importance of small alterations of pH to the marked changes of the whey proteins association with casein micelles as was suggested by others (Anema & Li, 2003; Vasbinder & de Kruif, 2003; Anema *et al.* 2004*a*).

The addition of P_{in} to 9% w/w MSNF milk solutions resulted a significant increase in the amount of caseins present in the soluble aggregates, in comparison without the additions. The amount of casein present in soluble aggregates of 9% w/w MSNF milk solutions at pH 6.8 at 90°C were 31.39 ± 0.70%, whereas 10mM P_{in} added 9% w/w MSNF milk solutions at pH 6.8 at 90°C were 59.94 ± 1.89%. The addition of EDTA resulted in the same trends (Table 3.4).

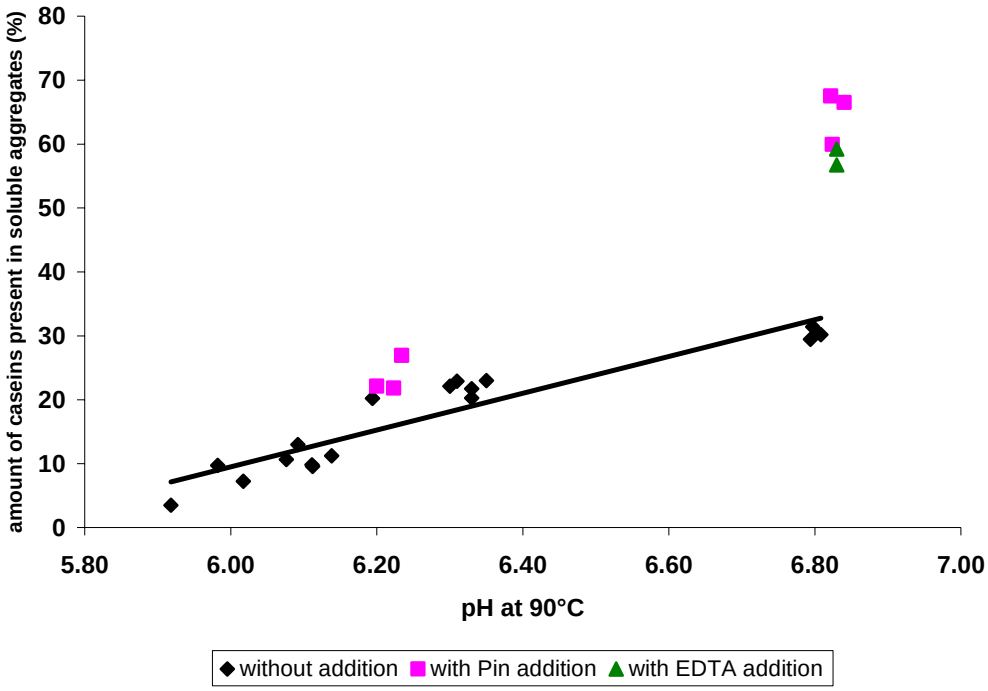


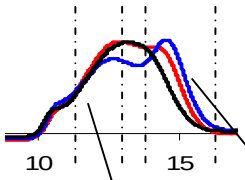
Figure (3.13): The amount of casein present in soluble aggregates obtained from heated 9% w/w MSNF milk solutions with/without addition of calcium chelating agents as a function of pH at 90℃

Figure (3.13) shows the relationship between pH at 90°C and the amount of caseins present in the soluble aggregates formed during heating to 90°C for 10 min. The amount of caseins present in the soluble aggregates increased linearly with increase in pH for milk systems without added salts (Figure 3.13). However, the addition of calcium chelating agents (P_{in} & EDTA) resulted an increase in the amount of caseins present in soluble aggregates for a given pH in comparison with milk solutions without the addition of P_{in} and EDTA. This effect is not that significant at lower pH values, whereas it becomes more prominent with increase in pH as can be seen in Figure (3.13). It highlights the importance of the

compositional changes towards the composition of the soluble aggregates formed during heat treatment.

The quantification studies through SDS-PAGE of the small and large soluble aggregates (Section 3.3.2.7- Figure 3.11) formed during heat treatment of 9% w/w MSNF milk solutions with added EDTA are shown in Table (3.5).

Table (3.5): The amount of caseins present in the small and large soluble aggregate fractions of 9% w/w MSNF milk solutions with added EDTA as defined by sections marked in the figure



Additions to milk (mmol/kg of milk)	pH at 25°C	pH at 90°C	Casein present in large soluble aggregates (%)	Casein present in small soluble aggregates (%)
10	6.68	6.26	2.30 ± 0.55	72.21 ± 0.64
20	6.54	6.23	12.14 ± 0.89	68.44 ± 0.72
10	6.68	6.26	2.30± 0.55	72.21 ± 0.64
20	6.66	6.37	22.79 ± 0.94	61.49 ± 1.02

Mean values & s.d. of (n=3) analyses

Pooled s.d. for pH = ± 0.02

The results showed that the small elution time aggregates contained less casein than the larger elution time aggregates. All of the aggregates were consisted primarily of denatured whey proteins. The addition of EDTA resulted an increase in formation of aggregates of larger elution times (as described in Section 3.3.2.7), which consisted more caseins. Hence, it clearly suggested that, if more casein is present in the supernatant; then the caseins present in the soluble aggregates were high. As the amount of EDTA addition increased, more of the casein dissociates, results more of casein rich soluble aggregates. These results suggested that it might be due to the higher affinity of denatured whey proteins to be associated with the caseins that were already been out of the micelle. The evidence obtained in Section (3.3.1.1) in the present study also highlighted this argument. However, this is in contrast with Donato & Dalgleish, 2007 who found the preferential reaction happening on the surface of the casein micelle.

3.4 Conclusion

The total protein content in supernatants of unheated milk solutions increased with increase in pH due to the increased dissociation of caseins as pH increased. This happened irrespective of milk concentration and the addition of calcium chelating agents. The increase in the amounts of total protein in the supernatants was much more pronounced with the addition of EDTA due to the stronger chelating power of EDTA than P_{in}.

The total protein content in the supernatant after heating to 90°C for 10 min increased for 9% w/w MSNF milk solutions with pH values greater than 6.65, whereas below this pH

value, there was a decrease in the protein content in the supernatant after heating in comparison with the unheated milk solutions. This is due to the distribution of protein components between colloidal and aqueous phases of milk; as the pH decreases, more of the denatured whey proteins are associated with the casein micelles, which will be then transferred to the colloidal phase of milk. On the other hand, as the pH increases the more of caseins dissociates from the micelles and forms complexes in the serum phase, where the majority of the complexes are in the serum phase. The addition of P_{in} to milk solutions resulted similar behavior in the total protein content of heated supernatants, in comparison with the milk solutions without the addition at a given pH. In contrast the total protein content in the supernatants of heated skim milk solutions with added EDTA was lower at all pH values studied with respect to the unheated milk solutions. This suggests a re-association of casein micelles on heating.

Soluble aggregates were formed upon heating, which consisted of denatured whey proteins and casein micelles.

The amount of soluble aggregates formed during heat treatment decreased as the milk concentration increases. However, increasing the milk concentration also resulted in a decrease in pH. Keeping the pH at 25°C & at 90°C constant, showed less marked changes in the HPLC-SEC elution profiles of the soluble aggregate peak as a function of milk concentration compared to the soluble aggregate peak obtained from milk solutions with varying pH values. The addition of calcium chelating agents resulted slight changes in the quantities and size distribution of the soluble aggregates. It suggests the importance of milk

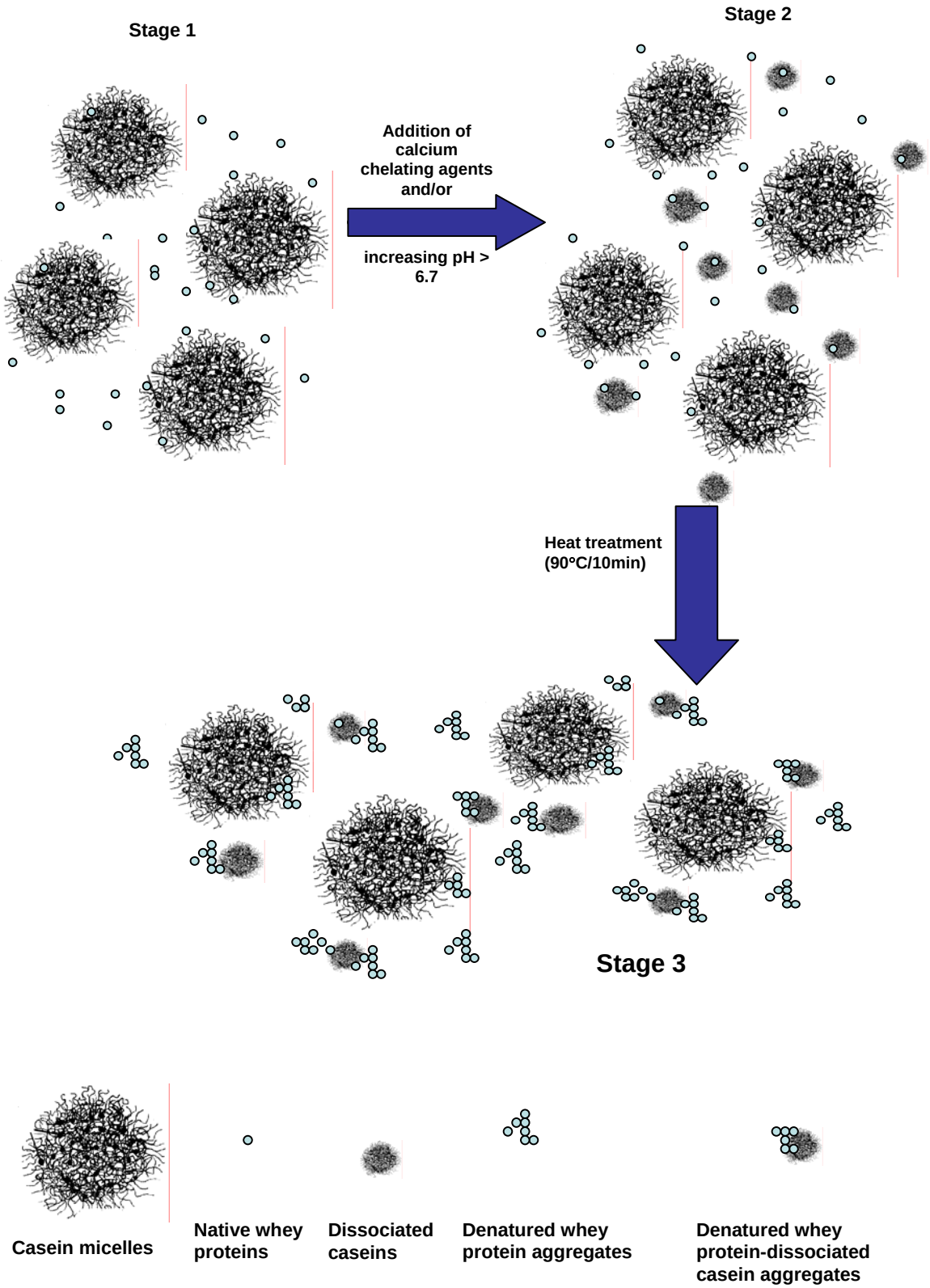
composition with respect to pH and mineral composition rather than the milk concentration in determining the quantity and size of the soluble aggregates formed during heating.

The soluble aggregates are predominantly disulfide bonded. The compositional analysis of the soluble aggregates showed that the amount of caseins present in the aggregates is dependent on the distribution of the proteins between the serum and micellar phases of milk and the heat treatment given. The distribution of proteins between phases is largely dependent on the distribution of the calcium and phosphates. The distribution of whey protein-casein aggregates in the serum and their composition as a function of high pH, the addition of calcium chelating agents and heat treatment is schematically depicted in Figure (3.14).

At high pH and the addition of EDTA/P_{in} resulted in an increased dissociation of caseins from the casein micelles (Stage 1 to Stage 2). However, the distribution of whey proteins did not change. Once the heat treatment is given, the whey proteins denature and associate with themselves and/or with casein micelles and dissociated caseins. At high pH values and/or with added calcium chelating agents, the amount of denatured whey proteins associated with the dissociated caseins increased, when there was an increased quantity of casein present in the serum phase rather than the association of the denatured whey proteins with the casein micelles. Hence, our results indicated that there is a high preference of denatured whey proteins to be associated with caseins that have already been dissociated from the casein micelles, although there is a smaller fraction of the denatured whey proteins associating with the casein micelles (Stage 3).

This chapter concludes that the formation and the composition of the soluble aggregates depend on the composition of the milk solutions. In chapter 4, the effect of physico chemical changes and the formation and composition of the soluble aggregates on heat stability is examined.

Figure (3.14): A schematic representation of the interaction between casein micelles and/or dissociated caseins with denatured whey proteins/whey protein aggregates in milk during heat treatment for 10 min at 90℃ at high pH values with/without addition of calcium chelating agents


Stage 1
Stage 2
Addition of calcium chelating agents and/or
increasing pH > 6.7
Heat treatment (90°C/10min)
Stage 3
Casein micelles
Native whey proteins
Dissociated caseins
Denatured whey protein aggregates
Denatured whey protein-dissociated casein aggregates

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Chapter 4

Heat Stability of Milk: Effect of pH at heating, milk concentration and added calcium chelating agents

4.1 Introduction

The ability of milk to withstand high processing temperatures without excessive thickening and coagulation is one of the key factors in the manufacturing of dairy products. Many physical and chemical changes that occur during these high temperature processes involving the milk proteins as was discussed in earlier chapters. These changes may manifest themselves as an increase in viscosity or as gelation of the milk system. The heat-induced gelation of the milk proteins and the excessive thickening in milk systems depend on the protein composition, the protein concentration, the processing conditions (heating and cooling rates), and environmental factors such as pH, ionic strength, and mineral composition (Panouille *et al.* 2004)**.**

This chapter examines the changes in the physical structure of milk as manifested by gelation and/or changes in viscosity on heating. These changes were examined as a function of initial pH at 25°C (pH 6.2-7.2), milk concentration (9%-21% w/w MSNF) and addition of various amounts of calcium chelating agents such as P_{in} and EDTA (10-30mM).

Two experimental techniques were used to monitor the increased viscosity and/or gelation during heat treatment. Viscosity measurements were carried out at 25°C prior to heating, at 90°C and at 25°C after cooling. The other technique used is Diffusing Wave Spectroscopy

(DWS), which was used to investigate the physical changes *in situ*. From the DWS measurements changes that occur in the product of the viscosity of the medium and particle size during heating are obtained. This technique allows the investigation of aggregation phenomena in a non-invasive manner and most importantly without need for dilution of the samples. The changes in bulk viscosity during heating were then compared with the DWS data, the two techniques being complementary to each other.

4.2 Materials and Methods

4.2.1 Preparation of Reconstituted Skim Milk Solutions

Reconstituted skim milk with and with out added calcium chelating agents were prepared and equilibrated as described in (Section 2.2.2).

4.2.2 pH Measurements

The pH measurements at 25°C and at 90°C were carried out as described in (Section 2.5).

4.2.3 Viscosity Measurements

The viscosity of the milk solutions were measured using a PAAR PHYSICA modular compact rheometer MCR 300 (Anton Paar GmbH, Anton-Paar-Straβe 20, 8054 Graz, Austria). The temperature was ramped from 25°C to 90°C (rate = 0.09°Cs^{-1}), then held at 90°C for 10 min and cooled to 25°C (rate = 0.09°Cs^{-1}). The viscosities were recorded every 30 seconds. At the end of the cooling cycle, the milk samples were held for a further 5 min

at 25°C before the measurements. A constant shear rate of 46.1/s was used on the assumption that all the milk solutions (≤ 21% w/w MSNF) behaved like Newtonian fluids (Hinrichs, 1999; Trinh *et al.* 2007).

4.2.4 DWS Measurements

DWS measurements were performed in the cross correlation mode in a transmission geometry using an instrument built in-house as described by McKinnon *et al.* (2008). The light source was a DL5038-021 operating at 635nm and delivering 35mW. It is controlled by a current controller(LDC205) and a laser diode temperature controller (TED200C) (Thorlabs Inc 435,Route 206 Newton,NJ 07860). The laser beam was expanded using a PS879-B Anamorphic prism to about 7mm diameter at the sample cell. The diffuse light was collected by a bifurcated ,Y configuration single mode optic fiber (Font Canada , 2242-173 street Surrey BC V3S 9Z7 Canada) operating at 633nm wavelength. The common end of the optic fiber was fitted with F230FC-B GRIN lence (Thorlabs Inc 435,Route 206 Newton, NJ 07860) which was used to collect the diffused light. The other two legs of the optic fiber were connected to two Hamamatsu H9305 photomultiplier detectors (Stantron Australia, Castle Hill NSW 2154) and a Flex03LQ-OEM correlator (Correlator.com , 15, Colart way, Bridge water, NJ 08807) was used to calculate the intensity correlation function. The sample cell was a 10mm depth, 10mm wide and 4.5cm in height made from special optical glass (Starna Pty Ltd , P.O.Box 6751,Baulkham Hills BC,NSW 2153). The sample cell was held in a TLC40E peltier temperature controlled cell holder (Quantum north west, 9723,W sunset Highway Spokane WA 99224-9426). In this work, the

correlation functions were accumulated over a period of 1 minute. The correlation functions and average intensities were collected at 1 min intervals over a time period of 15 min.

In an expanded beam mode DWS transmission experiment the field autocorrelation function $g_{(1)}(t)$, is obtained from the intensity autocorrelation function using the Siegert relationship:

$$g_{(2)}(t) = 1 + \left|g_{(1)}(t)\right|^2 \qquad [1]$$

And $g_{(1)}(t)$ is given by Weitz & Pine, 1993:

$$g_{(1)}(t) = \frac{\left(\frac{L/l^*+4/3}{z0/l^*+2/3}\right)\left\{\sinh\left[\frac{z0}{l^*}\sqrt{\frac{6t}{\tau}}\right] + \frac{2}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\frac{z0}{l^*}\sqrt{\frac{6t}{\tau}}\right]\right\}}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{\frac{6t}{\tau}}\right] + \frac{4}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\left(\frac{L}{l^*}\right)\sqrt{\frac{6t}{\tau}}\right]} \qquad [2]$$

where z_0 = the penetration depth

L = the sample thickness

τ = relaxation time

l* = photon transport mean free path - the length scale over which, in the multiply scattering regime, the direction of photon is fully randomized

l* is a function of both the scattering form factor F(q) and the structure factor S(q). The form factor is related to the particle size, indices of refraction of the particles and the dispersion medium and the wavelength of the laser light. The structure factor describes the positional correlation between particles. A change in l* reflects changes in both the form factor and the structure factor. For completely non interacting particles, which are fully

uncorrelated in space the value of l* depends only on the scattering form factor. In concentrated suspensions the spatial positions of the particles and their correlation may become significant. For interacting particles a change in l* will indicate some degree of change of interactions between particles since the structure factor is defined by inter particle interactions. When all other variables are constant changes in this parameter can be taken as indications of changing organization between the particles in a suspension. This enables us to gain some idea of the ordering of the system as the particles interact with their closet neighbors. These correlations in position affect the angular distribution of the scatter light and hence the turbidity and l*.

To calculate l*, the correlation functions of a range of latex samples were obtained (McKinnon *et al.* 2008) under conditions where inter particle interaction effects were negligible. Since these are well defined mono disperse systems with known diffusion coefficients (known values of τ), the values of l* could be obtained directly from the measured correlation function. The value of I_0 is obtained from the measured scattering intensity of a dilute latex sample with the form factor calculated from Mie theory. These calibrations were used to prepare a calibration curve of l* as a function of measured intensity I. The calibration curve was then used to determine the value of l* of an unknown sample from the measured transmitted light intensity I.

In the case of particles of radius r undergoing Brownian motion, the relaxation time (τ- Eq [2]) is given by,

$$\tau = \left(D_s\, k_0^2\right)^{-1}$$

where D_s = Diffusion coefficient

k = wave vector

and $D_s = k_B T \frac{1}{6\Pi\eta r}$

k_B = Boltzmann constant

T = Temperature

η = viscosity of the medium

r = radius of the particle

Thus knowing τ, (η*r) the product of viscosity of the medium and radius can be obtained.

* Theoratical background of DWS was obtained from Pine *et al.* 1988; Horne, 1989; Horne & Davidson, 1993; Van Keuren *et al.* 1993; Durian, 1995; Mason *et al.* 1997; Wijmans *et al.* 2000; Rega *et al.* 2001; Alexander *et al.* 2002; Rojas-Ochoa *et al.* 2002; Schffold, 2002; Hemar *et al.* 2003; Hohler *et al* 2003; Nicolas *et al*. 2003; Pinder *et al.* 2003; Dalgleish *et al.* 2004; Hemar *et al.* 2004; Alexander & Dalgleish, 2005; Eliot *et al.* 2005; Navabpour *et al*. 2005; Alexander *et al.* 2006; Pinder *et al.* 2006; Corredig & Alexander, 2007.

4.3 Results and Discussion

4.3.1 Skim Milk Solutions of 9 - 21% w/w MSNF

The viscosities at 25°C before heating, at 90°C (after 10 min) and after cooling to 25°C for 9-21% w/w MSNF milk solutions are given in Table (4.1). The pH unadjusted 9%-21% w/w MSNF milk solutions are given in bold italic in the table.

Table (4.1): The pH and viscosity changes of 9% - 21% w/w MSNF milk solutions

Sample	Initial pH at 25°C	pH at 90°C	Viscosity at 25°C before heating/ (cP)	Viscosity at 90°C/ (cP)	Viscosity at 25°C after heating/ (cP)	Increase ($Vis_{aft} > Vis_{bef}$) or Decrease ($Vis_{aft} < Vis_{bef}$)
	6.22	5.83	1.80	1.02	2.28	⇑
9%	6.40	5.97	1.79	0.69	1.84	⇑
	6.63	***6.15***	***1.89***	***0.71***	***1.64***	⇓
W/w	6.82	6.30	1.98	0.74	1.78	⇓
	7.02	6.46	2.05	0.73	1.80	⇓
MSNF	7.23	6.62	2.07	0.72	1.77	⇓
	7.45	6.80	1.98	0.62	1.66	⇓
	8.00	7.22	2.51	0.85	2.12	⇓
	8.52	7.61	3.39	0.94	3.24	⇓
	6.25	5.83	2.00	1.05	2.51	⇑
12%	6.39	5.94	2.04	1.03	2.32	⇑
	6.57	***6.07***	***2.22***	***0.89***	***2.19***	⇓
W/w	6.83	6.27	2.24	0.87	2.17	⇓
	7.01	6.41	2.42	0.83	2.17	⇓
MSNF	7.22	6.57	2.52	0.83	2.21	⇓
	7.55	6.82	2.66	1.80	2.16	⇓
	8.01	7.17	3.06	1.86	2.46	⇓
	8.50	7.55	5.64	2.46	4.07	⇓
	6.19	5.78	2.56	2.07	7.68	⇑
15%	6.40	5.93	2.56	1.53	4.00	⇑
	6.51	***6.00***	***2.62***	***1.43***	***3.56***	⇑
W/w	6.80	6.21	2.79	1.36	2.71	⇓
	6.98	6.34	2.88	1.17	2.83	⇓
MSNF	7.21	6.50	2.97	1.09	2.86	⇓
	7.61	6.79	4.25	1.63	3.47	⇓
	7.99	7.07	5.81	1.67	3.45	⇓
	8.50	7.43	9.65	1.84	5.49	⇓
	6.24	5.82	3.42	3.47	*40.1	⇑
18%	***6.47***	***5.98***	***3.66***	***1.88***	***5.35***	⇑
	6.62	6.09	3.57	1.57	4.01	⇑
W/w	6.83	6.24	3.83	1.35	3.8	⇑
	7.04	6.39	4.07	1.32	3.74	⇓
MSNF	7.21	6.51	4.31	1.26	3.88	⇓
	7.64	6.81	6.05	2.16	4.12	⇓
	8.03	7.07	7.67	2.30	4.44	⇓
	8.51	7.42	15.2	1.92	4.97	⇓

	6.19	5.75	4.28	15.0	*57.0	⇑
21%	***6.44***	***5.92***	***4.55***	***2.22***	****11.9***	⇑
	6.66	6.08	4.80	1.92	5.90	⇑
W/w	6.85	6.21	5.18	1.80	5.20	⇑
	7.02	6.33	5.66	1.41	4.64	⇓
MSNF	7.23	6.48	6.52	1.52	4.87	⇓
	7.68	6.80	9.60	2.08	5.63	⇓
	8.01	7.01	12.8	2.53	5.76	⇓
	8.51	7.36	23.8	2.16	5.66	⇓

Mean values & s.d. of (n=2) analyses
Pooled s.d. for viscosity = ± 0.120. * s.d. are ± 3.2
Pooled s.d. for pH = ± 0.02
The unadjusted milk solutions are in bold italic.

As the table demonstrates, the viscosity of unheated milk solutions increased with increasing milk concentration at a given pH. This is in accord with the results obtained by others (Trinh *et al.* 2007). The viscosity increase is mainly due to the increased disperse phase volume fraction of the milk systems with increase in milk concentration. There was also an increase in viscosity of unheated milk solutions as the pH increased (Table 4.1). This is due to the dissociation of caseins from the micelles, resulting in an increase in the disperse phase volume fraction in the system, and hence an increase in viscosity. Huppertz *et al.* 2008 had found that increasing milk pH up to pH = 10, reduced milk turbidity indicating the complete disintegration of the casein micelles.

Figure (4.1) shows the logarithmic change in viscosity of a skim milk solution as a function of the reciprocal of the temperature during heating to 90°C together with equivalent data from literature for water and a solutions of 5% lactose in water (Whitaker *et al.* 1927). Whitaker *et al.* (1927) showed similar behavior in heating skim milks to 80°C. Since the proteins and lactose are the main constituents of skim milk, the question rises as to whether

or not lactose would cause a change in viscosity as temperature is raised. The slopes of the functions in Figure (4.1) represent the activation energy for flow. It was clear that the presence of lactose increased the activation energy for flow compared with water whereas the proteins and minerals of the milk decreased the activation energy for flow and that in both cases the difference was relatively small.

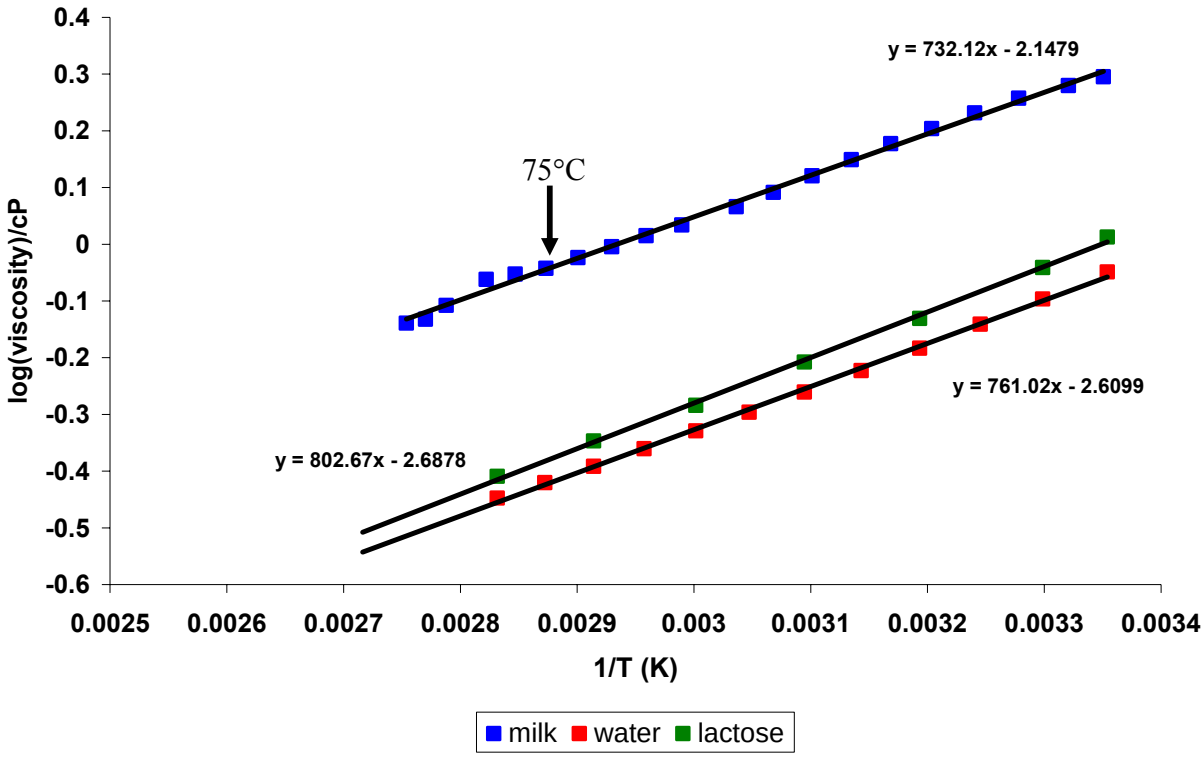


Figure (4.1) : The changes in viscosity as a function of temperature for 9% w/w MSNF milk solution at an initial pH of 6.8. The graph contain plots of the changes in viscosity of water and of a 5% lactose solution as a function of temperature

In Figure (4.1) the curve representing milk shows a decrease in slope at approximately 75°C, the temperature of the on set of whey protein denaturation. Jeurnink & de Kruif, (1993) had compared the viscosity for milk samples heated at 85°C and cooled to 25°C with the viscosity at 25°C before heating. For skim milk solutions with native whey proteins they observed an increase in viscosity after the heat treatment. However, they did not observe an increase in viscosity in the absence of whey proteins. Milk solutions where the native whey proteins had been replaced by the already denatured (and aggregated) whey proteins also showed no increase in viscosity after the heat treatment. Hence, it was found that the aggregated whey proteins did not lead to an increase in viscosity during heating. Hence, they concluded that the denaturation of the whey proteins during heating is primarily responsible for the increase in viscosity, where the unfolding of the whey proteins is one of the key reactions in increasing the viscosity during heat treatment (Jeurnink & De Kruif, 1993). The results in the present study confirm the importance of the contribution from the unfolding of the whey proteins during heat treatment.

Typical measured field auto correlation function $g_1(t)$ of 9% w/w MSNF milk solutions at pH 6.2 heated for 10 min at each different heating temperatures ranging from 25°C to 120°C are reported in Figure (4.2). At 50°C, the decay of the correlation function was faster than that at 25°C due to the increase in the particle mobility as a consequence in the decrease in the viscosity of the continuous phase upon heating. At 60°C, the correlation function moved further to the left. However, at 75°C and 90°C the correlation functions moved towards the right of the correlation function at 60°C, but still lay left to the correlation function at 50°C. However, at 120°C, the decay of the correlation function was

much slower than that at 75°C, 90°C or 25°C. This is due to the increase in particle size and the viscosity of the milk solutions as described by McKinnon *et al.* 2008. During heat treatment at temperatures >75°C, the major whey proteins denature and begin to interact with each other and with casein micelles changing the colloidal properties of the casein micelles (Dalgleish *et al.* 1987; Singh & Creamer, 1991; O'Connell & Fox, 2001; Vasbinder *et al.* 2001; Anema *et al.* 2006). This will lead either to an increase in particle size (due to the layer of whey proteins attached to the casein micelles) or to an increase in viscosity of the medium (due to an increase in volume fraction due to the denaturation of whey proteins). As a consequence, the decay of the correlation functions should be expected to be slower. At the same time, the viscosity of the medium decreased with increase in temperature up to 90°C as was indicated in Figure (4.1). This would lead the decay of the correlation functions to be faster. However, the correlation functions for 75°C and 90°C lie in between the correlation functions obtained at 50°C and 60°C as was discussed before. At 120°C, the decay of the correlation function was much slower with the correlation function shifted towards the right, indicating the considerable increase in particle size and/or particle interactions. It can be clearly seen that the 60°C correlation function had only one observable relaxation, while at 120°C the correlation function was clearly different and it is characteristic of a gel formation. However, at longer correlation times at high temperatures, the correlation function is less defined due to the slow relaxation of the gelled system, results in a reflection of fewer scattering events due to network formation. However, 9% w/w MSNF milks showed no signs of visible coagulation at 10 min nor on removal after 15 min.

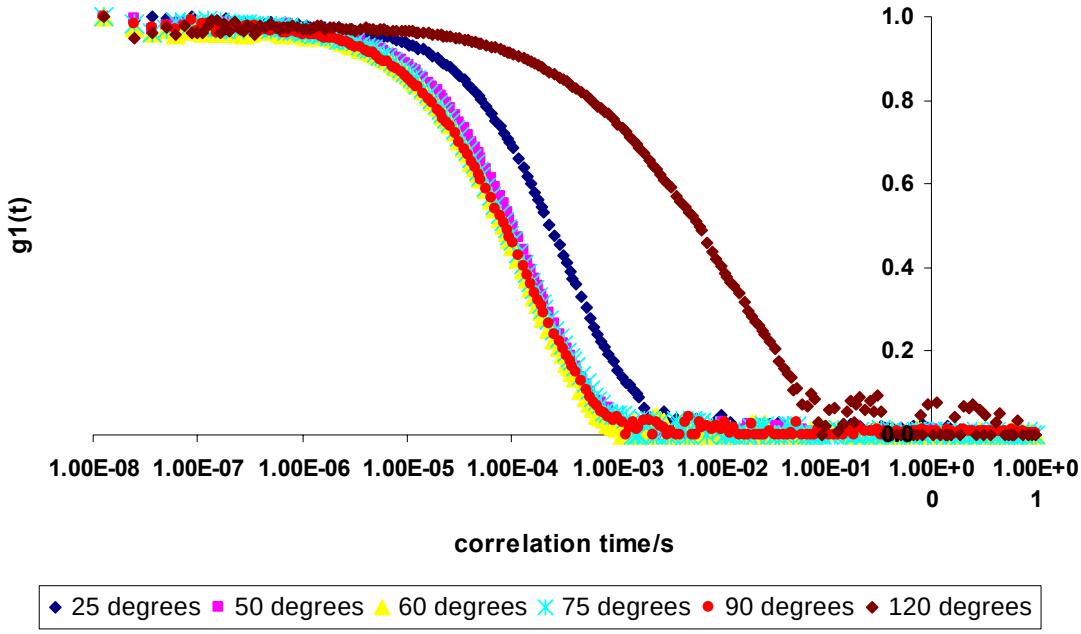


Figure (4.2) : The correlation function plots of 9% w/w MSNF milk solution at pH 6.2 as a function of different temperatures heated from 25℃ to 120℃ (Fresh portions of the prepared milk solution were heated at each temperature for 10 min)

The 10 minute correlation functions of the 21% w/w MSNF milk solution at pH 6.2 at temperatures of 60°C and 120°C are shown in Figure (4.3). The correlation function for the 21% w/w MSNF milks at 120°C is very similar to that of 9% w/w MSNF milks indicating signs of extensive aggregation, but no gelation. However, the correlation functions after 12 minutes (not shown) exhibited clear signs of gel formation. Samples on removal after 15 minutes showed visible strong gelation.

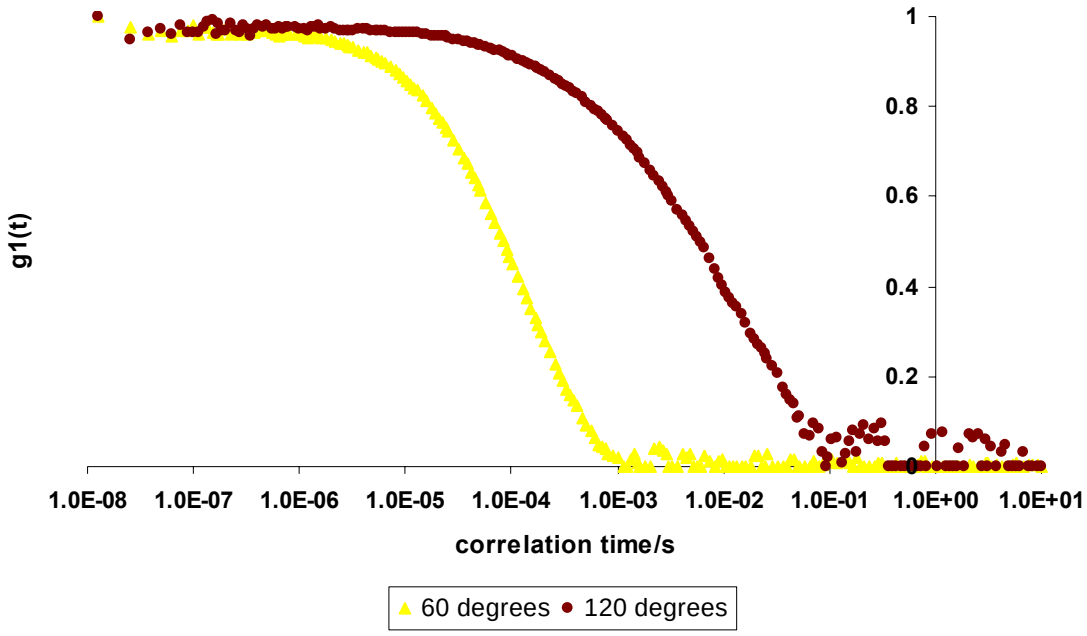
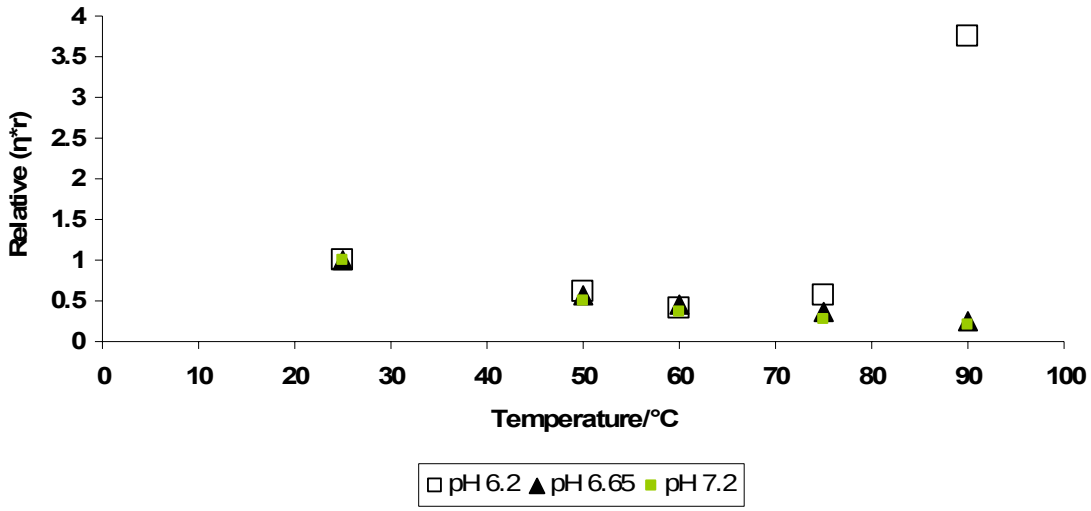


Figure (4.3): The correlation function plots of 21% w/w MSNF milk solution at pH 6.2 as a function of temperatures heated at 60°C and 120°C

Comparison of the effect of temperature on different milk solutions is most easily made using the relative ηr, which is defined as ηr at a given temperature divided by the value of ηr at 25°C. The relative product of viscosity*particle radius (ηr) as a function of heating temperature for 9% and 18% MSNF milk solutions at three different initial pH values of 6.20, 6.65 and 7.20 at 25°C are depicted in Figure (4.4*a* & 4.4*b*) respectively. Up to a heating temperature of around 60°C for all milk concentrations ηr decreased, mainly due to the decrease in viscosity of the continuous phase with increase in temperature. With 9% & 18% w/w MSNF milk solutions at pH 6.2, there was an increase in ηr when heated at temperatures ≥ 75°C (Figure (4.5*a* & 4.5*b*)). This is due to the denaturation of whey proteins and their subsequent interaction with the casein micelles and with themselves.

These results are in accordance with the proposed model by Vasbinder & de Kruif, (2003), where at lower pH values most of the denatured whey proteins/whey protein aggregates are associated with the casein micelles. Anema & Li, (2003) found that for milk solutions that have been subjected to heat treatment and then cooled back to 25°C larger increases in casein micelle size at lower pH values and smaller changes in casein micelle size as the pH of the milk was increased were observed. The single strength (9% w/w MSNF) milk solutions at high pH values of pH 6.65 and 7.2 showed a continual decrease in ηr at all heating temperatures, in contrast the concentrated (18% w/w MSNF) milk solutions with pH values ≥ 6.65 showed a small increase in the relative ηr at 90°C.

(a)



(b)

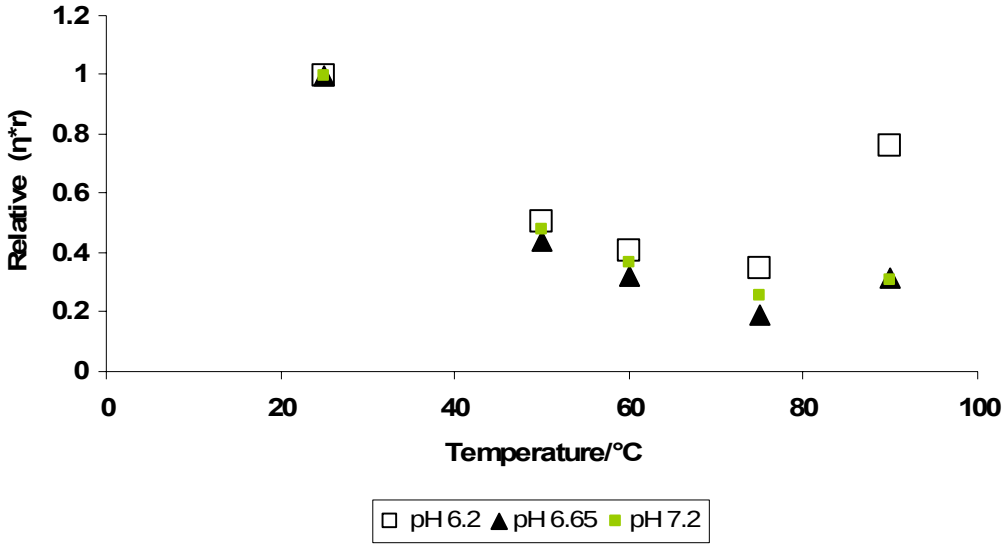


Figure (4.4): Relative ηr as a function of heating temperature for (a) 9% (b) 18% w/w MSNF milk solutions. The initial pH values of milks at 25℃ are 6.2, 6.65 and 7.2

The observed increases in the relative ηr heating at temperatures ≥ 75°C for milk solutions at pH 6.2 were quite larger than the expected size increase due to the association of whey proteins to the casein micelles. Jeurnink & de Kruif, (1993) found that the increase in viscosity due to heating skim milk could not be explained by an increase in volume fraction alone. The application of the Batchelor Russel theory to their work indicated that there is also an increase in attraction between micelles. However, the attractions between micelles are weak. Hence, they suggested that there is no permanent clustering of the micelles but instead temporary clusters of micelles are present. Bienvenue *et al.* (2003) had found that the association of the denatured or aggregated whey proteins at the micelle surface of

concentrated (≥ 40%) heat treated milks led to an increase in effective volume fraction. In addition, due to the other changes during heating such as changes in pH, led to association of denatured whey proteins with the casein micelles and further interactions of casein micelles through their adsorbed denatured whey proteins. Therefore the large increase in viscosity is a combination of the effects of increased volume fraction and increased interactions between micelles. The results obtained in the present study also confirms the fact that the increase in the relative ηr at temperatures ≥ 75°C for low pH milk solutions is not merely to the observed size changes of the casein micelles, but a combination effect of the size increase and the increased interactions between denatured whey proteins associated casein micelles.

The product of viscosity*particle radius (ηr) at 90°C as a function of time for a period of 13 min for 9% w/w MSNF milk solution at an initial pH value of 7.20 at 25°C is shown in Figure (4.5). According to chapter 3, it was found that at higher pH values the denatured whey proteins tend to form soluble aggregates with the caseins that are already dissociated to the serum phase rather than attaching to the casein micelles. The data from Anema, (2007), which suggested that the size of the casein micelles at 25°C after heat treatment decreased at high pH values, showed a decrease by a factor of 0.86. At the same time, if the viscosity of the serum decreases by the same factor as water, then ηr would be expected to change by the factor 0.35. However, the present data showed a decrease in ηr of a factor of approximately 0.2. It clearly suggests either a larger decrease in particle size or either a larger decrease in the viscosity of the medium. The first two points are the time taken for thermal stabilization of the milk solution.

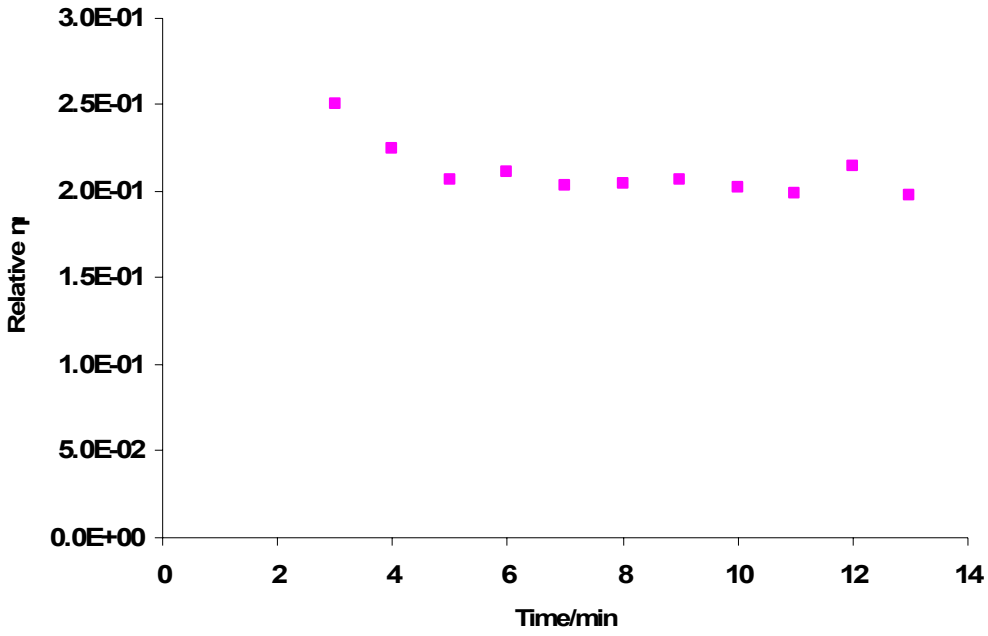


Figure (4.5): Relative ηr as a function of time for heated (at 90℃) 9% w/w MSNF milk solution with an initial pH at 25℃ of 7.2

In order to investigate the on set of irreversible denaturation of the proteins within milk systems, the heat treated milk solutions at different temperatures ranging from 50°C to 90°C for 10 min were equilibrated overnight after the heat treatment and DWS measurements were done on re-equilibrated milk solutions. The skim milk solutions heated at 50°C,60°C and 75°C did not show significant differences from the initial values at 25°C before heating. However, the product of ηr at 25°C after the heat treatment at 90°C showed a marked increase in comparison to the ηr at 25°C before the heat treatment indicating the irreversible behavior of the milk proteins heated at temperatures greater than 90°C. This is in accordance with Panouille *et al.* (2004) where they found decreased degree of

reversibility with increasing heating time and temperature obtained through dynamic light scattering.

The data in Table (4.1) shows considerable variations in viscosity with respect to pH and milk concentration at 25°C and also the effects of heat treatment on viscosities as a function of pH and milk concentration. Those data are represented in figures for each of the milk concentrations (9%-21% w/w MSNF) studied. Figures (4.6*a*– 4.6*e*) show the viscosity measurements at 25°C before heating, at 90°C and after cooling to 25°C for 9%- 21% w/w MSNF milk solutions as a function of initial pH of 6.2 to 8.5 at 25°C.

The viscosities at 25°C before heat treatment for higher pH milk solutions increased with increase in pH irrespective of the milk concentration. This is mainly due to the increased dissociation of caseins from the casein micelles with increase in pH as was discussed in Chapter 3- Section (3.3.1.1). In general, the viscosity at 90°C was as expected lower than the viscosity at 25°C before heating. In contrast, at pH=6.2 where the denatured whey proteins were mostly associated with the casein micelles giving rise to increased interactions between the particles, the viscosity at 90°C of 21% w/w MSNF milk solution was larger than its value at 25°C prior to heating (Figure 4.6*e*). On cooling to 25°C after heat treatment concentrated milk solutions at low pH values showed a marked increase in viscosity compared with the values before heating (Figure 4.6*e* & 4.6*d*). At lower concentrations the increase in viscosity is much less marked (Figure 4.6*a* to 4.6*c*). At high pH values, where the denatured whey proteins form soluble aggregates with the caseins that were already dissociated from the casein micelles giving rise to increased amounts of

soluble aggregates the dependence on milk concentration of the increase in the viscosity on cooling ($\eta_{\text{after cooling to 25°C}}/\eta_{\text{at 90°C}}$) was relatively small.

At an initial pH =8.5;

η (9% MSNF, after cooling to 25°C)/η(9% MSNF, at 90°C) = 3.4

η (21% MSNF, after cooling to 25°C)/η(21% MSNF, at 90°C) = 2.5

(Data taken from Table 4.1)

This is in contrast with the change in viscosity on heating, where the dependence on milk concentration of the decrease in the viscosity was large.

At an initial pH =8.5;

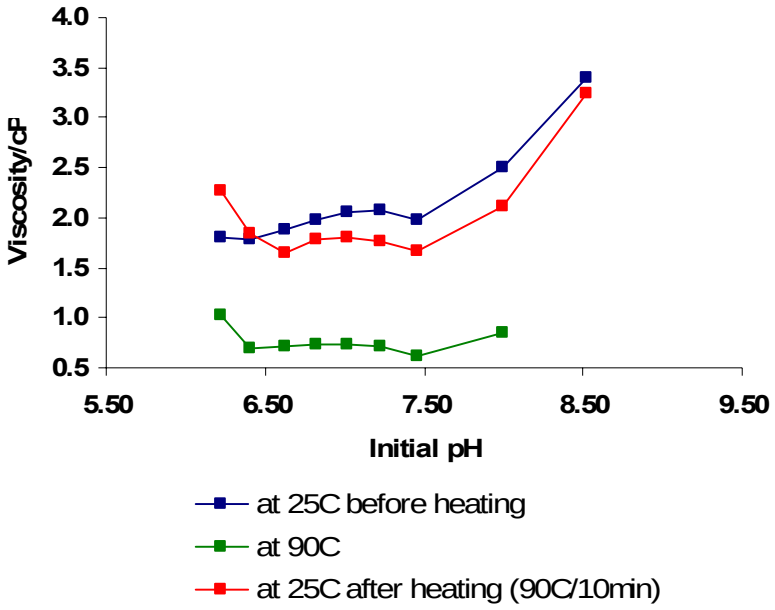
η (9% MSNF, 25°C prior to heating)/η(9% MSNF, at 90°C) = 3.6

η (21% MSNF, 25°C prior to heating)/η(21% MSNF, at 90°C) = 10.8

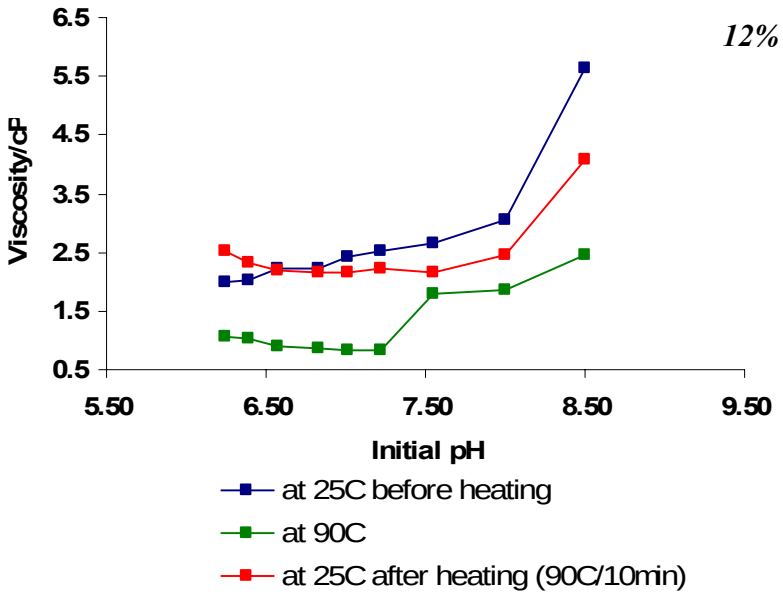
(Data taken from Table 4.1)

(a)

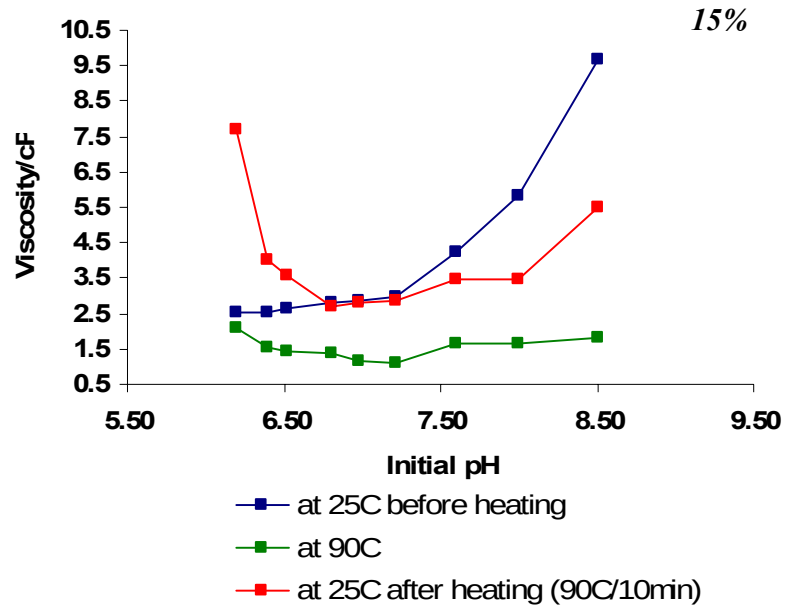
9%


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Viscosity/cP
5.50
6.50
7.50
8.50
9.50
Initial pH
at 25C before heating
at 90C
at 25C after heating (90C/10min)

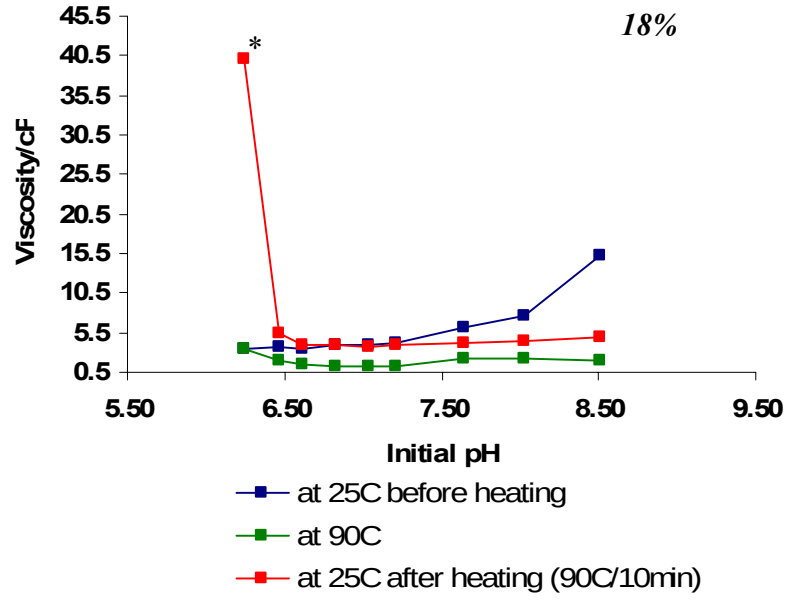
(b)


12%
6.5
5.5
4.5
3.5
2.5
1.5
0.5
Viscosity/cP
5.50
6.50
7.50
8.50
9.50
Initial pH
at 25C before heating
at 90C
at 25C after heating (90C/10min)

(c)


15%
Viscosity/cF
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
5.50
6.50
7.50
8.50
9.50
Initial pH
at 25C before heating
at 90C
at 25C after heating (90C/10min)

(d)


18%
*
Viscosity/cF
45.5
40.5
35.5
30.5
25.5
20.5
15.5
10.5
5.5
0.5
5.50
6.50
7.50
8.50
9.50
Initial pH
at 25C before heating
at 90C
at 25C after heating (90C/10min)

(e)

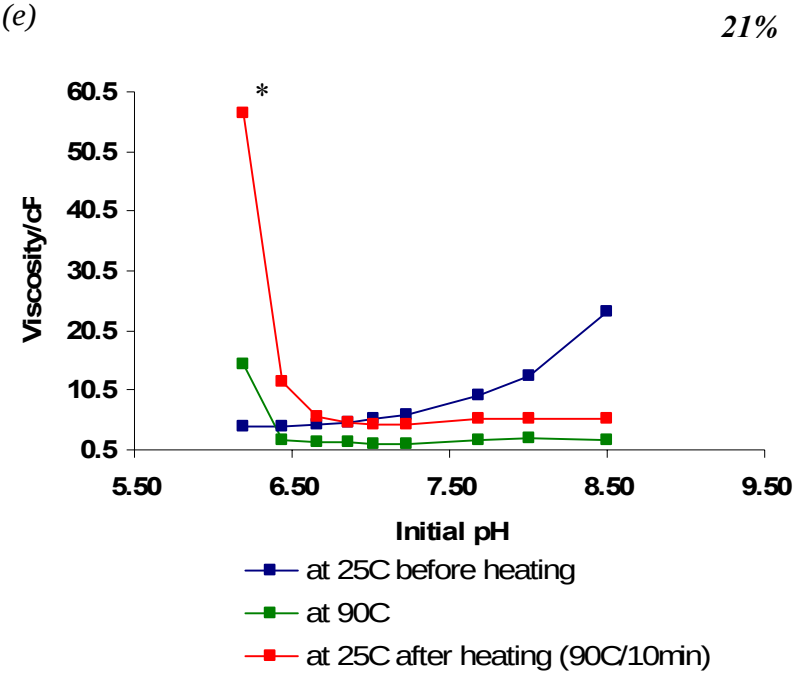
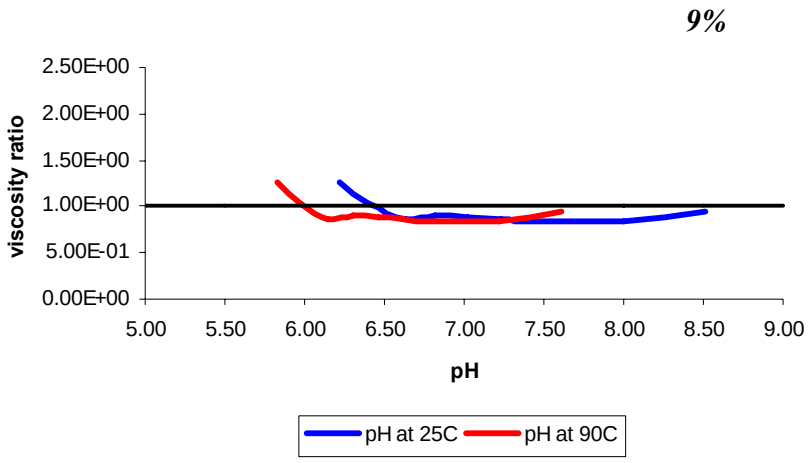


Figure (4.6) : The changes in viscosity as a function of initial pH for 9% to 21% w/w MSNF milk solutions (a-e respectively) at 25℃ before heating, at 90℃ and at 25℃ after heating

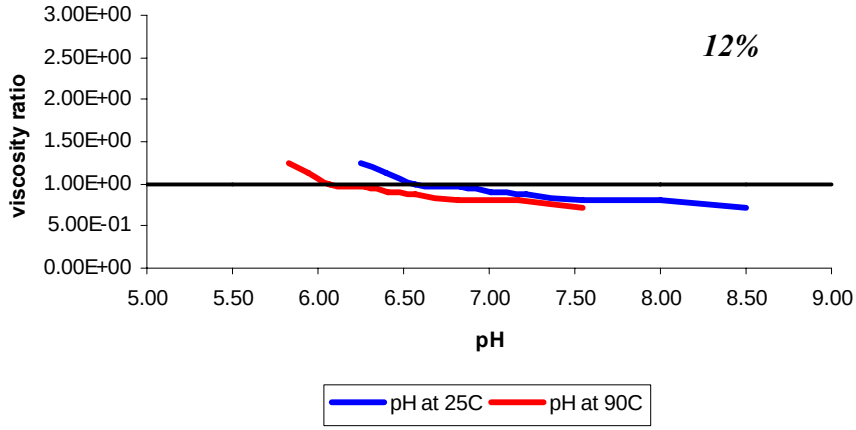
In the present study, the heat stability was assessed using the ratios of the viscosities before heating (η_{before}) and on cooling to 25°C after heat treatment (η_{after}). It was assumed when ($\eta_{after}/\eta_{before}$)> 1 indicated that the milk solutions were susceptible to thickening. A second indicator, was if the milk solutions gelled on cooling to 25°C. In Figures (4.6*d*) & (4.6*e*) these milks are denoted by *. One means of comparing the heat stability of milk solutions between various milk systems was to use the "minimum pH of thermal stability". This was taken as the minimum pH (initial pH at 25°C or pH at 90°C) at which ($\eta_{after}/\eta_{before}$) > 1. The curves in Figures (4.6*a*–4.6*e*) represent the viscosity ratio ($\eta_{after}/\eta_{before}$) of 9%-21% w/w MSNF milk solutions as a function of pH. In each graph, the blue curve is the viscosity

ratio as a function of initial pH at 25°C and the red curve the viscosity ratio as a function of the pH at 90°C.

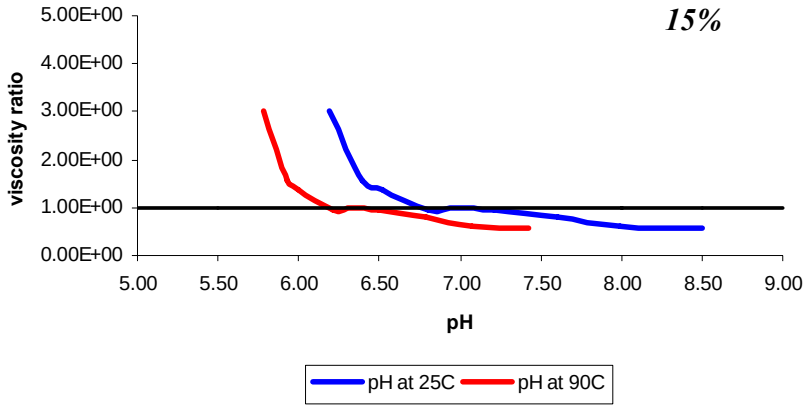
(a)



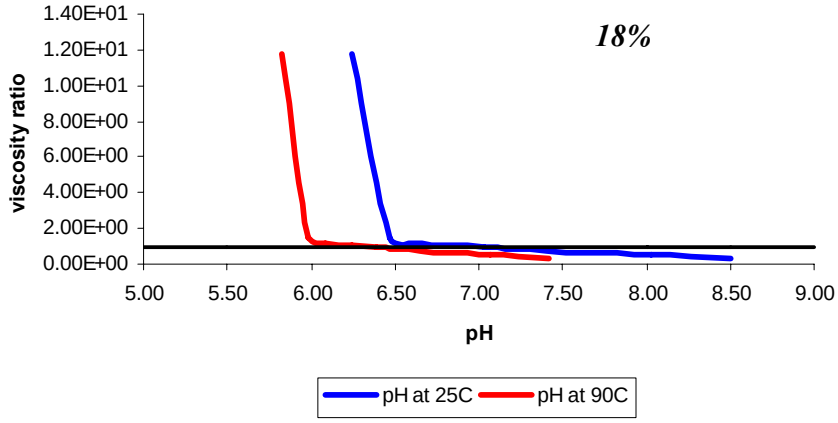
(b)



(c)



(d)



(e)

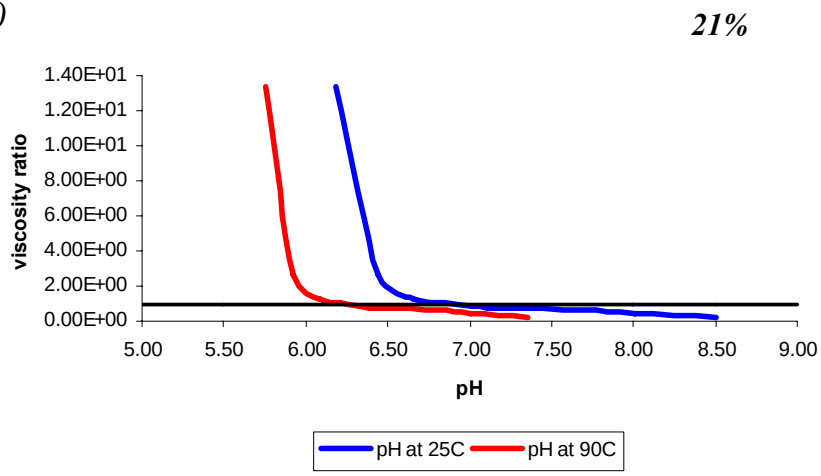


Figure (4.7) : The relative viscosity as a function of initial pH at 25℃ and at 90℃ for 9% to 21% w/w MSNF milk solutions (a-e respectively)

The viscosity ratio profiles of 9%-21% w/w milk solutions (Figures (4.6*a*– 4.6*e*)) had similar features. The "minimum pH at 25°C/90°C of thermal stability" for the concentrated milks (15%,18% & 21% w/w MSNF) were similar with values of pH=6.8 at 25°C and pH=6.2 at 90°C. For more dilute milks (9% & 12% w/w MSNF) the "minimum pH at 25°C/90°C of thermal stability" was pH=6.4 at 25°C and pH=6.0 at 90°C. A possible explanation is through consideration of the denaturation of the whey proteins and their subsequent association with the casein micelles and the interaction between casein micelles coated with denatured whey proteins. As the pH increases the fraction of denatured whey proteins attached to the casein micelles decreases with the remainder forming the soluble aggregates. The decrease in the extent of the attachment between the denatured whey proteins and the casein micelles leads to lesser interaction between the micelles. In the more concentrated milks with the increased number of micelles and increased amount of whey proteins the interactions between the micelles are significant at higher pH values.

4.3.2 9% & 18% w/w MSNF solutions with added Calcium Chelating Agents (P_{in} & EDTA)

In previous work, it was shown that the addition of calcium to milk increased the drop in pH on heating and that the minimum pH for stability of the milks studied was found to be correlated with pH at 90°C rather than the initial pH at 25°C of the milk prior to heating (McKinnon *et al.* 2008). The viscosity measurements for milk solutions with added calcium chelating agents are presented in Tables (4.2*a* & 4.2*b*). In the tables, columns 2 and 3 report the pH values at 25°C and at 90°C respectively. The columns 4,5 and 6 reports the

viscosity measurements at 25°C before heating, at 90°C and viscosity on cooling to 25°C after heating for 9% & 18% w/w MSNF milk solutions with added P_{in} (Figure 4.2*a*) and EDTA (Figure 4.2*b*) respectively.

The viscosities at 25°C before the heat treatment for milk solutions with added EDTA increased irrespective of the milk concentration in comparison with the viscosities at 25°C before the heat treatment for milk solutions without the additions at a given pH (Table 4.2*a* & 4.2*b*). Addition of EDTA complexes the calcium ions present in the serum phase, resulting in colloidal calcium phosphate to dissolve and thereby dissociate some caseins from the casein micelles (Van boekel *et al.* 1989). This will lead to an increase in disperse phase volume fraction which will result in an increase in viscosity. However, the viscosity of milk solutions with the addition of P_{in} behaves in a much more complicated manner than the addition of EDTA.

Table (4.2): The pH and viscosity changes of 9% & 18% w/w MSNF milk solutions with/without added (a) P_{in} (b) EDTA

(a)

Sample	Additions to milk / (mmol/kg of solution)	Initial pH at 25°C	pH 90°C	Viscosity at 25°C before heating/ (cp)	Viscosity at 90°C/ (cP)	Viscosity at 25°C after heating/ (cP)
9%	0	6.23	5.83	1.80	1.02	2.28
	10	6.23	5.87	2.57	1.16	2.18
w/w	20	6.23	5.89	2.20	1.65	2.05
	30	6.21	5.91	1.85	1.20	1.91
MSNF						
	0	6.66	6.15	1.89	0.71	1.64
	10	6.62	6.26	2.62	1.88	1.79
	20	6.59	6.29	2.34	1.10	1.73
	30	6.58	6.29	2.38	0.94	1.75
	0	7.20	6.62	2.07	0.95	1.82
	10	7.17	6.83	2.84	0.81	1.84
	20	7.18	6.82	2.78	0.94	1.78
	30	7.18	6.81	2.68	1.08	1.87
18%	0	6.22	5.82	3.42	3.47	40.1*
	10	6.25	5.87	3.00	5.09	30.7*
w/w	20	6.25	5.90	2.89	8.36	28.9*
	30	6.25	5.95	3.09	10.4	27.0*
MSNF	0	6.63	6.09	3.57	1.57	4.01
	10	6.63	6.14	3.78	2.39	3.90
	20	6.64	6.20	4.05	2.55	4.14
	30	6.63	6.22	4.33	2.01	4.07
	0	7.19	6.51	4.31	1.26	3.88
	10	7.22	6.58	6.81	2.43	4.93
	20	7.20	6.60	7.34	1.98	4.73
	30	7.18	6.61	8.80	1.80	4.21

Mean values & s.d. of (n=2) analyses
Pooled s.d. for viscosity = ± 0.161 * s.d. are ± 2.8
Pooled s.d. for pH = ± 0.02

(b)

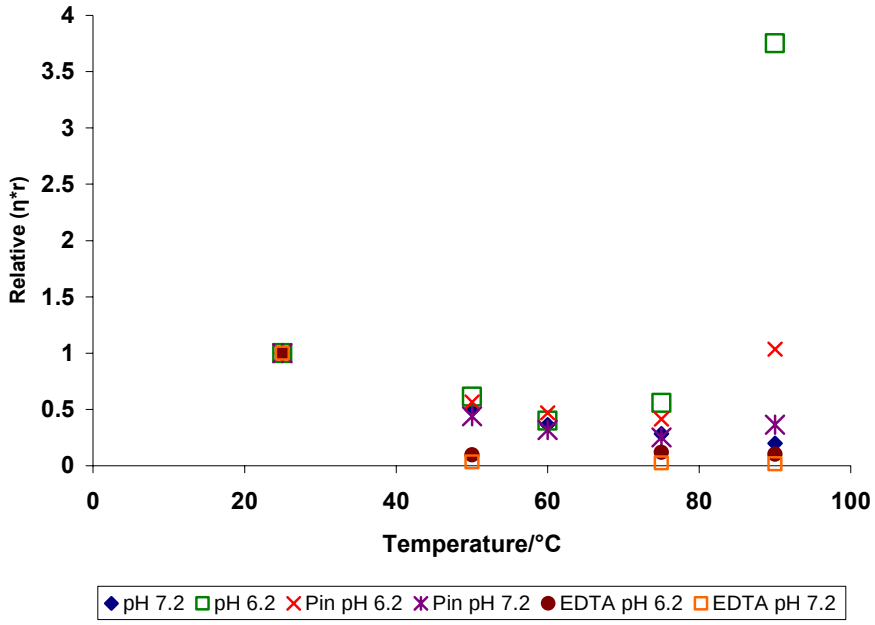
Sample	Additions to milk / (mmol/kg of solution)	Initial pH at 25°C	pH 90°C	Viscosity at 25°C before heating/ (cp)	Viscosity at 90°C/ (cP)	Viscosity at 25°C after heating/ (cP)
9%	0	6.23	5.83	1.80	1.02	2.28
	10	6.28	5.83	1.97	0.86	2.73
w/w	20	6.23	5.95	2.15	0.83	2.43
	30	6.23	5.98	2.12	0.86	2.30
MSNF	0	6.66	6.15	1.89	0.71	1.64
	10	6.62	6.25	2.00	0.68	1.90
	20	6.62	6.37	2.17	0.73	1.87
	30	6.63	6.33	2.27	0.75	1.85
	0	7.20	6.62	2.07	0.95	1.82
	10	7.24	6.81	2.05	0.79	1.92
	20	7.25	6.83	2.19	0.68	1.94
	30	7.22	6.82	2.41	0.75	1.90
18%	0	6.22	5.82	3.42	3.47	40.1*
	10	6.21	5.88	3.95	13.1	40.3*
w/w	20	6.24	5.89	3.98	6.05	22.9*
	30	6.22	5.91	5.17	5.53	12.9*
MSNF	0	6.63	6.09	3.57	1.57	4.01
	10	6.66	6.15	4.02	1.52	4.80
	20	6.63	6.19	4.77	1.74	5.11
	30	6.64	6.17	5.41	1.50	5.64
	0	7.19	6.51	4.31	1.26	3.88
	10	7.24	6.54	5.90	1.44	4.18
	20	7.20	6.60	6.86	2.06	4.55
	30	7.25	6.63	7.90	2.20	5.98

Mean values & s.d. of (n=2) analyses
Pooled s.d. for viscosity = ± 0.152 * s.d. are ± 3.0
Pooled s.d. for pH = ± 0.02

The relative product of viscosity*particle radius (ηr) (DWS-Section 4.2.4) as a function of heating temperature for 9% (Figure 4.8*a*) and 18% (Figure 4.8*b*) MSNF milk solutions at two different pH values of 6.2 and 7.2 with /without addition of 20mM calcium chelating

agents (P_{in} and EDTA) are shown. At a given pH, the relative ηr is defined as in Section (4.3.1). All the 9% w/w MSNF milk solutions with added P_{in} and EDTA showed a decrease in relative ηr up to a heating temperature of around 60°C. In the present study, it was found that 9% w/w MSNF milk solutions at pH 6.2 without the addition of P_{in} or EDTA showed an increase in relative ηr at temperatures ≥ 75°C as was described in Section (4.3.1). The milks containing the added salts had a lesser increase in the relative ηr at temperatures ≥ 75°C (Figure 4.8*a*). The addition of EDTA disrupts the casein micelle structure (Montilla & Calvo, 1997) and hence favors the association of denatured whey proteins with caseins that are already dissociated from the casein micelles leaving few denatured whey protein left in the serum phase as was described in Chapter 3 in the present study. This will lead no noticeable effects of the size of casein micelles and reduce the interaction between casein micelles coated with the denatured whey proteins, resulting in a lesser increase in ηr. This phenomena will be discussed in Section (4.4) with the use of a schematic representation.

(a)



(b)

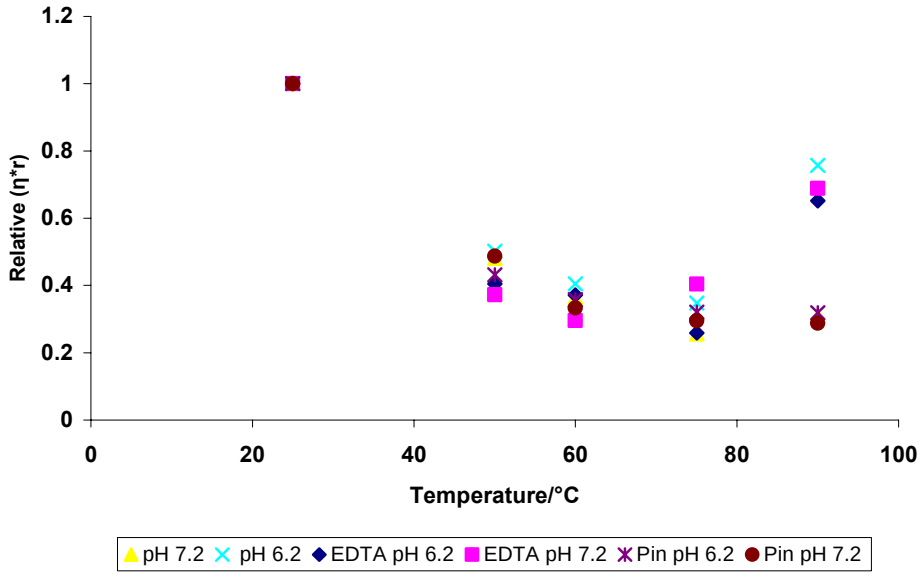


Figure (4.8): ηr as a function of heating temperature for (a) 9% (b) 18% w/w MSNF milk solutions with/without addition of P_{in} and EDTA. The initial pH values of milks at 25℃ are 6.2 and 7.2

In the present study, the heat stability was assessed using the ratio of $(\eta_{after}/\eta_{before})$. It was assumed when $(\eta_{after}/\eta_{before})> 1$ indicated that the milk solutions were susceptible to thickening as was discussed before. It was found in the present study that the "minimum pH at 25°C/90°C of thermal stability" for the concentrated milks (15%,18% & 21% w/w MSNF) were similar with values of pH=6.8 at 25°C and pH=6.2 at 90°C. For more dilute milks (9% & 12% w/w MSNF) the "minimum pH at 25°C/90°C of thermal stability" was found to be pH=6.4 at 25°C and pH=6.0 at 90°C as was described in Section (4.3.1). However, the "minimum pH at 25°C of thermal stability" for 18% w/w MSNF milk solutions with added calcium chelating agents was found to be ~pH 6.65, although the "minimum pH at 90°C of thermal stability" was found as pH 6.2 at 90°C. The "minimum pH at 90°C of thermal stability" for milk solutions with the additions was consistent with the milk solutions without the additions in the present study. This is accordance with Augustin & Clark, (1990), where they suggested a need of proper adjustment of the pH of the systems to obtain maximum thermal stabilization. However, the viscosities of 9% w/w milk solutions with added P_{in} did not show a "minimum pH value of thermal stability" in the pH range of 6.2 to 7.2 at 25°C. It clearly highlights the more complicated behavior of P_{in}. Nevertheless, the results obtained highlighted importance of the mineral composition of the milk systems, which controls the pH at the temperature of heating for the thermal stability of milk systems. The pH dependencies of the viscosity and the particle volume are a consequence of the pH dependence of the interaction of denatured whey proteins with the micelles which is supported by the inter relationships between viscosity, micelle volume and heat induced whey protein association with the micelles (Anema, 2004). Hence, there

will be increased further association of the casein micelles consisting denatured whey proteins via sulfhydryl-disulfide, hydrophobic interactions and H bonding leading to increased crosslinking bridging between the micelles (Bieunvenue *et al.* 2003; Surel & Famelart, 2003). In the present study, it was found that these interactions are mainly governed by the pH at the temperature of heating. The following table (4.3) summarizes the "minimum pH value of thermal stability" for all the milk systems studied.

Table (4.3): The "minimum pH at 25°C/90°C of thermal stability" for all the milk systems studied

Sample	"minimum ~pH at 25°C of thermal stability"	"minimum ~pH at 90°C of thermal stability"
9% w/w MSNF	6.40	6.0
12% w/w MSNF	6.45	6.0
15% w/w MSNF	6.80	6.2
18% w/w MSNF	6.83	6.2
21% w/w MSNF	6.85	6.2
9% w/w MSNF with P_{in}	Stable	Stable
9% w/w MSNF with EDTA	6.25	6.0
18% w/w MSNF with P_{in}	6.65	6.2
18% w/w MSNF with EDTA	6.65	6.2

4.4 Conclusion

The viscosity of unheated milk solutions increased with increase in milk concentration and milk pH. This is due to the larger number of particles and dissociation of caseins from the casein micelles which leads to increased volume fraction. The addition of EDTA to milk solutions increased the viscosity at 25°C before heating relative to the milk solutions without the EDTA. This effect is due to the dissociation of caseins from the casein micelles caused by removal of micellar calcium resulting in an increased volume fraction of solutes within the serum.

DWS measurements allowed the examination of the particle size changes along with the viscosity changes of the medium during heating of milk solutions. The correlation functions obtained at each heating temperatures of ≤ 90°C, showed increased particle mobility due to the decrease in the viscosity of the continuous phase upon heating. However, The correlation functions obtained at temperatures of greater than 90°C, showed decreased particle mobility due to the interactions between the micelles resulting in from the adsorption of the denatured whey proteins on to casein micelles. Gel formation can be clearly identified from the correlation plots as initial relaxation and a long non-exponential decay. The rheological measurements are performed by applying a stress and measuring the resulting in deformation. This deformation may disrupt structures held together by very weak forces (Hemar *et al.* 2004). DWS is a non disruptive technique giving effectively values of ηr at zero strain. Hence, DWS is more suitable in monitoring the formation of weak structures *in situ*.

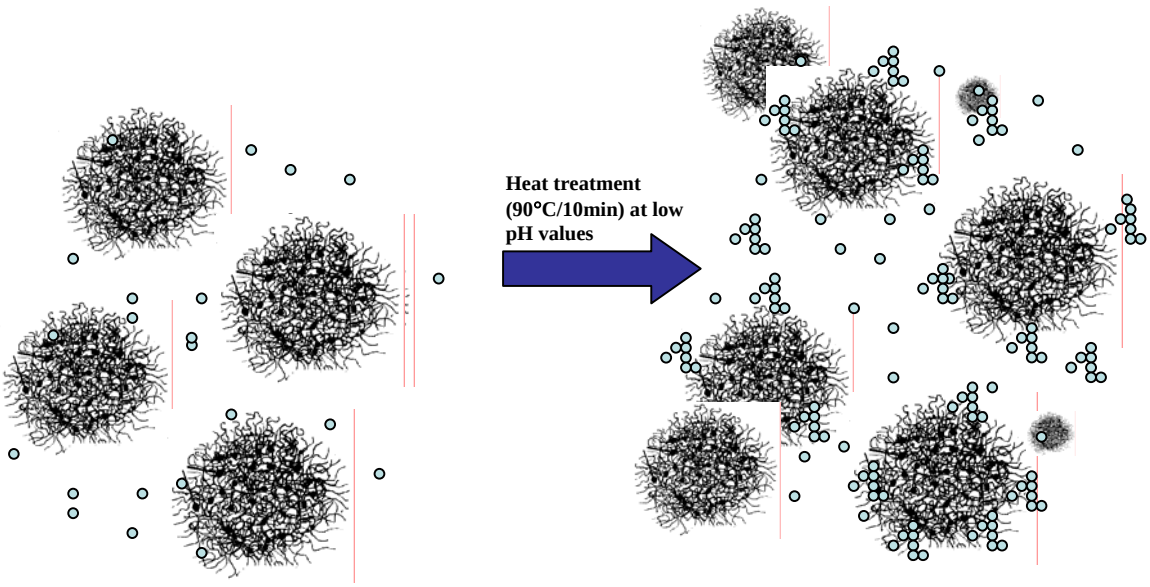
The relative ηr showed an increase for milk solutions at pH 6.2 and at temperatures ≥ 75°C. This highlights the increased association of denatured whey proteins to casein micelles and interaction of casein micelles coated with denatured whey proteins as pH decreases. However, at high pH values (pH ≥ 6.65), the relative ηr showed a gradual decrease with increase in temperature. The addition of calcium chelating agents at lower pH values showed a lesser ηr increase compared to that of the ηr of the milk solutions at that particular pH without the additions. The main reason will be explained in terms of the schematic representation in Figure (4.8).

Heat treatment at low pH values, will result more casein micelles associating with denatured whey proteins or whey protein aggregates as illustrated in Figure (4.8*a*). This will result an increase in casein micelles size due to the increased layer of denatured whey proteins attached. However, at the same time there will be increased interactions between casein micelles coated with the denatured whey proteins. This will lead to increased disperse phase volume fraction leading to increased viscosity. Heat treatment at high pH values, will result more denatured whey proteins associated with the dissociated caseins from the casein micelles resulting in the formation of soluble aggregates. This will lead to an decrease in size of the casein micelles due to the increased dissociation of caseins. At the same time, there will be decreased interactions between the casein micelles (Figure 4.8*b*), leading to decreased viscosity.

It can be concluded that the heat stability of a particular milk system is predominantly governed by the pH at the temperature of heating, which is primarily determined by the

milk composition particularly the milk concentration and free calcium. The use of added Pin and calcium chelating agents affects the pH at the temperature of heating and control the extent of association of denatured whey proteins with the casein micelles. The two experimental techniques used in the present study to monitor the increased viscosity and/or gelation during heat treatment has found to be complimentary with each other.

(a)



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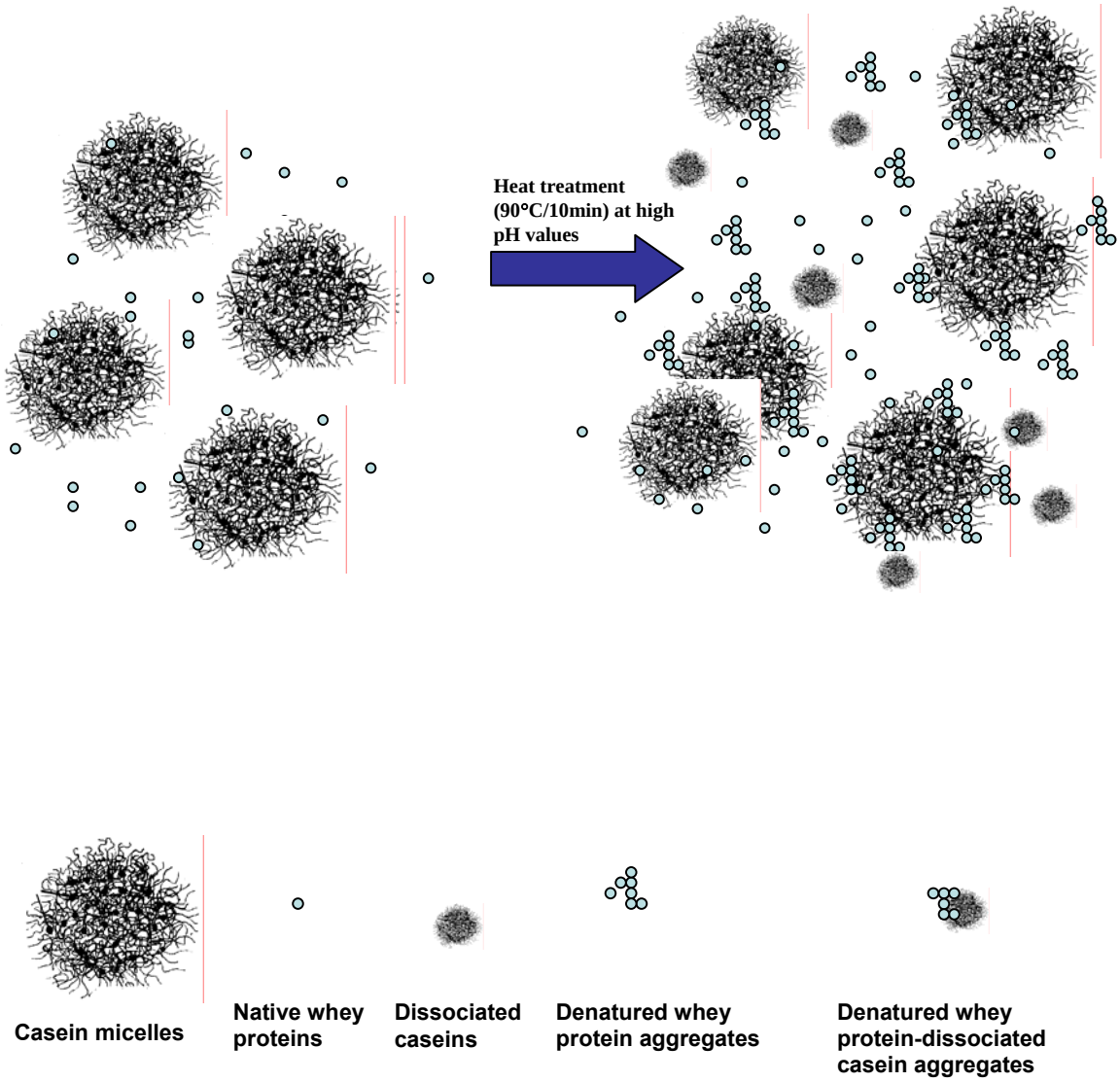

Figure (4.9): A schematic representation of the interaction between casein micelles and/or dissociated caseins with denatured whey proteins/whey protein aggregates in milk during heat treatment for 10 min at 90℃ at low & high pH values

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Chapter 5

Conclusions and Future Directions

5.1 Conclusions

The changes occurring in milk systems during heating are dependent on environmental factors such as temperature, pH, speciation of milk components and milk concentration as discussed in early chapters. As a result when investigating physicochemical properties and their effect on the stability of the milk systems, it is of outmost importance to maintain the milk systems under the conditions of interest. This approach would help to achieve better understanding of the milk systems at the times when actual changes are taking place. Hence, the ultimate goal of this work was to determine the heat induced changes in relation to the physicochemical parameters at the temperature of heating.

The pH and calcium activity decreased as temperature increased for all the milk solutions investigated. This is mainly associated with the formation of calcium phosphate during heating as defined by the following approximate chemical equilibria.

$$Ca^{2+}{}_{(aq)} + 0.7\,HPO_4^{2-}{}_{(aq)} + 0.2PO_4^{3-}{}_{(aq)} \leftrightarrow Ca\left(HPO_4^{2-}\right)_{0.7}\left(PO_4^{3-}\right)_{0.2(s)} \qquad \text{Eq 1}$$

$$H_2PO_4^{-}{}_{(aq)} \leftrightarrow HPO_4^{2-}{}_{(aq)} + H^{+}{}_{(aq)} \qquad \text{Eq 2}$$

$$HPO_4^{2-}{}_{(aq)} \leftrightarrow H^{+}{}_{(aq)} + PO_4^{3-}{}_{(aq)} \qquad \text{Eq 3}$$

An upfield shift of the P_{in} peak in the ^{31}P NMR spectrum of skim milk solutions was observed with increase in temperature which correlates to the change in pH on heating from 25°C to 90°C. The P_{in} peak became much broader but with constant area as temperature increased. It indicated that a constant amount of P_{in} contributing to the NMR signal although the mobility of some of the P_{in} present in the serum phase became restricted. The pH decrease on heating from 25°C to 90°C was greater at higher values of initial pH (pH at 25°C before heating) and higher milk concentration (9%-21% w/w MSNF). The calcium activity behaved the opposite way, with the magnitude of the decrease in calcium activity heating from 25°C to 60°C being less at higher values of initial pH (pH at 25°C before heating) and at higher milk concentration. The decrease in pH on heating from 25°C to 90°C in skim milk solutions with added calcium chelating agents was less than the skim milk solutions without the addition of chelating agents at the same initial pH at 25°C. The decrease in calcium activity upon heating from 25°C to 60°C in skim milk solutions with added calcium chelating agents was also less than without the addition at the same pH at 25°C. The changes in pH and calcium activity were largely reversible provided enough time was given for equilibration.

The total true protein content in the supernatants obtained from mild centrifugation (33,000g) of the milk was greater for heat treated (90°C/10min) 9% w/w MSNF milk solutions at pH values ≥ 6.65 than for non heat treated milks, whereas below this pH value, the total true protein content in the supernatants of heat treated milks was less than unheated. This difference is due to differences in distribution of protein components between colloidal and aqueous phases of milk depending on the pH of the milk solution. As

the pH decreases, more of the denatured whey proteins are associated with the casein micelles and hence are pelleted with the casein micelles on centrifugation at 33,000g. On the other hand, as the pH increases, dissociation of caseins from the casein micelles occurs. This results in preferential reaction of the denatured whey proteins with the caseins in the serum to form "soluble aggregates" and remain in the aqueous phase on centrifugation. The soluble aggregates are predominantly disulfide bonded. From the results, it was concluded that the formation and the composition of the soluble aggregates is dependent on the distribution of the proteins and minerals between the serum and micellar phases of milk, which in turn dependent on the variations in the milk composition and pH.

Two complementary experimental techniques (DWS & rheology) were used to monitor the increase in viscosity and/or occurrence of a gelation during heat treatment. The correlation functions obtained from DWS at each heating temperature, showed the decreased particle mobility at temperatures ≥ 75°C due to the denatured whey protein association with the casein micelles. Gel formation was identified clearly from the correlation plots as a initial relaxation along with a non-exponential decay. The relative ηr showed an increase for lower pH milk solutions at pH 6.2 at temperatures ≥ 75°C. This highlighted the increased association of denatured whey proteins to casein micelles and the subsequent interactions between the casein micelles coated with the denatured whey proteins at low pH values. However, at higher pH values, the relative ηr showed a gradual decrease with increase in temperature indicating both the increased formation of soluble aggregates as was concluded in chapter 3 and the decrease in the size of casein micelles. The addition of calcium chelating agents at lower pH values showed a lesser increase in relative ηr at 90°C

compared to that of the relative ηr at 90°C of the milk solutions at that particular pH without the additions. With the addition of EDTA, the formation of casein rich soluble aggregates was favored. Hence, this will lead to lesser interactions between casein micelles coated with whey proteins.

In the present study, thermal instability was defined as gel formation and/or an increase in viscosity on returning to 25°C after heat treatment compared with the initial viscosity at 25°C. The "minimum pH at 90°C of thermal stability" was found as pH 6.2 at 90°C for concentrated skim milk solutions (≥15% w/w MSNF) with/without the addition of calcium chelating agents. It concludes that during heating at lower pH values, more of the denatured whey proteins associated with the casein micelles and the resulting increased interactions between casein micelles creates the observed thermal instability. On the other hand, during heating at higher pH values, more of the denatured whey proteins associated with the caseins dissociated from the casein micelles and resulting increased amounts of soluble aggregates which leads to lesser interactions between casein micelles creates the observed thermal stability. The heat stability of a particular milk system is predominantly governed by the pH at the temperature of heating, which itself is primarily determined by the composition of the milk.

The hypotheses tested in the present study were;

(a) The chemical and structural changes of milk during heat treatment are primarily determined by the pH at the temperature of heating,

(b) The changes in milk concentration and addition of mineral salts, through their effect on the pH at the temperature of heating, influence the chemical and structural changes of milk during heat treatment and hence the heat stability of milk.

It was found that the changes in milk concentration and addition of mineral salts is to influence the speciation of the milk components (especially minerals and proteins) and subsequently influences the pH at the temperature of heating and thereby influence the chemical and structural changes during heating. The heat stability of milk was found to be primarily determined by the pH at the temperature of heating. Control the pH at the temperature of heating and hence controlling the extent of interaction between denatured whey proteins and casein micelles is of utmost importance in controlling the behaviour of milk systems during heating.

5.2 Future Directions

There are few directions that will lead in answering some of the questions that was raised at the end of this work. One of the most important directions is to investigate what is actually happening to the casein micelles and to the soluble aggregates at high temperatures. There is a possibility of using techniques such as micro filtration at high temperatures to separate casein micelles and/or soluble aggregates at a particular temperature. By examining the amount of whey proteins associated with the casein micelles at the temperature of heating, it is possible to determine the optimum conditions of the extent of association of the denatured whey proteins with the casein micelles and the subsequent interactions between the casein micelles in order to obtain a more heat stable milk product. This might open

doors in using some of the new technological options such as high pressure treatment in controlling the denaturation of the whey proteins without affecting the minerals.

Appendix A

PRIMARY STRUCTURES OF 2 MAJOR WHEY PROTEINS

1 **11**
Leu Ile Val Thr Gln Thr Met Lys Gly Leu Asp Ile Gln Lys Val Ala Gly Thr Thr Trp
21 **31**
Ser Leu Ala Met Ala Ala Ser Asp Ile Ser Leu Leu Asp Ala Gln Ser Ala Pro Leu Arg
41 **Gln in Varient D** **51** **Varient C His**
Val Tyr Val Glu Glu Leu Lys Pro Thr Pro Glu Gly Asp Leu Glu Ile Leu Leu **Gln** Lys
61 **Gly in Varients B, C** **71**
Asp Glu Asn **Asp** Glu Cys Ala Gln Lys Lys Ile Ile Ala Glu Lys Thr Lys Ile Pro Ala
81 **91**
Val Phe Lys Ile Asp Ala Leu Asn Glu Asn Lys Val Leu Val Leu Asp Thr Asp Tyr Lys
101 **111** **Varients B, C Ala**
Lys Thr Leu Leu Phe Cys Met Glu Asn Ser Ala Glu Pro Glu Gln Ser Leu **Val** Cys Gln
121 **131**
Cys Leu Val Arg Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys Phe Asp Lys Ala Leu
141 **151**
Lys Ala Leu Pro Met His Ile Agr Leu Ser Phe Asn Pro Thr Gln Leu Glu Glu Gln Cys
161 **162**
His Ile OH

Figure (A.1) : The primary structure of β-Lactoglobulin (http://class.fst.ohio-state.edu/FST822/lectures/Milk2.htm).

Appendix A

1 **Arg in Varient B**

Glu Gln Leu Thr Lys Csy Glu Val Phe **Gln** Glu Leu Lys Asp Leu Lys Gly Tyr Gly Gly

21 **31**

Val Ser Leu Pro Glu Trp Val Cys Thr Thr Phe His Thr Ser Gly Tyr Asp Thr Glu Ala

41 **51**

Ile Val Glu Asn Asn Gln Ser Thr Asp Tyr Gly Leu Phe Gln Ile Asn Asn Lys Ile Trp

61 **71**

Cys Lys Asn Asp Gln Asp Pro His Ser Ser Asn Ile Cys Asn Ile Ser Cys Asp Lys Thr

81 **91**

Leu Asn Asn Asp Leu Thr Asn Asn Ile Met Cys Val Lys Lys Ile Leu Asp Lys Val Gly

101 **111**

Ile Asn Tyr Trp Leu Ala His Lys Ala Leu Cys Ser Glu Lys Leu Asp Gln Trp Leu Cys

121 **123**

Glu Lys Leu OH

Figure (A.2) : The primary structure of α-Lactalbumin (http://class.fst.ohio-state.edu/FST822/lectures/Milk2.htm).

Appendix B

PRIMARY STRUCTURES OF 4 TYPES OF CASEINS

1 11
Glu Glu Gln Asn Gln Glu Gln Pro Ile Arg Cys Glu Lys Asp Glu Arg Phe Phe Ser Asp
21 31
Lys Ile Ala Lys Tyr Ile Pro Ile Gln Tyr Val Leu Ser Arg Tyr Pro Ser Tyr Gly Leu
41 51
Asn Tyr Tyr Gln Gln Lys Pro Val Ala Leu Ile Asn Asn Gln Phe Lue Pro Tyr Pro Tyr
61 61
Tyr Ala Lys Pro Ala Ala Val Arg Ser Pro Ala Gln Ile Leu Gln Trp Gln Val Leu Ser
81 81
Asp Thr Val Pro Ala Lys Ser Cys Gln Ala Gln Pro Thr Thr Met Ala Arg His Pro His
101 105 106 111
Pro His Leu Ser Phe Met Ala Ile Pro Pro Lys Lys Asn Gln Asp Lys Thr Glu Ile Pro
121 131 Ile Varient B
Thr Ile Asn Thr Ile Ala Ser Gly Glu Pro Thr Ser Thr Pro Thr Thr Glu Ala Val Glu
141 Varient B has Ala **P** 151
Ser Thr Val Ala Thr Leu Glu Asp **Ser** Pro Glu Val Ile Glu Ser Pro Pro Glu Ile Asn
161 169
Thr Val Gln Val Thr Ser Thr Ala Val

Figure (B.1) : The primary structure of κ- casein (Advanced Dairy Chemistry:

Volume 1: Proteins – Fox P.F. & McSweeney P.L.H. - 2003).

Appendix B

									10							**P**	**P**	**P**	**20**
Arg	Glu	Leu	Glu	Glu	Leu	Asn	Val	Pro	Gly	Glu	Ile	Val	Glu	Ser	Leu	Ser	Ser	Ser	Glu
				In G 1 Casein, split here					**30**					**P**	**Lys in varient E**				**40**
Glu	Ser	Ile	Thr	Arg	Ile	Asn	Lys	**Lys**	Ile	Glu	Lys	Phe	Gln	**Ser**	**Glu**	**Glu**	Gln	Gln	Gln
									50				**In varient C, lys**						**60**
Thr	Glu	Asp	Glu	Leu	Gln	Asp	Lys	Ile	His	Pro	Phe	Ala	Gln	Thr	Gln	Ser	Leu	Val	Tyr
	In varients B, A1 & C his								**70**										**80**
Pro	Phe	Pro	Gly	Pro	Ile	**Pro**	Asn	Ser	Leu	Pro	Gln	Asn	Ile	Pro	Pro	Leu	Thr	Gln	Pro
									90										**100**
Pro	Val	Val	Val	Pro	Pro	Phe	Leu	Gln	Pro	Glu	Val	Met	Lys	Val	Ser	Lys	Val	Lys	Glu
In G 3 Casein, split here						Split here in G 2 Casein													**120**
Ala	Met	Ala	Pro	**Lys**	His	**Lys**	Glu	Met	Pro	Phe	Pro	Lys	Tyr	Pro	Val	Gln	Pro	Phe	Thr
	Arg in varient B								**130**										**140**
Glu	**Ser**	Gln	Ser	Leu	Thr	Leu	Thr	Asp	Val	Glu	Asn	Leu	His	Leu	Pro	Pro	Leu	Leu	Leu
									150										**160**
Gln	Ser	Trp	Met	His	Gln	Pro	His	Gln	Pro	Leu	Pro	Pro	Thr	Val	Met	Phe	Pro	Pro	Gln
									170										**180**
Ser	Val	Leu	Ser	Leu	Ser	Gln	Ser	Lys	Val	Leu	Pro	Val	Pro	Glu	Lys	Ala	Val	Pro	Tyr
									190										**200**
Pro	Gln	Arg	Asp	Met	Pro	Ile	Gln	Ala	Phe	Leu	Leu	Tyr	Gln	Gln	Pro	Va;	Leu	Gly	Pro
													209						
Val	Arg	Gly	Pro	Phe	Pro	Ile	Ile	Val	OH										

Figure (B.2) : The primary structure of β-casein (Advanced Dairy Chemistry:

Volume 1: Proteins – Fox P.F. & McSweeney P.L.H. - 2003).

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Appendix B

Arg Pro Lys His Pro Ile Lys His Gln Gly Leu Pro Gln (Glu Val Leu Asn Glu Asn Leu
(Absent in Varient A) 30 40
Leu Arg Phe Phe Val Ala) Pro Phe Pro Gln Val Phe Gly Lys Glu Lys Val Asn Glu Leu
P P 50 ThrP in varient D 60
Ser Lys asp Ile Gly Ser Glu Ser Thr Glu Asp Gln Ala Met Glu Asp Ile Lys Glu Met
P P P P 70 P 80
Glu Ala Glu Ser Ile Ser Ser Ser Glu Glu Ile Val Pro Asn Ser Val Glu Gln Lys His
90 100
Ile Gln Lys Glu Asp Val Pro Ser Glu Arg Tyr Leu Gly Tyr Leu Glu Gln Leu Leu Arg
110 P 120
Leu Lys Lys Tyr Lys Val pro Gln Leu Glu Ile Val Pro Asn Ser Ala Glu Glu Arg Leu
130 140
His Ser Met Lys Gln Gly Ile His Ala Gln Gln Lys Glu Pro Met Gly Val Asn Asn Gln
150 160
Glu Leu Ala Typ Phe Tyr Pro Glu Leu Phe Arg Gln Phe Tyr Gln Leu Asp Ala Tyr Pro
170 180
Ser Gly Ala Trp Tyr Tyr Val Pro Leu Gly Thr Gln Tyr Thr Asp Ala Pro Ser Phe Ser
190 Gly in varient C 199
Asp Ile Pro Asn Pro Ile Gly Ser Glu Asn Ser Glu Lys Thr Thre Met Pro Leu Trp OH

Figure (B.3) : The primary structure of α_{S-1} casein B (Advanced Dairy Chemistry:

Volume 1: Proteins – Fox P.F. & McSweeney P.L.H. - 2003).

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Appendix B

Figure (B.4) : The primary structure of the α_{S-2} casein (Advanced Dairy

Chemistry: Volume 1: Proteins – Fox P.F. & McSweeney P.L.H. - 2003).

Appendix C

X-RAY DIFFRACTION TECHNIQUE

X-ray diffraction is a non destructive technique and requires a very small amount of sample for analysis. X-ray diffraction has been a new research tool for the dairy industry. Every crystalline substance gives an X-ray pattern. The same substance always gives the same pattern and in a mixture of substances each produces its pattern independently of the others. Hence, the X-ray diffraction pattern of a pure substance is like a finger print of a particular substance.

There are mainly two types of solid matters; amorphous and crystalline. Atoms are arranged in a random way in the former whereas in the latter the atoms are arranged in a regular pattern. An electron in an alternating electromagnetic field will oscillate with the same frequency as the field. When an X-ray beam hits an atom in an amorphous state, the electrons around the atom start to oscillate with the same frequency as the incoming beam. In all directions will result a destructive interference where the combining waves are out of phase and there is no energy leaving the solid sample. This will result a one broad peak. However the atoms in a crystal are arranged in a regular pattern and in a very few directions can result a constructive interference where the waves will be in phase and there will be well fined X-ray beams leaving the sample at various directions. This will result in a sharp peak characteristic to the substance. Hence a diffracted beam may be

described as a beam composed of a large number of scattered rays mutually reinforcing one another. It depends on several factors. They are;

a) The distance between similar atomic planes - d spacing
b) The angle of diffraction- θ
c) The wave length of the incident X-ray radiation - λ

They obeys the Bragg's law which states; $n*\lambda = 2d\text{Sine}\ \theta$

n = Integer

λ = Wavelength

d = Inter atomic spacing

θ = Diffraction angle

A typical diffraction pattern records the X-ray intensity as a function of 2θ angle.

Appendix D

Table (D.1): The changes in pH at 120°C for 10 min for 9%-21% w/w MSNF milk solutions as a function of initial pH at 25°C

Sample	Initial pH at 25°C	pH at 120°C	Δ pH (pH at 25°C–pH at 120°C)
9%	6.21	5.56	0.652
	6.42	5.64	0.787
w/w	6.62	5.92	0.809
	6.80	5.88	0.909
MSNF	6.98	6.08	0.919
	7.21	6.08	1.128
	6.21	5.47	0.743
12%	6.44	5.77	0.870
	6.57	5.64	0.934
w/w	6.78	5.67	1.109
	7.04	5.78	1.255
MSNF	7.18	5.84	1.34
	6.21	5.42	0.789
15%	6.44	5.72	0.916
	6.56	5.56	1.003
w/w	6.77	5.67	1.105
	6.98	5.73	1.252
MSNF	7.19	5.82	1.368
	6.22	5.58	0.844
18%	6.47	5.49	0.930
	6.59	5.60	0.994
w/w	6.80	5.71	1.097
	6.99	5.92	1.178
MSNF	7.21	5.85	1.353
	6.22	5.50	0.925
21%	6.41	5.60	1.014
	6.61	5.66	1.154
w/w	6.77	5.75	1.215
	6.96	5.82	1.344
MSNF	7.24	5.91	1.434

Mean values of (n = 3) analyses.
s.d. for pH = ± 0.02; s.d. for change in pH = ± 0.015

Appendix E

The Speciation and distribution of mineral components between the colloidal and serum phases

The Speciation and distribution of mineral components between the colloidal and serum phase values were obtained by running a mineral speciation program modified by Ian McKinnon using Carl Holt's basic mineral speciation program (Personal communication). The most important values are taken in to the following table (E.1).

Table (E.1): The amounts of free P_{in} ions in the serum phase

Sample	Free P_{in} concentration/mmol per kg of milk
9% w/w MSNF- pH 6.65	8.09
18% w/w MSNF- pH 6.65	11.63

The total concentrations of calcium and P_{in} in 9% w/w MSNF milk solution were used as 30 and 18.22 mmol/kg of solution respectively. The total concentrations for 18% w/w MSNF milk solutions was taken as double the amounts for 9% w/w MSNF milk solutions.

For 9% w/w MSNF milk solutions, the amount of free P_{in} ions was ~8.09 mmol/kg of solution, whereas for 18% w/w MSNF milk solutions, the amount of free P_{in} ions was ~ 11.63 mmol/kg of solution. Hence, the amounts of free P_{in} ions in the serum phase for re-equilibration was more with increase in milk concentration.

Appendix E

Table (E.2): The fraction of calcium ions in the serum phase at 25°C

Sample	Fraction of calcium ions
9% w/w MSNF- pH 6.65	0.44
18% w/w MSNF- pH 6.65	0.28

For 9% w/w MSNF milk solutions, the fraction of calcium ions in the serum phase was 0.44, whereas for 18% w/w MSNF milk solutions, the fraction of calcium ions in the serum phase was 0.28. Hence, the amount of calcium ions in the serum phase available for calcium phosphate precipitation during heating was much less with increase in milk concentration.

The program out puts for 9% an 18% w/w MSNF milk solutions at pH 6.65 at 25°C are attached.