A study on the mechanisms of calcium-induced gelation in skim milk

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Abstract

The destabilisation and aggregation of milk proteins is the first step towards the gelation of milk. The addition of calcium to milk is known to destabilise milk proteins and may result in gelation on heating. However, the mechanisms involved in gelation induced by heating calcium-added milk was not well understood. Therefore, this project aimed to determine the fundamental mechanisms involved in the development of a calcium-induced skim milk gel.

Skim milk was selected as the model system and gelation was induced in-situ by heating the calcium-added skim milk at the rheometer. The changes in the storage modulus, G', were monitored to study the development of the gel network. This project examined the impact of the following factors on the rheological properties of a calcium-induced skim milk gel: the type of soluble calcium salt added (calcium chloride, calcium lactate, calcium gluconate, calcium lactobionate and calcium iodide), pH, holding temperature during gelation (70°C to 90°C), preheat treatment, ionic strength (by addition of sodium chloride) and the contribution of casein and whey proteins.

A higher calcium ion activity ($a_{Ca^{2+}}$), which indicated a higher calcium ion (Ca^{2+}) concentration, and a lower pH favoured the formation of a stronger gel. An increase in ionic strength by addition of sodium chloride decreased the final G' of the calcium-induced skim milk gel due to reduced calcium bridging and increased hydration repulsion. A higher heating temperature also resulted in gels with higher final G' due to more frequent particle collisions.

Casein micelles and whey proteins were both responsible for the structure of the gel network. The contribution of whey proteins towards the gel network was dependent on

if they were denatured prior to heating, on the concentration of calcium ions available, and on the ratio between the casein and whey proteins present. At lower added calcium concentrations (10 mmol L^{-1}) where the available calcium ions were limited, interactions and aggregation amongst denatured whey proteins via hydrophobic and disulphide bonds may have resulted in the formation of a stronger gel. However, at higher added calcium concentrations (20 and 40 mmol L^{-1}), where sufficient calcium ions may be available for binding, interactions between casein and calcium dominated over the self-aggregating effect of denatured whey proteins. In conclusion, the results demonstrated that the final gel properties of a calcium-induced skim milk gel were dependent on the net effect of all the factors involved in the stability and interactions of the milk proteins, including the calcium salt concentration, pH, preheat treatment, ionic strength, and the protein composition in solution. These findings provide alternative methods for texture modification in milk.

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List of publications and presentations

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H.E. Oh, L. Lin, H.C. Deeth, M. Wong (2016). Factors affecting calcium-induced gelation in milk. Oral presentation, NIZO conference, Singapore.

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G'	Storage modulus
G''	Loss modulus
tan δ	Loss tangent, ratio G'/G''
$a_{Ca^{2+}}$	Calcium ion activity
$c_{Ca^{2+}}$	Calcium ion concentration
$\gamma_{Ca^{2+}}$	Activity coefficient of calcium ion

List of abbreviations

CCP	Colloidal calcium phosphate
pI	Isoelectric point
HA	Hard-anodised aluminium
SS	Stainless steel
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
Ig	Immunoglobulin
SH	Thiol group
Tris-HCl	Tris-hydrochloride
Tris-base	Tris(hydroxymethyl)aminomethane
GDL	Glucono-delta-lactone
CMP	Caseinomacropeptide
VCF	Volume concentration factor
WPI	Whey protein isolate

Chapter 1 - Introduction

Calcium is a divalent cation and is the most abundant mineral in the human body (Medeiros & Wildman, 2015). It is known for its importance in bone health and prevention of osteoporosis. However, the rise in cases of osteoporosis has highlighted the prevalence of inadequate calcium intake, thus leading to global programs focusing on increasing dietary intake of calcium (World Health Organization, 2004). As a result, calcium fortification continues to be a major area of interest. Milk and dairy products are the richest natural sources of calcium and contributes as much as 50 to 70% of dietary calcium (Dror & Allen, 2014; Medeiros & Wildman, 2015). Consequently, the most common medium for calcium fortification is milk and dairy products.

Besides enhancing its nutritional value, the addition of calcium to milk and dairy products also changes its physicochemical properties due to the functionality of calcium in milk (Deeth & Lewis, 2015). For example, the addition of calcium to milk was found to destabilise the milk proteins, which may influence the structure and textural properties of milk protein gels (Dalgleish & Corredig, 2012; Lucey, 2009). The destabilising effect of the addition of calcium to milk can also be exploited to induce gelation in milk with heat (Ramasubramanian, D'Arcy, Deeth & Oh, 2014). However, while heat- (Ji, Lee & Anema, 2016; O'Connell & Fox, 2003; Oztop, Mccarthy, Mccarthy & Rosenberg, 2012), acid- (Anema, Lowe & Lee, 2004b; Hyslop, 2003; Ozcan, Horne & Lucey, 2015) and rennet-induced (Hyslop, 2003; Malacarne et al., 2014) milk protein gels have been studied extensively, there is limited information on the mechanisms involved in the formation of calcium-induced milk gels. A fundamental understanding of factors affecting the gelation of a calcium-induced milk gel could lead to the production of a novel type of calcium-enriched milk gel with various textures for a range of different applications.

The aim of this study was to determine the mechanisms involved in the development of a calcium-induced skim milk gel through a fundamental understanding of the factors affecting gelation in milk. Skim milk was used to investigate the interactions between milk proteins and calcium ions to minimise any possible interactions and effect of milk fat. The mechanisms involved in the gelation of the calcium-added skim milk was investigated by applying different treatments to the skim milk to alter the interactions between the milk proteins (casein and whey proteins) and calcium ions. The effect of these treatments on the calcium ion activity (a_{Ca}^{2+}), pH, distribution of the proteins and calcium between serum and colloidal phases, particle size, zeta-potential and rheological properties (G') of the calcium-induced skim milk gel were determined.

The objectives of this study were to:

1. Determine the effect of different types of soluble calcium salts on the resulting rheological properties of the skim milk gel.
2. Determine the effect of pH, temperature and preheat treatment on the resulting skim milk gel.
3. Investigate the interactions between casein, whey proteins and calcium ions and the impact of the interactions on the gelation of skim milk.
4. Compare the effect of divalent ions (Ca^{2+}) vs monovalent ions (Na^{+}), and the effect of ionic strength on the gelation of skim milk.
5. Evaluate possible intermolecular forces involved in the association of the milk proteins that induce gelation in a calcium-added skim milk.

Chapter 2 - Literature review

This literature review will cover the fundamentals of milk protein chemistry including its structure, stability and the mineral equilibrium in milk. The factors that destabilise milk proteins and induce aggregation and gelation will also be discussed. This literature review attempts to highlight some of the known mechanisms behind milk protein gelation and the gaps involved in the role of calcium in milk gelation. The principles of rheological measurements for studying of the gelation of milk proteins will also be discussed in the last section of this literature review.

2.1 Overview of milk

Milk is a biological fluid secreted by females of all mammals for the purpose of meeting the nutritional requirements of the neonate of the species. The principal constituents of milk are water, lipids, sugar (lactose) and proteins. In addition, milk also contains trace levels of minerals, vitamins, hormones, enzymes and miscellaneous compounds (Fox, 2009). The composition varies between the different mammalian species. For instance, human milk contains approximately 1% protein and 7% lactose while bovine milk contains 3.4% protein and 4.8% lactose (Fox & McSweeney, 1998). For the purpose of this study, this literature review will cover only bovine milk.

The principal constituents of bovine milk are shown in Table 2-1. Understanding the role of each constituent is important in identifying its effects on milk when subjected to different conditions. In the following sections, the basic properties of the major components in milk will be described.

Table 2-1: Composition of milk (Walstra, Geurts, Noomen, Jellama & Van Boekel, 1999).

Component	Average content in milk (% *w/w*)	Range (% *w/w*)	Average content in dry matter (% *w/w*)
Water	87.1	85.3 - 88.7	
Solids-non-fat	8.9	7.9 - 10.0	
Lactose	4.6	3.8 - 5.3	36
Fat	4	2.5 - 5.5	31
Protein	3.3	2.3 - 4.4	25
- Casein	2.6	1.7 - 3.5	20
Mineral substances	0.7	0.57 - 0.83	5.4
Organic acids	0.17	0.12 - 0.21	1.3
Miscellaneous	0.15		1.2

Lactose is the primary carbohydrate found in milk. It is composed of galactose and glucose linked by β1-4 glycosidic bond and is only known to be found in milk. Nutritionally, lactose serves as a source of energy for the neonates, providing 30% of the calories and acting as an alternative to energy-dense lipids (Fox, 2009).

Milk fats are primarily present in milk as a source of energy for the neonate. They are also a source of essential fatty acids and fat soluble vitamins. From a sensory point of view, milk fats are also important in providing the flavour and mouthfeel of milk and dairy products (Fox & McSweeney, 1998). Commercial milks are homogenised to reduce the size of fat globules to increase stability during storage. This is achieved by pumping the milk through a small orifice at high pressure and at temperatures above 37°C. Homogenised milk does not form a cream layer upon storage and has improved flavour and textural characteristics (Chandan, 2011).

Bovine milk contains about 3.5% protein (Fox & McSweeney, 1998). The function of milk protein is to supply essential amino acids required for growth in the young neonates. Proteins play an important role in determining the characteristics of the milk through the changes in its physical properties and interactions with other components in the milk. Proteins in milk are subdivided into two groups, casein and whey. Casein is

defined as the protein precipitated at pH 4.6 (isoelectric point of casein). The proteins in the remaining liquid after isoelectric precipitation of casein are called whey or serum proteins. Each of these proteins exhibits different characteristics and behaves differently when subjected to various conditions.

Bovine milk contains about 7.6 g L^{-1} (0.7% *w/w*) of minerals (Flynn & Cashman, 1997; Walstra et al., 1999). Many of these are considered essential for human health. Minerals also influence the properties of milk, which determines the final quality of dairy products. They are known to interact strongly with casein. Therefore, changes in concentration or equilibrium of the minerals may induce changes in the casein structure and the overall properties of milk (Fox & McSweeney, 1998; Holt, 1997).

The typical composition of the major salts in milk is shown in Table 2-2. The milk salts in bovine milk are typically in the concentration range of 5 to 40 mmol L^{-1}. Ionic strength is a function expressing the effect of the charge of ions in a solution, and can be expressed in both molarity or molality (Rennie, 2016; van Boekel, 2008a) The ionic strength in the milk serum as exerted by the milk salts is estimated to be approximately 80 mmol L^{-1} (Fox & McSweeney, 1998; Walstra et al., 1999). Seasonal variations in the milk changes the salt and pH balance in milk. Pouliot & Boulet (1995) studied the pH and salt balance of milk in Canada for one year. As shown in Figure 2-1, the pH of milk is generally high from September to February (autumn and winter), but drops slightly from March to August (spring and summer). The average calcium in milk was the highest in winter, between December to January (Figure 2-1). It should be noted that because the seasons in the Northern Hemisphere are the opposite of those in the Southern Hemisphere, the calcium content of milk from Australia and New Zealand is the highest from June to August, the winter months in the Southern Hemisphere (Auldist, Coats, Rogers & McDowell, 1995).

Table 2-2: Typical mineral composition in milk (Holt, 1997).

Component	Concentration of mineral in skim milk (mmol L^{-1})
Calcium (Ca)	30.1
Magnesium (Mg)	5.1
Sodium (Na)	25.5
Potassium (K)	36.8
Phosphate	20.9
Citrate	9.8
Chloride (Cl)	30.3

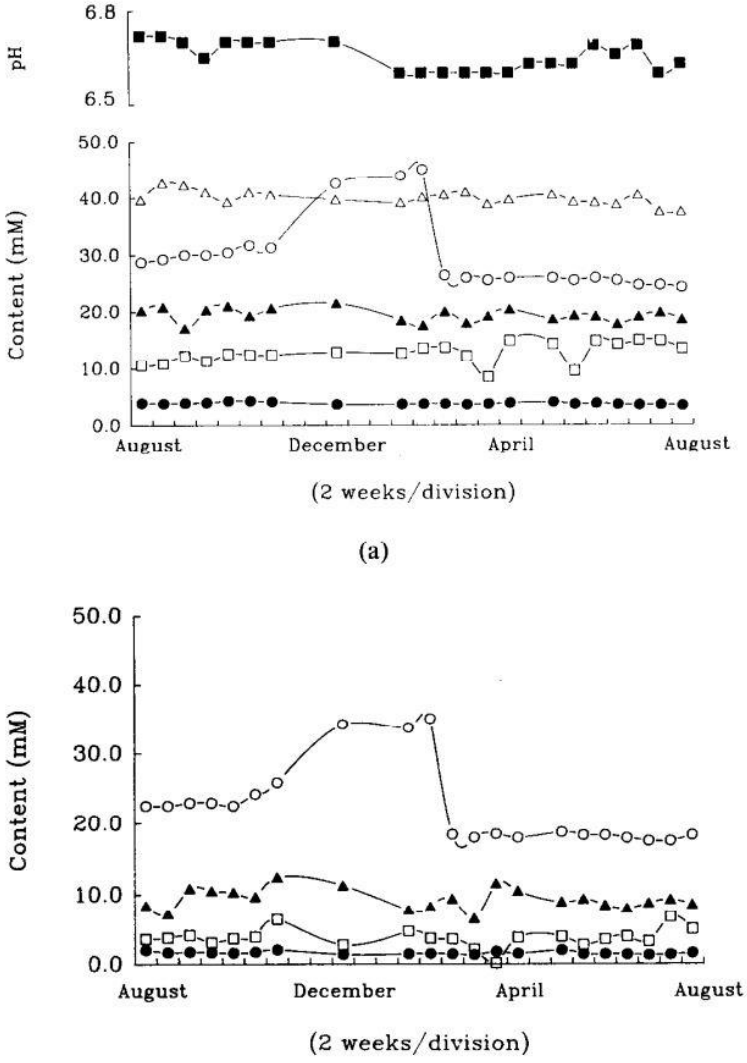


Figure 2-1: Seasonal changes in the composition of milk in Canada with respect to pH (■), Ca (○), PO_4 (▲), Mg (●), citrate (□), and K (△). (a) Total composition (b) composition of colloidal phase (Pouliot & Boulet, 1995).

2.2 Casein

Caseins are phosphoproteins that make up about 80% of the proteins in milk. They exist in milk as complex aggregates known as micelles (Holland, 2009; Horne, 2009). Electron microscopy showed that the casein micelles are generally spherical in shape, with diameters in the range of 40 - 500 nm and molecular weight of 10^6 - 10^9 Da (Walstra, Wouters & Geurts, 2006).

The unique structure of casein micelles allows milk to be supersaturated with respect to calcium. About two-thirds of total calcium exists in colloidal form associated with the micelles, either as colloidal calcium phosphate (CCP) or as calcium ions bound to the phosphoserine residue (Deeth & Lewis, 2015; Flynn & Cashman, 1997). Phosphates exist in the micelle as CCP (inorganic phosphate) or covalently bound to caseins as phosphate groups (organic phosphate) (Fox & McSweeney, 1998; Malacarne et al., 2014).

2.2.1 Primary structure and chemical composition of casein

Caseins can be divided into four genetic variants, α_{s1}-, α_{s2}-, β- and κ-casein which represent 38, 10, 35 and 12% respectively of whole bovine casein (Pritchard & Kailasapathy, 2011; Swaisgood, 2003). The different chemical composition of each subunit gives rise to different functionality.

α_{s1}-casein

α_{s1}-casein is the major component of the α_s- casein fraction (Swaisgood, 2003). α_{s1}-casein consists of 199 amino acids and its molecular weight is approximately 23.6 kDa. It has the highest charge of all casein molecules (Pritchard & Kailasapathy, 2011). α_{s1}-casein consists of eight phosphoserine residues (8P/ mol). The minor component, previously known as α_{s0}- casein, has an additional phosphorylated serine residue at position 41 (9P/ mol) (Holt, 1992).

α_{s2}-casein

α_{s2}-casein consists of 207 amino acids and its molecular weight is approximately 25.4 kDa (Pritchard & Kailasapathy, 2011). It is the most hydrophilic of the caseins as it contains three clusters of anionic phosphoseryl and glutamyl residues in its structure, at residues 8-12, 56-63, and 129-133 (Farrell et al., 2004; Swaisgood, 2003). Two areas of the structure are relatively hydrophobic due to their apolar amino acids, at residues 160-207 of the C-terminal, and residues 90-120 of the central structure (Pritchard & Kailasapathy, 2011; Swaisgood, 2003). However, the C-terminal sequence, although relatively hydrophobic, also possesses a large net positive charge of + 9.5 at pH 6.6, while the more hydrophilic N-terminal 68 residues has a net negative charge of -21. Hence, the α_{s2}- casein structure is characterised by distinct domains of high net charge, particularly the three phosphoseryl residues, which results in the α_{s2}- casein being very sensitive to ionic strength and cations such as protons and calcium (Farrell et al., 2004).

β-*casein*

β-casein consists of 209 amino acids and its molecular weight is approximately 24 kDa (Pritchard & Kailasapathy, 2011). β- casein is the most hyrophobic of all caseins (Swaisgood, 2003). Its N-terminal contains an anionic phosphoserine cluster which is highly charged, but the C-terminal is largely hydrophobic with no charge. This results in the highly amphiphilic character of β-casein (Swaisgood, 2003).

κ-casein

κ-casein consists of 169 amino acids and its molecular weight is approximately 19 kDa, and it contains both glycosylated and phosphorylated residues (Pritchard & Kailasapathy, 2011). It can exist as a dimer up to a decamer with the subunits held together by disulphide linkages (Pritchard & Kailasapathy, 2011). It contains a hydrophilic C-terminal, which carries a high negative charge, particularly when

glycosylated, and a predominantly hydrophobic remainder (Holt, 1992). κ- casein lacks the presence of phosphoserine clusters and therefore does not bind to calcium to the same extent as the other caseins (Swaisgood, 2003).

Variability in the casein subunit affects its behaviour and interactions. Fox (2009) classified the variability into five forms.

Variability in phosphorylation- All caseins are phosphorylated (P) but to varying degrees: α_{s1}- 8 or 9P; α_{s2}- 10, 11, 12 or 13P, β- 4 or 5P, and κ- 1 or 2P per molecule. The number of phosphate residues is thus expressed as α_{s1}-CN 8P, β-CN 5P etc. As calcium binds to the phosphate residues, highly phosphorylated α_{s2}-casein is the most calcium sensitive casein with almost complete precipitation occurring at a concentration of 2 mmol L^{-1} Ca^{2+}, followed by α_{s1}-casein, which precipitates at 6 mmol L^{-1} of Ca^{2+} (Farrell et al., 2004). Comparatively, β-casein is less sensitive to Ca^{2+} ions while κ-casein is the least sensitive and does not precipitate with excess calcium ions. Calcium-mediated interactions via clusters of phosphoserine groups are important in the polymerization of casein molecules and the solubilisation of the calcium phosphate within the casein micelles (Fox, 2009; McMahon & Oommen, 2008; Walstra et al., 2006). The high phosphate content also accounts for the hydrophilicity of the caseins and allows them to be reasonably water soluble (Pritchard & Kailasapathy, 2011).

Disulphide bonding- The two principal caseins, α_{s1}- and β-casein do not contain cysteine and cystine, but the two minor caseins, α_{s2}- and κ-casein each contains two ½ cysteine residues which occur as intermolecular disulphide bonds. In dimeric α_{s2}-casein, the two chains are linked by two disulphide-bonds, aligned either in a parallel (common amino-to-carboxyl-terminus direction) or anti-parallel (opposing amino-to-carboxyl-terminus direction) configuration (Figure 2-2). For κ-casein, up to 10 chains (multimer)

may be linked by disulphide bonds (Figure 2-3) (Fox, 2009; Rasmussen, Højrup & Petersen, 1992a; Rasmussen et al., 1999).

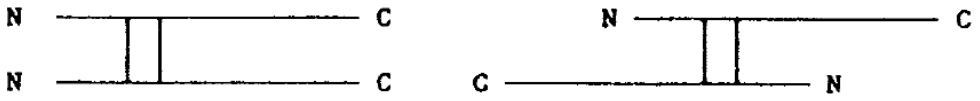


Figure 2-2: Assignment of disulphide bridges and orientation of the polypeptide chains in dimers of bovine α_{s2}-casein in parallel (left) and anti-parallel (right) conformation (Rasmussen et al., 1992a).

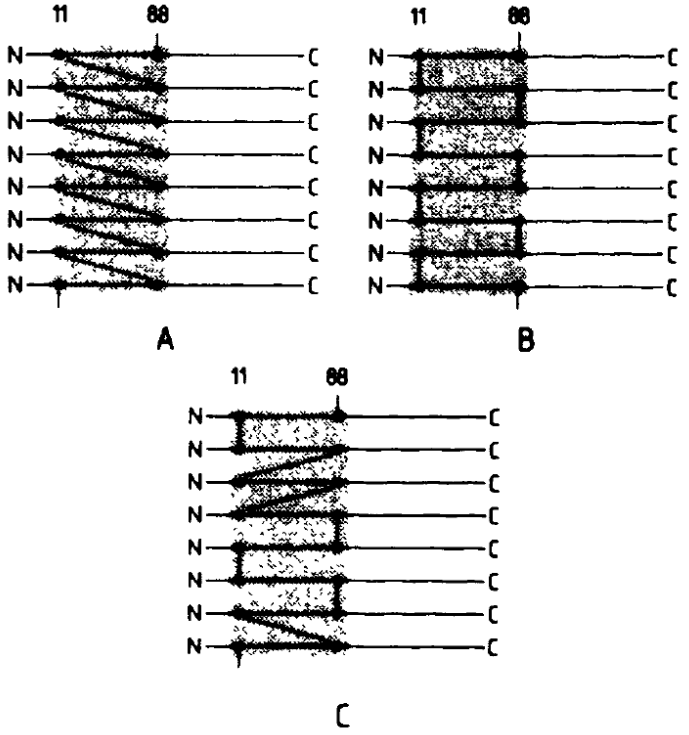


Figure 2-3: Schematic representation of possible disulphide-binding patterns in multimers of κ-casein. (A) Regular pattern involving one type of inter-chain disulphide linkage. (B) Regular pattern involving two types of inter-chain disulphide linkage. (C) Random pattern involving three types of inter-chain disulphide linkage. Since all three types of inter-chain disulphide bridges are found in multimers, the disulphide-bonding pattern of κ-casein is best represented by (C) (Rasmussen, Højrup & Petersen, 1992b).

Variations in the degree of glycosylation- κ-casein is the only casein that is glycosylated. It contains galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic (sialic) acid, which occur as tri- or tetrasaccharides (Fox, 2009).

Genetic polymorphism- Genetic polymorphism is used to describe the genetic variants in milk. All milk proteins exhibit genetic polymorphism and at least 45 variants have been detected by electrophoresis. Detection of genetic variants by electrophoresis has been possible because most of the mutants identified involve a change in the net charge of the protein. Important properties of milk such as rate and extent of coagulation by rennet, heat stability, and proportions of milk proteins are affected by the genetic polymorphs of milk proteins present (Fox, 2009; Ng-Kwai-Hang & Grosclaude, 2003).

Hydrolysis of caseins by plasmin- Plasmin is the principal proteinase found in milk. The preferred casein substrates for plasmin are α_{s2}- and β-casein. α_{s1}-casein is also hydrolysed, but κ-casein is very resistant, as are whey proteins. Hydrolysis of β-casein by plasmin produces C-terminal peptides, γ-caseins, and N-terminal peptides, which are the principal components of proteose peptones. Although α_{s2}-casein in solution is also quite susceptible to plasmin, α_{s2}-casein-derived peptides have not been detected in milk. Lastly, members of the minor casein fraction, λ- casein, are N-terminal fragments of α_{s1}-casein produced by plasmin (Fox, 2009).

2.2.2 Structure of casein micelle

Caseins are generally considered hydrophobic proteins, even though the amino acid composition indicates they are not particularly so. As they lack the secondary and tertiary structure as seen in globular proteins, their hydrophobic groups are exposed at the surface (Fox & McSweeney, 1998). Their open, flexible structure also makes them susceptible to enzymatic hydrolysis, which is to fulfil their primary role as a source of

amino acids for the neonate. It has also been suggested that the unfolded conformation was advantageous in inhibiting calcium phosphate precipitation from solution so that it did not progress beyond the nucleation stage (Thorn, Ecroyd, Carver & Holt, 2015).

Even though milk has been studied extensively over the last century, the exact structure of the casein micelles is still a subject of debate. However, some common features are agreed across all models. These include:

- the hydrophilic, negatively-charged C-terminal of κ-casein located mainly on the outer layer of the micelle, providing steric and electrostatic stability (De Kruif & Holt, 2003; Walstra et al., 2006);
- association of the casein subunits by hydrophobic interactions (Horne, 2009; Ingham et al., 2015)
- the presence of nanoclusters of colloidal calcium phosphate (CCP) which contain inorganic phosphates and calcium in the micelles, and the organic phosphate of the serine residue (SerP). The micelle structure is kept by the nanoclusters cross-linking between the peptide chains (Ingham et al., 2015; Thorn et al., 2015; Walstra et al., 2006)

A number of models have been proposed since the 1960s and have been refined over the years with more new information about the structure of the casein micelle. The proposed models can be classified into several categories: (1) core-coat; (2) submicelle; and (3) internal structure.

Core-coat model

The core-coat structure was first proposed by Waugh & Noble Jr. (1965). The core-coat structure suggests that the micelles have a composition which changes, from the surface to the centre. It is further proposed that the hydrophilic characteristics of κ-casein

suggest that the surface is κ-casein rich; while the core consists mainly of α_s- and β-caseins so that they are not in contact with the environment. The coat subunits are required to have certain interaction properties, such as strong interaction with core α_{s2}-casein. The surface subunits exposed have little tendency to interact with themselves or other constituent proteins, but are in exchange with similar components in solution (De Kruif & Holt, 2003).

Submicelle model

There has been a strong support for the submicelle model for many years, although there has also been some criticism. The model suggests that the micelle consists of submicelles which are about 10^6 Da and 10 - 15 nm in diameter. They associate by hydrophobic bonds, but CCP also acts as linkage between the submicelles (Figure 2-4) (Fox & McSweeney, 1998).

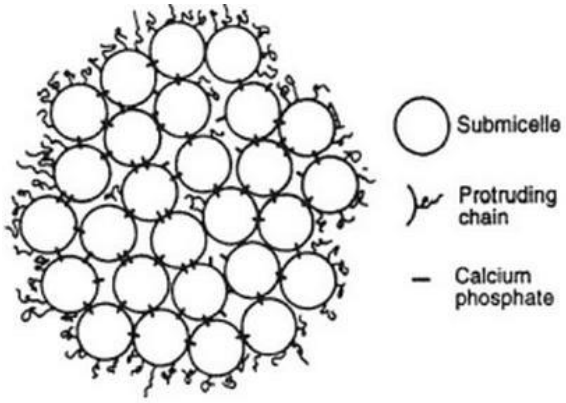


Figure 2-4: Submicelle model of the casein micelle proposed by Morr (O'Mahony & Fox, 2013).

The submicelle model was first proposed by Morr (1967) and has been refined over the years, most notably by Slattery & Evard (1973) and Schmidt (1980). The submicellar spheres were generally considered to be composed of α_{S1}- and β-casein cores surrounded by a layer that is rich in κ-casein. These spheres are held together by bridges

of calcium ions or of calcium and phosphate ions between casein organic phosphate esters on adjacent spheres.

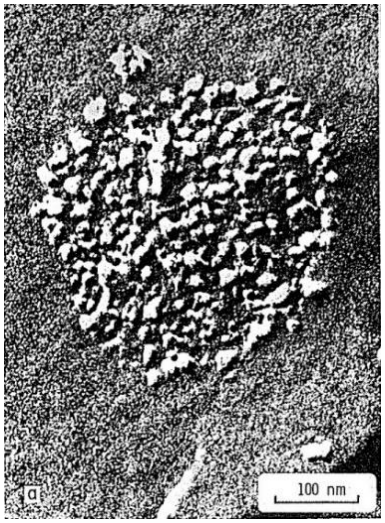


Figure 2-5: Electron micrograph of casein micelles made from a freeze-fractioned preparation by Schmidt (1980).

Schmidt (1980) captured an electron micrograph of the casein micelle and proposed that the small spherical units forming the bumpy surface of the micelle (Figure 2-5), were the submicelles. Schmidt (1982) also postulated that a casein micelle comprises a hydrophobic core covered with a hydrophilic surface in which the polar moieties of κ-casein molecules accumulated in one area. The other parts of the surface consist of polar parts of the other caseins, specifically the phosphoserine. It was proposed that the caseins thus aggregate via CCP, which is bound to the α_{s1}-, α_{s2}-, and β-casein via their phosphate side-chains, but not to the phosphoserine-deficient κ-casein (Figure 2-6). Essentially, submicelles with low or no κ-casein would be buried in the core of the micelle, while micellar growth would eventually cease when the whole micelle surface is covered with κ-casein.

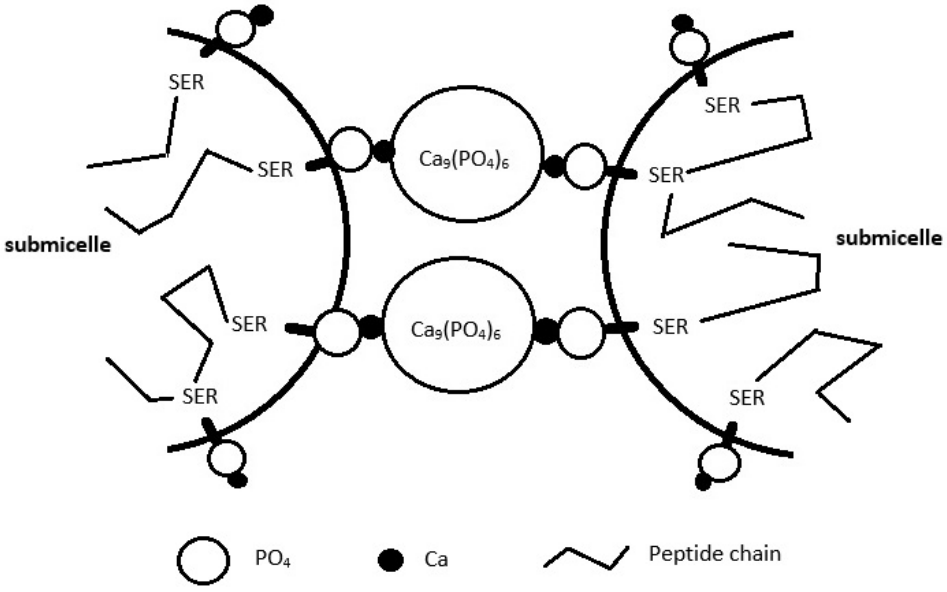


Figure 2-6: Schematic diagram of the binding of two submicelles via calcium phosphate cluster at the phosphoserine residue (adapted from Schmidt (1982)).

However, the submicelle model has never enjoyed unanimous support. Horne (2006) criticised the submicelle model because it does not explain what drives the segregation of κ-casein, or why the κ-casein, having preferred to associate with its own kind, would then associate with the other caseins. McMahon & McManus (1998) studied the micelle structure by transmission electron microscopy (TEM) and concluded that the micelles do not appear to contain submicelles, or that if the micelles consist of submicelles, then those micelles must be smaller than the 20 nm previously postulated as most of the electron dense particles were only 2-3 nm. They also proposed that if submicelles existed, they would be less densely packed than assumed, so that in the micrographs the constituent proteins of the submicelles appear as individual proteins.

Internal structure model

The internal structure model consist of the nanocluster model from Holt (1992) and the dual-binding model from Horne (1998). The nanocluster model incorporates the caseins cross-linked by calcium phosphate nanoclusters into a tangled-web structure without

subunits (Figure 2-7). Unlike the submicelle model, the nanocluster model does not contain a distinct hairy layer. Holt & Sawyer (1993) described casein as rheomorphic proteins, which have an open and mobile conformation. Under the nanocluster model, the rheomorphic polypeptide chains provide steric stabilization in the outermost limits of the particle, and the individual polypeptide chains with two or more phosphate centres could provide a network of strong interactions that could link together all or some parts of the calcium-sensitive caseins in a micelle (De Kruif & Holt, 2003). Holt, Carver, Ecroyd & Thorn (2013) also proposed that the biological function of casein micelle is to prevent calcium phosphate precipitation.

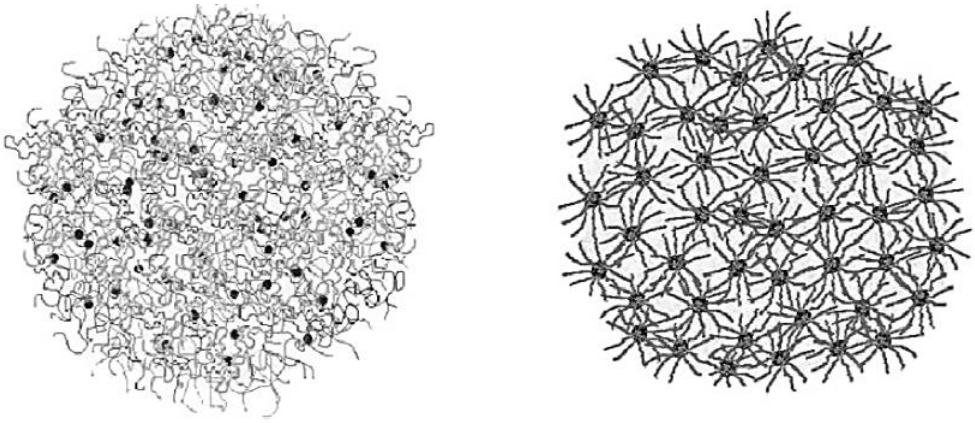


Figure 2-7: Representations of the nanocluster model of the casein micelle proposed by Holt. The black circles (•) represents the calcium phosphate nanocluster linking the caseins (De Kruif, Huppertz, Urban & Petukhov, 2012).

Horne (1998) proposed the dual-binding model (Figure 2-8) which differs from the nanocluster model of Holt (1992) in several ways. Firstly, the dual-binding model involves two distinct forms of binding, namely, cross-linking through hydrophobic regions of the caseins and bridging across calcium phosphate nanoclusters. Other differences from the nanocluster model are in the size of the nanocluster, and in the number of phosphate clusters that the surface of the nanocluster can accommodate (Horne, 2006).

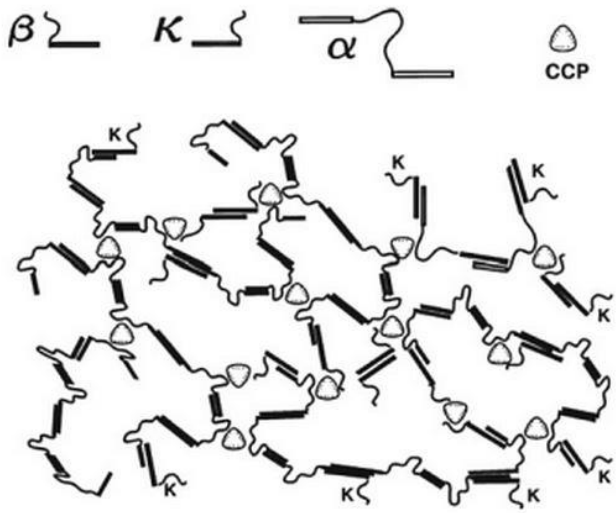


Figure 2-8:Dual-binding model of structure of casein micelle proposed by Horne (Horne, 1998).

The dual-binding model by Horne (2009) describes the bond formation, which supports the micelle integrity and stability, as obtained by stronger hydrophobic attraction over electrostatic repulsion (from the phosphoserine). Each casein functions as a block copolymer, with the hydrophobic region offering a multitude of weak, hydrophobic interactions. Figure 2-9 illustrates the initiation of self-association between the α_{s1}-, and β-casein where the polypeptide chains bind at the hydrophobic region. The hydrophilic region contains the phosphoserine cluster (with the exception of κ-casein, which has no cluster) which offers multiple functionality for cross-linking.

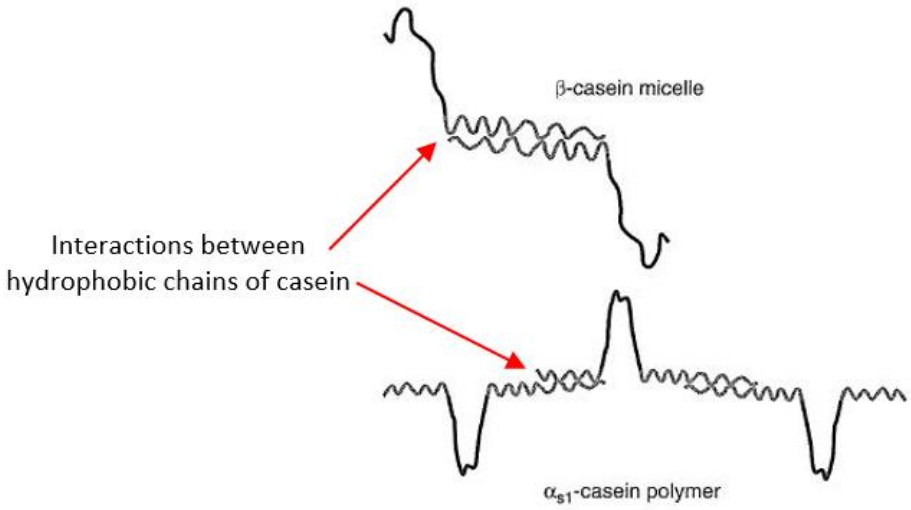


Figure 2-9: Polymeric structures generated when the hydrophobic chains of the caseins interact (Horne, 2009).

In the dual-binding model, κ-casein plays an important role as it is essential in terminating the casein micelle growth. Its hydrophobic N-terminal can link onto growing chains while the C-terminal block is hydrophilic and cannot sustain growth. As κ-casein does not possess a phosphoserine cluster, it is thus unable to extend the polymer chain through a nanocluster link. With that, the chain and network ends, leaving casein micelles with an outer layer of κ-casein (Horne, 2009).

A recent study was reported by De Kruif et al. (2012) where the authors used small-angle neutron, X-ray scattering and static light scattering spectrum (SANS, SAXS, SLS) to model the internal structure of the casein micelle according to the submicelle model, nanocluster model and dual-binding model. De Kruif et al. (2012) used a series of model calculations on the scattering data and concluded that only the nanocluster model was capable of accounting for the experimental scattering contrast variation data. However, compared to the earlier model proposed by Holt (1992), De Kruif et al. (2012) postulated that the casein molecules are not only attached to the nanoclusters at the phosphoserine residues, the hydrophilic tails are also associated by a collection of

weak interactions including hydrophobic interaction, hydrogen bonding, ion bonding, weak van der Waals attraction and other factors.

Recent developments in casein micelle structure

While the exact structure of casein micelle continues to be debated, recent studies involving the hydration and salt partitioning of casein micelles provided new insights into the casein micelle structure. Huppertz et al. (2017) investigated the reformation of casein micelles from sodium caseinate through introduction and supersaturation of calcium phosphate. Huppertz et al. (2017) studied the physical properties (e.g. size, protein and mineral distribution, zeta-potential, hydration) of the casein particles prepared from sodium caseinate and found that the casein particles reformed from sodium caseinate were very similar to casein micelles present in milk. The authors proposed that the caseinate particles, termed primary casein particles (PCP), were the building blocks for casein micelles and were not spherical in shape. Huppertz et al. (2017) further proposed that the core of the casein micelle is formed by a matrix of PCPs consisting of α_{S1}-, α_{S2}- and β-casein that are cross-linked by calcium phosphate nanoclusters, and the stabilisation of the surface of the micelles is provided by κ-casein (Figure 2-10). In addition, hydration studies of the PCP also suggested that the internal structure of the casein micelle would contain areas of high and low protein density where the protein-rich domains will be relatively poorly hydrated. However, the aqueous void spaces between the PCP (moisture-rich domains) as well as the hydrated surface of the casein micelles will contribute to the overall hydration of the casein micelles.

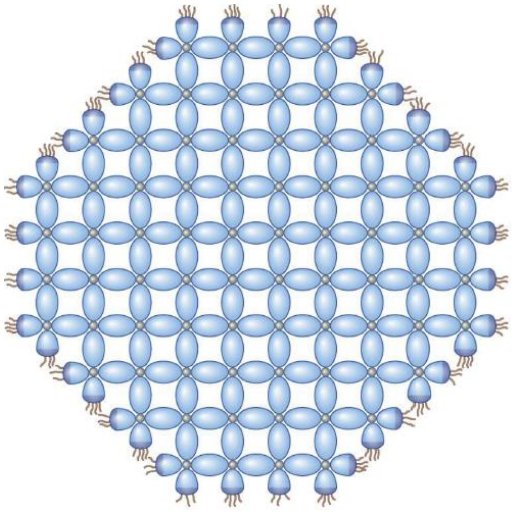


Figure 2-10: Illustration of transversal section through a casein micelle with a diameter of ~200 nm. Grey- colloidal calcium phosphate nanoclusters; blue: primary casein particle, with areas consisting of α_{S1}-, α_{S2}- and β-casein in light blue and κ-casein in dark blue/brown (Huppertz et al., 2017)

The association of casein molecules with the calcium phosphate nanocluster within a casein micelle had also been reinvestigated recently by Bijl, Huppertz, van Valenberg & Holt (2019). Through quantitative modelling of the ion equilibria and calcium phosphate sequestration by individual casein molecules, the authors examined two models used to predict the salt partitioning between the colloidal and serum phase in milk. In Model 1, previously proposed by Holt (2004), a casein molecule with several phosphate centres will form a distribution of unbound, partially bound, and fully bound states to the calcium phosphate nanoclusters. For example, a casein molecule with two phosphate centres can be free of any linkage to the calcium phosphate or can be bound through only one or both. In Model 2, all single and grouped phosphorylated residues on a given casein molecule cooperate together to react with a calcium phosphate nanocluster after an initial binding event, so that individual casein molecules are either fully bound through all their phosphorylated residues or not bound at all. (Bijl et al., 2019) reported that Model 2 provided better agreement with experimental results of the

partition of caseins between free and bound states, and partition of salts. The study provided a better understanding of how the individual casein molecules may interact with the calcium phosphate nanoclusters within a casein micelle and may support further understanding of the structure of casein micelles.

2.3 Whey proteins

About 20% of protein in milk belongs to a group of proteins referred to as whey proteins. They exist in the serum phase of milk as monomers, dimers, or as small quaternary structures (Fox, 2009). Whey proteins differ from casein proteins in a number of ways. Upon addition of rennet to milk or reducing the pH to 4.6, caseins aggregate and precipitate as they reach their isoelectric point. Whey proteins remain soluble at pH 4.6 or after rennet coagulation of casein. They are globular and highly structured proteins that contain hydrophobic inner regions and disulphide linkages (Pritchard & Kailasapathy, 2011; Thorn et al., 2015).

Whey proteins are known to have well-developed secondary, tertiary and quaternary structures but lower heat stability than casein proteins. Generally, they are destabilised at temperatures above 70°C, although this may be time and pH-dependent. Whey protein denaturation can be split into two processes; the unfolding of the protein molecules, and the aggregation of the unfolded proteins (De Wit, 1990). It should be noted that the unfolded proteins may be refolded back to their native state depending on the severity of the heat treatment, but the aggregation process is irreversible (Anema, 2009b).

The two principal whey proteins in milk are β-lactoglobulin and α-lactalbumin. β-lactoglobulin represents about 50% of whey (12% of total protein in milk, or 2 to 4 g L^{-1} milk), while α-lactalbumin represents 20% of whey protein (3.5% of total protein in

milk, or 1 to 1.5 g L^{-1} milk) (Swaisgood, 2003). Other whey proteins include bovine serum albumin (BSA), immunoglobulins (Ig), lactoferrin, and smaller amounts of proteose peptones (Fox, 2003).

2.3.1 β-lactoglobulin

β-lactoglobulin has a compact globular structure which consists of 162 residues per monomer. It has a molecular mass of approximately 18 kDa and isoelectric point of approximately 5.2. One monomer of 18 kDa contains two intramolecular disulphide bonds and one mole of cysteine. β-lactoglobulin exists as monomers, dimers, and oligomers at equilibrium, depending on pH, protein concentration, and ionic conditions, with the dimer being the prevalent form under physiological conditions. At pH between 5.5 and 7.5, β-lactoglobulin forms dimers of approximately 36 kDa which are linked by one to three disulphide bonds. Between pH 3.5 to 5.5, especially at close to pH 4.6, β-lactoglobulin forms octamers with molecular weight of about 144 kDa. However, below pH 3.5 and above 7.5, it dissociates into monomers (Edwards, Creamer & Jameson, 2009; Fox, 2003, 2009; Pritchard & Kailasapathy, 2011).

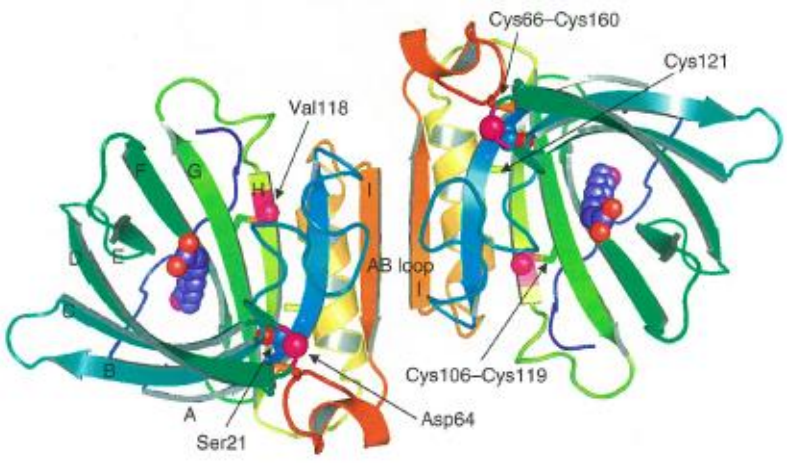


Figure 2-11: Dimeric structure of β-lactoglobulin. The structure is rainbow coloured, beginning with blue at the N-terminus and ending with red at the C-terminus (Edwards et al., 2009).

β-lactoglobulin denatures at around 70°C. During heat denaturation, dimers of β-lactoglobulin dissociate at between 30 to 55°C. Monomer unfolding occurs at higher temperature to permit more sulphydryl reactivity that can lead to disulphide interchange and aggregation, although aggregation also can occur without the involvement of sulphydryl. At temperatures below 70°C before aggregation occurs, the effects are generally reversible (Loveday, 2016; Sawyer, 2003).

The thiol group of cysteine that is exposed during heating is also responsible for interactions with the disulphide of κ-casein and significantly affects rennet coagulation and the heat stability properties of milk. The presence of κ-casein reduces aggregation of β-lactoglobulin, presumably due to intermolecular repulsion from the charge of the κ-casein. The interaction between β-lactoglobulin and κ-casein results in increased heat stability and rennet clotting time of milk (Fox, 2003; Kinsella & Morr, 1984; Vasbinder & De Kruif, 2003).

2.3.2 α-lactalbumin

α-lactalbumin is a compact, globular protein with molecular weight of approximately 14 kDa. It contains four intramolecular disulphide bonds per mole, which renders it relatively heat stable (Kinsella & Morr, 1984). Unlike β-lactoglobulin, α-lactalbumin does not contain a free thiol group. It is a metalloprotein, which binds one calcium ion per mole. The binding of calcium to native α-lactalbumin is coordinated by five oxygen atoms and two water molecules (Figure 2-12). The role of bound calcium appears to be to confer stability to the tertiary structure (Brew, 2003). The calcium ions also aid in the refolding and native disulphide formation of the denatured proteins (Brew, 2003; Edwards et al., 2009; Fox & McSweeney, 1998).

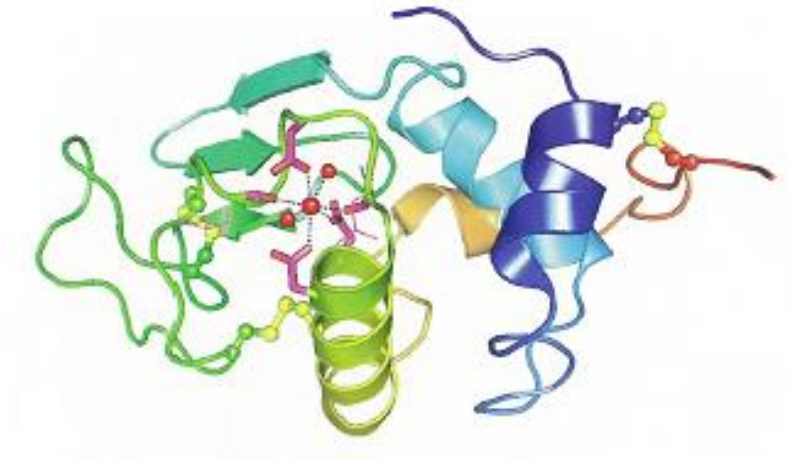


Figure 2-12: Structure of α-lactalbumin showing the calcium ion binding site (coordinated by seven bonds – five oxygen atoms and two water molecules). The peptide chain is rainbow coloured, beginning at the N-terminus in blue and progressing to the C-terminus in red (Edwards et al., 2009).

2.3.3 Bovine serum albumin (BSA)

Bovine serum albumin (BSA) contains 582 amino acids, making it the largest whey protein. It is approximately 66 kDa and makes up 1 to 5% of total whey protein (Fox & McSweeney, 1998). BSA molecules contain 17 disulphide and one sulphydryl groups. All the disulphide-linked cysteines are relatively close together in the polypeptide chain, therefore, it exits as a series of relatively short loops. In blood, BSA serves various functions but in milk, due to its low concentration, it has little effect on the physico-chemical properties of milk (Fox, 2003).

2.3.4 Immunoglobulin (Ig)

Immunoglobulins (Ig) form a diverse family of proteins whose members, when in milk, act as antibodies that provide offspring with protection against pathogenic microorganisms (Edwards et al., 2009). The main immunoglobulins in milk are IgG, IgG2, IgA, and IgM. In bovine milk, the predominant species of Ig proteins are IgG. Colostrum contains up to 10% Ig, a level which decreases rapidly post-partum. Similar

to BSA, the concentration of Ig proteins in milk is too low to exert a significant effect on the physico-chemical properties of milk (Pritchard & Kailasapathy, 2011).

2.3.5 Lactoferrin

Lactoferrin is a monomeric, globular, glycol protein consisting of approximately 680 amino acids (Edwards et al., 2009). It has a molecular weight of 80 kDa and has a high isoelectric point of between 8 to 9, making it a positively charged protein (García-Montoya, Cendón, Arévalo-Gallegos & Rascón-Cruz, 2012). Lactoferrin is known for its iron-binding ability, although other metal ions such as copper, chromium, manganese and aluminium can also be bound to lactoferrin (Lönnerdale, 2003). Studies on lactoferrin mostly involve its role in immunity and antibacterial effects. In milk, the low concentration means that it is unlikely to exert major physico-chemical effects (Anema, Singh & Creamer, 1993).

2.4 Calcium in milk

One of the most well-known micronutrients in milk is calcium. Milk contains about 1200 mg L^{-1} or 30 mmol L^{-1} of calcium (Deeth & Lewis, 2015). The concentration of calcium in milk is higher than the concentration that can be maintained in an aqueous solution at the pH of native milk, with excess calcium being present in the colloidal phase. Consequently, it exists partly in soluble form (serum) and partly in an insoluble form (colloidal) associated with casein (Fox & McSweeney, 1998). Two-thirds of the calcium (~ 20 mmol L^{-1}) exists as colloidal calcium phosphate (CCP) in the casein micelles, or as calcium ions bound to the phosphoserine residues. The remaining one-third (~ 10 mmol L^{-1}) of the calcium is soluble in the serum phase as calcium citrate, phosphate or free ions. Ionised calcium accounts for about 10% of the total calcium (~ 2 mmol L^{-1}), with very small amounts (0.15%) bound to α-lactalbumin (Deeth & Lewis, 2015; Flynn & Cashman, 1997; Ramasubramanian, Webb, D'Arcy & Deeth, 2013).

Calcium, in its colloidal, soluble non-ionic and soluble ionic forms exists in a dynamic equilibrium.

2.4.1 Calcium equilibrium in milk

The calcium in the serum and colloidal phases exists in a dynamic equilibrium. Figure 2-13 summarises the equilibrium relationships of calcium, phosphate and citrate ions between the serum and colloidal phases in native milk (pH 6.6 to 6.7). Structures of the free ions and calcium salts are shown in Figure 2-14.

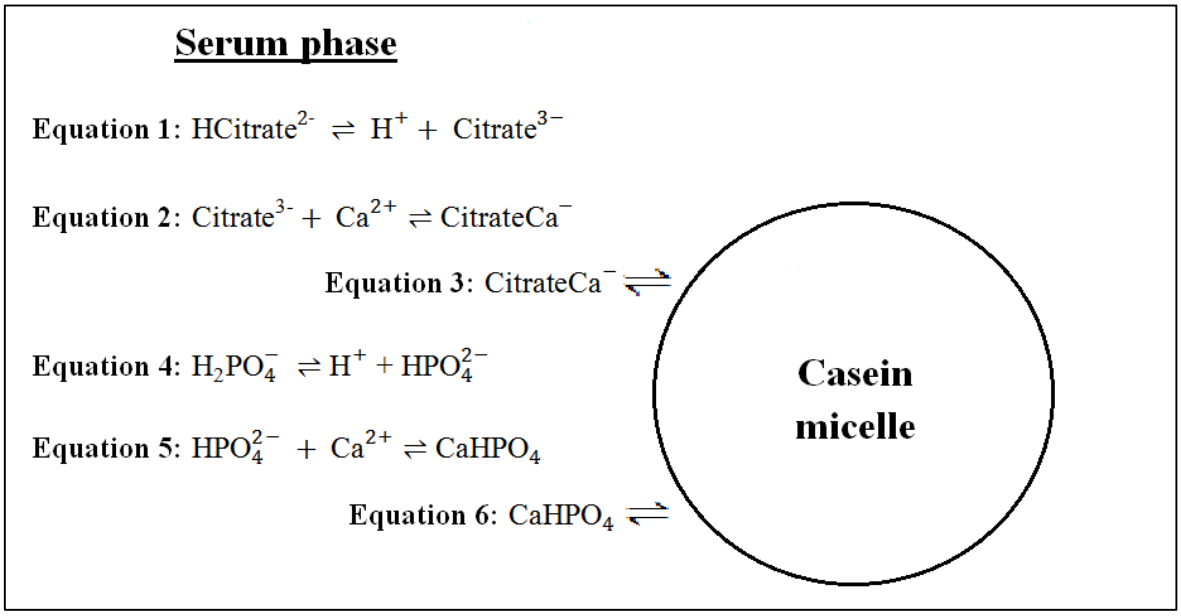


Figure 2-13: Summary of the equilibrium between calcium, citrate and phosphate ions in the serum and colloidal phase in native milk (pH 6.6 to 6.7). Equations 1 to 3 show the equilibrium between the calcium and citrate ions; Equations 4 to 6 show the equilibrium between the calcium and phosphate ions (adapted from Croguennec, Jeantet & Schuck (2016).

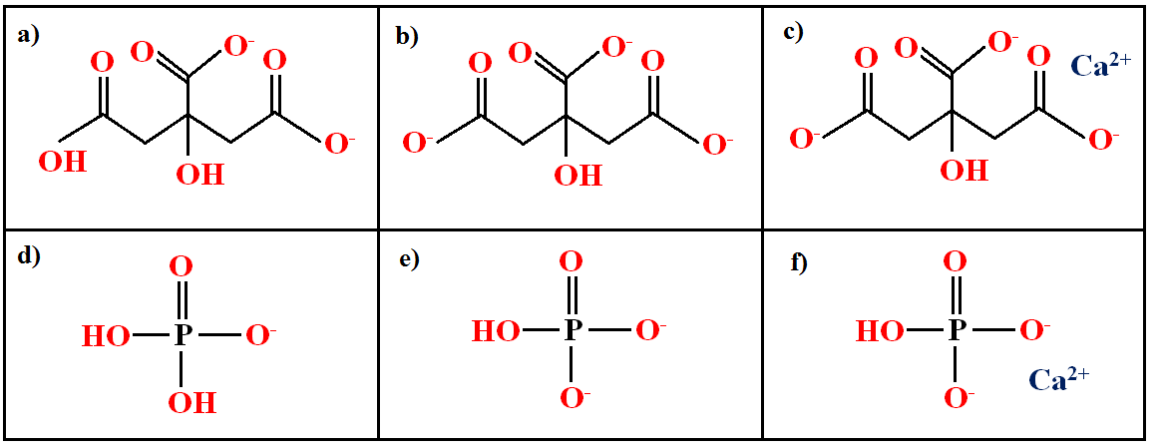


Figure 2-14: Structures of citrates (a to c) and phosphates (d to f) at different states of ionisation and association with calcium. a) $HCitrate^{2-}$; b) $Citrate^{3-}$; c) $CaCitrate^{-}$; d) $H_2PO_4^-$; e) HPO_4^{2-} ; f) $CaHPO_4$.

In the serum phase of milk, the association between the calcium and the anions (such as citrates and phosphates) is dependent on the association constants (or affinity) (Croguennec et al., 2016; Gaucheron, 2005). For example, $H_2PO_4^-$ has a low affinity for calcium and therefore remains mostly in its ionic form or in equilibrium with HPO_4^{2-} (Figure 2-13- Equation 4). In contrast, HPO_4^{2-} has a relatively high affinity for calcium and therefore able to associate with calcium ions (Mekmeme, Graët & Gaucheron, 2009). Milk is supersaturated with respect to calcium phosphate (Walstra et al., 1999). The presence of casein micelles prevents the precipitation of calcium phosphate by the integration within the micelle structure as colloidal calcium phosphate (CCP) (Croguennec et al., 2016). The equilibrium in native milk shown in Figure 2-13 is easily disrupted by a change in the conditions of the milk such as pH, temperature and addition of salts (Dalgleish & Law, 1988; Horne, 2009).

2.4.2 Different forms of calcium phosphate in milk

Calcium phosphates exist in different Ca/P ratio and in different structures (amorphous or crystallised) (Gaucheron, 2005). Possible forms of calcium and its complexes in milk are shown in Table 2-3. In the colloidal phase, the composition of CCP is difficult to

determine experimentally as calcium bound to organic or inorganic phosphates are not separable. Holt (1982) proposed a modified dicalcium phosphate structure of $Ca(HPO_4)_{0.7}(PO_4)_{0.2}$ which is in near-equilibrium with the colloidal phase. Holt (1982) further proposed that the calcium phosphates in milk contain a mixture of salts, with dicalcium phosphate as a precursor of more basic salts such as octacalcium phosphate and hydroxyapatite.

Table 2-3: Chemical formulas and Ca/P ratios of different potential calcium salts in the soluble phase of milk (Gaucheron, 2005).

Compound	Formula	Ca/P ratio
Dicalcium phosphate	$CaHPO_4$	1.0
Dicalcium phosphate dihydrate	$CaHPO_4.2H_2O$	1
Micellar calcium phosphate	$Ca(HPO_4)_{0.7}(PO_4)_{0.2}.xH_2O$	1.1
Octacalcium phosphate	$Ca_8H_2(PO_4)_6.5H_2O$	1.33
β-tricalcium phosphate	$\beta\text{-}Ca_3(PO_4)_2$	1.5
Hydroxyapatite	$Ca_5OH(PO_4)_3$	1.67
Amorphous calcium phosphate	$Ca_3(HPO_4)_{0.2}(PO_4)_{1.87}.xH_2O$	1.45
Tricalcium citrate dihydrate	$Ca_3(Cit)_2.2H_2O$	N.A.

2.4.3 Effect of pH on calcium equilibrium

Acidification increases the ionic calcium concentration and dissolved calcium in the soluble phase of milk (Deeth & Lewis, 2015; Faka, Lewis, Grandison & Deeth, 2009; Lucey et al., 1996; McMahon & Brown, 1984). Decreasing pH increases the H^+ ions in the soluble phase of milk, thus inducing changes in the calcium equilibrium. For instance, in the case of calcium phosphate equilibrium which is shown in Equations 4 to 6 (Figure 2-13), the following shifts in the equilibrium may occur following acidification of milk. Increase in H^+ may result in an equilibrium shift towards the protonation of HPO_4^{2-} to $H_2PO_4^-$ (Gaucheron, 2005).

From Equation 4 (Figure 2-13): $H^+ + HPO_4^{2-} \rightleftharpoons H_2PO_4^-$

The decreased concentration of HPO_4^{2-} thus results in the soluble calcium phosphate ($CaHPO_4$) in the serum phase being converted to HPO_4^{2-} and Ca^{2+} to restore the balance.

From Equation 5 (Figure 2-13): $CaHPO_4 \rightleftharpoons HPO_4^{2-} + Ca^{2+}$

The calcium phosphate in the serum phase is reduced, and as a result, the CCP in the casein micelles is solubilised into the serum phase to re-establish the equilibrium (Figure 2-13- Equation 6). Thus, the overall effect of acidification is the reduction in CCP and an increase in the concentration of calcium ions in the serum phase. The increase in calcium ion concentration with decreasing pH was shown in a study by Tsioulpas, Lewis & Grandison (2007) where the pH of nine individual milk samples was altered and a strong negative correlation was found ($R^2 = 0.975$) between the log free Ca^{2+} and pH (Figure 2-15). Moreover, the slope for each sample was very similar, which suggested that when the pH of milk was altered, the changes in the log free Ca^{2+} were similar in the different milk samples tested.

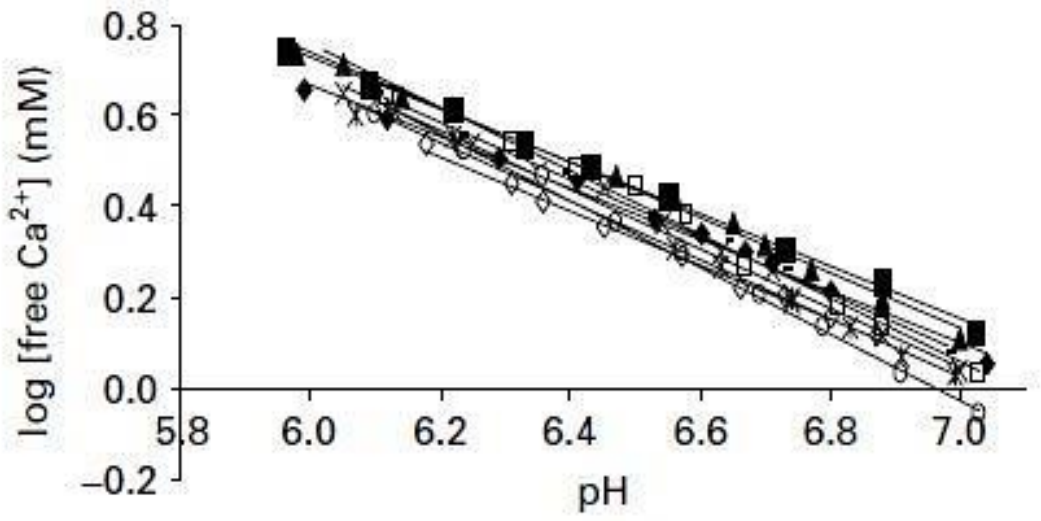


Figure 2-15: Effect of pH on log of free calcium ion concentration of milk samples from nine individual cows (Tsioulpas et al., 2007). Different symbols indicate the different milk samples from the nine individual cows.

An increase in milk pH may result in the reverse of the above described reactions, where the reduction in H^+ ions leads to the formation of HPO_4^{2-} , and then $CaHPO_4$ with any free Ca^{2+} present in the serum, and hence a decrease in the calcium ion concentration in the serum will be observed (Horne, 2009). Fox (2003) stated that raising the pH up to pH 9 increases the level of CCP, implying the increased $CaHPO_4$ will react with the casein micelles to form CCP. However, further increase in the pH results in disintegration of the casein micelles (Huppertz, Vaia & Smiddy, 2008; Pan & Zhong, 2013). This was possibly due to the disturbance of the balance of electrostatic repulsion and hydrophobic attraction (Horne, 2009).

Similar to the calcium and phosphate equilibrium, the equilibrium involving calcium citrate could also take place with the change in milk pH (Equations 1 to 3, Figure 2-13). However, most literature primarily considers the equilibrium between calcium and phosphate as the changes in the phosphate concentration in the serum phase are considerably larger compared to changes in citrates when pH of milk is altered (Figure 2-16).

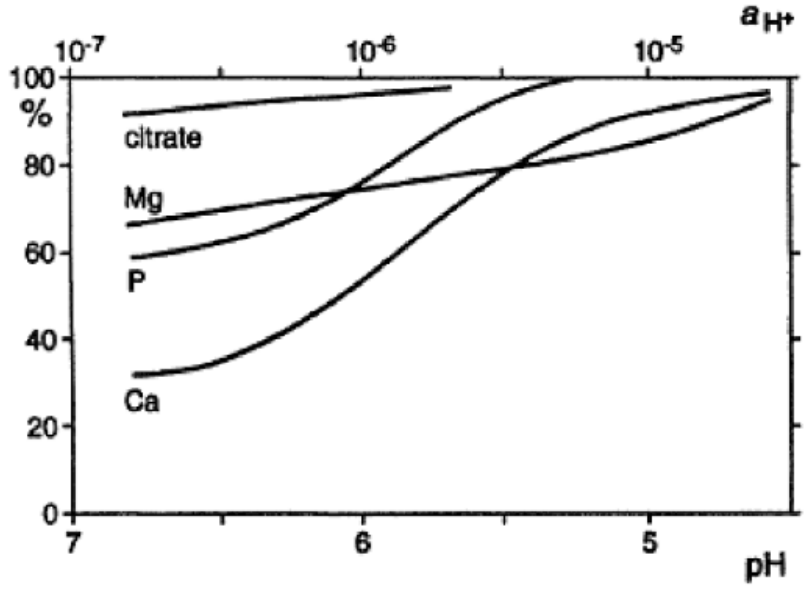


Figure 2-16: Changes in the percentages of calcium, phosphate, magnesium and citrate in the serum phase as a function of pH in milk (Walstra et al., 1999).

2.4.4 Effect of heating on distribution of calcium in milk

Heating is known to reduce the pH of milk mainly as a result of formation of formic acid due to the degradation of lactose (Berg & van Boekel, 1994). However, the reduction in pH during heating is not accompanied by a corresponding increase in ionic calcium. Sauer & Moraru (2012) found that the soluble calcium in the serum phase of reconstituted micellar casein decreased with increasing temperature across the pH range of 6.7 to 7.3 (Figure 2-17). This was due to decreased solubility of the calcium phosphate as temperature increased (Fox & McSweeney, 1998). Since milk is supersaturated with respect to calcium phosphate in the serum phase, the precipitated calcium phosphate is subsequently transferred to the colloidal state (Jeurnink & De Kruif, 1995; van Boekel, Nieuwenhuijse & Walstra, 1989; Walstra et al., 1999). On heating, the calcium equilibrium shifts towards $CaHPO_4$ entering the colloidal phase (Figure 2-13- Equation 6). Calcium ions and phosphates in the serum phase will form $CaHPO_4$ to restore the balance (Figure 2-13- Equation 5). This in turn results in the reduction in calcium ion concentration in the serum phase (Geerts, Bekhof & Scherjon, 1983). It has also been proposed that $H_2PO_4^-$ may also react with calcium ions to form $CaHPO_4$ with heat (Equation 7). However, the precise nature of the precipitated calcium phosphate formed on heating is uncertain, but the formation of tricalcium phosphate ($Ca_3(PO_4)_2$), with the release of H^+ ions, had been proposed (Equations 8).

Equation 7: $Ca^{2+} + H_2PO_4^- \rightleftharpoons CaHPO_4 + H^+$

Equation 8: $CaHPO_4 / Ca(HPO_4)_2 \rightleftharpoons Ca_3(PO_4)_2 + 3H^+$

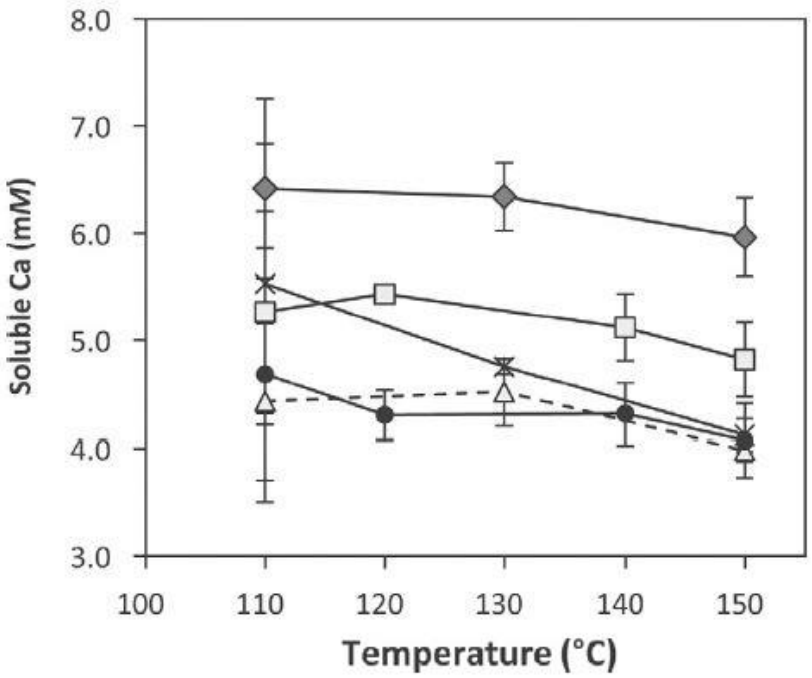


Figure 2-17: Calcium concentration in the soluble phase of heat-treated micellar casein concentrates. pH 6.5 (◆); pH 6.7 (■); pH 6.9 (▲); pH 7.1 (●); pH 7.3 (∗) (Sauer & Moraru, 2012).

Depending on the severity of the heat treatment, the change in the calcium equilibrium may be reversible. For instance, Pouliot, Boulet & Paquin (1989) reported that on heating milk at 85°C for 40 min, calcium recovery in the milk serum was 90 to 95% on cooling, while on heating milk rapidly to 130°C, 16% of a mixture of calcium and magnesium was lost through deposition on the heating system which were unrecoverable (Dalgleish, Pouliot & Paquin, 1987).

2.4.5 Effect of calcium addition to calcium equilibrium in milk

The calcium ions in the serum phase are free to react and can influence the calcium equilibrium, therefore playing a major role in the heat stability, rennetability and rheological properties of milk (de la Fuente & Juarez, 2015). Addition of soluble calcium salts, such as calcium chloride, calcium lactate and calcium gluconate, leads to an increase in concentration of calcium ions in milk (Crowley, Kelly & O'Mahony, 2014; Omoarukhe, On-Nom, Grandison & Lewis, 2010; Sievanen, Huppertz, Kelly &

Fox, 2008). This shifts the equilibrium towards formation of $CaHPO_4$ (Figure 2-13-Equation 5) which increases the concentration of $CaHPO_4$ in the serum of milk. As a result, the equilibrium shifts towards $CaHPO_4$ entering the casein micelles (Equation 6) (Croguennec et al., 2016; Fox & McSweeney, 1998; Gaucheron, 2005). The addition of calcium chloride was reported to result in a decrease in inorganic phosphate and citrate in the serum phase and an increase in CCP (deduced from the difference between the total calcium concentration and soluble calcium concentration). This suggested that the added calcium ions may have interacted with the available phosphates and citrates in the serum phase to form $CaHPO_4$ and calcium citrate salts which may then enter the casein micelles (Philippe, Gaucheron, Graët, Michel & Garem, 2003; Udabage, McKinnon & Augustin, 2000). However, it should be noted that insoluble calcium salts, such as calcium carbonate and calcium phosphate, caused no noticeable changes to the ionic calcium level (Crowley et al., 2014; Omoarukhe et al., 2010).

2.4.6 Effect of changes in calcium concentration on stability of casein micelle

The integrating role of calcium, in the form of CCP, on casein micelle structure has briefly been discussed in Section 2.2.2. Thus, this section will focus on discussing the effect of changes in the calcium concentration on the stability of casein micelles.

As discussed in Sections 2.4.4 and 2.4.5, pH alterations, and the addition of calcium salts can result in changes to the calcium equilibrium in milk. Calcium in the colloidal state can complex with the phosphate ester and carboxyl groups of micellar casein, or with phosphate, and perhaps citrate (Figure 2-18) (Aoki, Yamada, Tomita, Yoshitaka & Imamura, 1987; McMahon & Brown, 1984; Swaisgood, 2003). The changes in both ionic calcium and CCP can have an effect on the stability of casein micelles. Ionic calcium can form calcium bridges which link the casein micelles (Crowley et al., 2014; Fox, 2003; Walstra et al., 1999) or bind to the phosphate esters of the caseins,

diminishing the electrostatic repulsion between the casein molecules (van Boekel et al., 1989). Philippe et al. (2003) also reported an increase in casein hydrophobicity on addition of calcium, which may promote hydrophobic interactions between the casein micelles. The increase in CCP would increase crosslinking of the casein molecules, possibly with the soluble caseins in the serum phase (Horne, 2002; van Boekel et al., 1989), or reduce the steric repulsion between the casein micelles, leading to fusion of the micelles and aggregation (Walstra et al., 1999). As the addition of soluble calcium increases the level of ionic calcium in milk, this results in destabilisation of casein micelles. Calcium fortification of heat-processed milk products is achieved commercially by the use of fine powders of insoluble calcium salts such as calcium citrate, calcium carbonate and calcium phosphate (Deeth & Lewis, 2015; van Boekel et al., 1989).

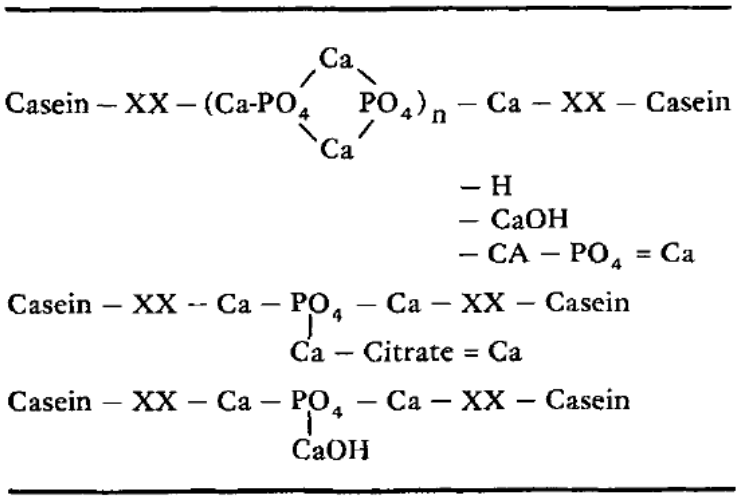


Figure 2-18: Possible linkages in colloidal calcium phosphate. XX = carboxylate or phosphoserine (McMahon & Brown, 1984).

2.5 Molecular interactions between milk proteins

The stability of casein micelles and whey proteins in milk is dependent on the attractive and repulsive forces between the protein molecules. In whey proteins, intermolecular

forces may also govern the conformation of the protein. An understanding of the intermolecular forces that may occur between proteins is therefore essential in the determination of the aggregation and gelation properties of the milk proteins. Table 2-4 summarises several types of bonds that may occur between two proteins. The principles of some of the forces involved in protein interactions are briefly discussed in this section.

Table 2-4: General characteristics of molecular interactions between two protein molecules in aqueous solution (Bryant & McClements, 1998).

Type of bond	Sign	Strength	Range
Hydrophobic	Attractive	Strong	Long
Electrostatic	Repulsive	Weak → Strong	Short → Long
• Salt bridging	Attractive		
Hydrogen bonding	Attractive	Weak	Short
Hydration	Repulsive	Strong	Short
van der Waals	Attractive	Weak	Short
Steric repulsion	Repulsive	Strong	Short
Disulphide bonds	Attractive	Very strong	Strong

2.5.1 Hydrophobic interactions

Long-range attractive interactions known as hydrophobic interactions were proposed to exist in the vicinity of the neutral charge surfaces (Eriksson & Yoon, 2007). Hydrophobic effect arises from unfavourable interactions between the water molecules and the non-polar surfaces of a protein (Nakai & Li-Chan, 1988). When a non-polar molecule is introduced into water, it causes the water molecules in its immediate vicinity to rearrange themselves to decrease the entropy of the system. As these changes are thermodynamically unfavourable, the non-polar surfaces of the molecule are thus forced to coalesce together to minimise the contact between the non-polar surface and water. Weak van der Waals attractive forces may also be formed between the two

interacting non-polar groups (Bryant & McClements, 1998; Nakai & Li-Chan, 1988). This may result in structural changes to a protein molecule, or interactions between two neighbouring proteins.

2.5.2 Electrostatic interactions

Electrostatic interactions can occur between atoms and molecules which carry an electrical charge, such as dipole-dipole, ion-dipole, or ionic interactions (Bryant & McClements, 1998). When the charges are the same sign, the interaction is repulsive, but when they have different signs, the interaction is attractive. For example, casein micelles carry a net negative charge at the native pH of milk, thus resulting in repulsive interactions with each other. Dipole-dipole interactions can occur between two polar molecules which contain a partially positive and partially negative end (Figure 2-19). The partial negative end of one polar molecule can interact with the partial positive end of another polar molecule by dipole-dipole interactions (Ravve, 2012).

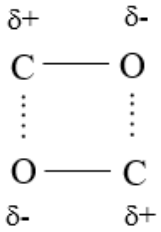


Figure 2-19: Schematic diagram depicting dipole-dipole interactions between two polar molecules. Dipole-dipole interactions can occur between the partial positive end (carbon) of one polar molecule and the partial negative end (oxygen) of another polar molecule.

Electrostatic interactions between oppositely charged ions and proteins can result in ion bridging of the protein. It occurs when a polyvalent ion (such as Ca^{2+}) simultaneously binds to the surface of two protein molecules which have an opposite charge to the ion. Polyvalent ions are able to form ion bridges and simultaneously reduce the magnitude

of electrostatic interactions through a charge screening effect (Bryant & McClements, 1998).

Electrostatic interactions are dependent on the pH and ionic strength of the solution as they influence the charge on the protein (Oakenfull, Pearce & Burley, 1997). At pH values above or below the isoelectric point, the protein carries a greater net charge, and thus greater electrostatic repulsion between proteins. In solutions with high ionic strength, the presence of electrolytes may reduce the electrostatic repulsions due to electrostatic screening by the counter ions (Bryant & McClements, 1998; Totosaus, Montejano, Salaza & Guerrero, 2002)

2.5.3 Steric repulsion

On close approach between two atoms or molecules, a strong repulsive force known as steric repulsion may occur due to overlapping of the electron cloud (Israelachvili, 2011). Steric repulsion may also occur between two approaching casein micelles due to the presence of the κ-casein on the surface of the micelle (Bhat, Dar & Singh, 2016). The hydrophilic tail of κ-casein on the surface of casein micelles may provide steric stability due to unfavourable entropy associated with restrictions in the configurational freedom (volume restriction) on close approach between the κ-casein tails on the surface of two casein micelles (Israelachvili, 2011).

2.5.4 Hydration interactions

Hydration interactions are fairly short range repulsive interactions that arise when two hydrated molecules closely approach each other (Bryant & McClements, 1998). Ions dissolved in solution have a number of water molecules orientated around them. Such ions are called hydrated ions, and the shell of water molecules surrounding a solvated ion is referred to as the hydration shell (Israelachvili, 2011). Interactions between

proteins in milk may be influenced by the hydration of the proteins, which in turn is dependent on the ionic strength of the solution. For instance, binding of strongly hydrated ions to protein surfaces may prevent protein aggregation due to increased hydration repulsive forces (Bryant & McClements, 1998; Oakenfull et al., 1997). Hydration repulsion may explain why native whey proteins remain soluble at its isoelectric point. The exposed surface of native whey proteins contain a high ratio of hydrophilic to hydrophobic groups. Although the proteins are electrically neutral at their isoelectric point which may result in aggregation via hydrophobic interactions, the hydration of the hydrophilic groups on the surface create hydration repulsion strong enough to offset the attractive hydrophobic interactions (Damodaran, 1997). However, it should be noted that when whey proteins are denatured, the surface hydrophobicity and hydrophilicity is altered, which could then lead to aggregation via hydrophobic interactions (Damodaran, 1997).

2.5.5 Disulphide bonds

The main covalent bonds crosslinking proteins are the disulphide bonds (-S-S-) of cysteine residues (Oakenfull et al., 1997). Disulphide bonds between cysteine residues can be intramolecular, which is important in stabilising the structure of the protein, or intermolecular, which can result in crosslinking of protein molecules (Holland, Deeth & Alewood, 2008). In the native state, the cysteine residue in β-lactoglobulin, which contains a thiol group (-SH), is hidden in the protein molecule. Upon heating, denaturation of the protein exposes the thiol groups, leading to formation of disulphide bonds via thiol oxidation or thiol/ disulphide interchanges (Famelart, Le, Croguennec & Rousseau, 2013). κ-casein also contains two cysteine residues per molecule and is thus also able to participate in thiol/ disulphide interchanges with β-lactoglobulin during heat treatment (Holland et al., 2008).

2.5.6 The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory

The DLVO theory is based on the theory that colloidal stability is dependent on the electrostatic repulsion and attractive van de Waals forces between two colloidal particles (Tadros, 2007). It was proposed that according to the DLVO theory, the electrostatic repulsion between casein micelles is not strong enough to compensate for the van der Waals attraction and prevent flocculation (Tuinier & De Kruif, 2002; Walstra et al., 1999). However, casein micelles do not flocculate and are stable under the physiological condition of milk. The counteracting repulsive forces that prevent aggregation is attributed to steric stabilisation provided by κ-casein on the surface of casein micelles (Tuinier & De Kruif, 2002). However, the other forces described in this section are also crucial and the stability of casein micelles and whey proteins is dependent on the overall attractive and repulsive forces.

2.6 Gelation of milk

A gel is a continuous, three-dimensional network of connected molecules or particles in a continuous liquid phase (Walstra, Van Vliet & Bremar, 1991). In milk gels, the occurrence of protein–protein, and protein–solvent interactions in an ordered manner results in the immobilization of a large amount of water by a small proportion of proteins (Mulvihill & Kinsella, 1987). The first step towards the formation of milk gels involve the destabilisation of the milk proteins, which allows interaction and aggregation of the proteins (De Kruif et al., 1995; Schmidt, 1981). A gel is formed when the extent of aggregation exceeds a critical level for the formation of a self-supporting network that is able to entrap the solvent (Lucey, 2009). Schmidt (1981) noted that it is critical that the aggregation step proceed at a slower rate than the unfolding step in proteins, and that the degree of crosslinking must be optimal for the formation of an ordered gel matrix.

The formation of a gel network is dependent on the balance between the attractive and repulsive forces among the protein molecules, which in turn is influenced by a number of factors such as pH, ionic strength and temperature (Kinsella, Rector & Phillips, 1994; Lucey, 2009). Generally, excessive attractive forces results in a random network that is unable to hold water, known as a coagulum. In contrast, if repulsive forces predominate, no network will be formed (Mulvihill & Kinsella, 1987). Therefore, a critical balance between the attractive and repulsive forces must be present for successful network formation and stabilization.

The gel structure formed by milk proteins can be divided into two types as shown in Figure 2-20: particulate gels which are opaque or turbid, and fine-stranded gels which appear transparent or translucent (Doi, 1993; Lucey, 2009; Nicolai & Durand, 2013). Fine-stranded gels are formed by an ordered association of molecules, followed by crosslinking of the strands (Phan-Xuan et al., 2013; Tombs, 1975) The dimensions of the strands are often so small that these gels appear transparent (Hermansson, 1994). Particulate gels are a result of random aggregation of the protein particles and the size of the aggregates can vary considerably depending on the type of protein and gelling conditions (Doi, 1993; Hermansson, 1994). Whey proteins can form either fine-stranded or particulate gels while casein gels are usually classified as particulate gels (Doi, 1993; van Vliet, Lakemond & Visschers, 2004). In both types of gels, the strength of the gel is dependent on the number of effective bonds, as well as distribution of the particles in the network (Fox & Mulvihill, 1990).

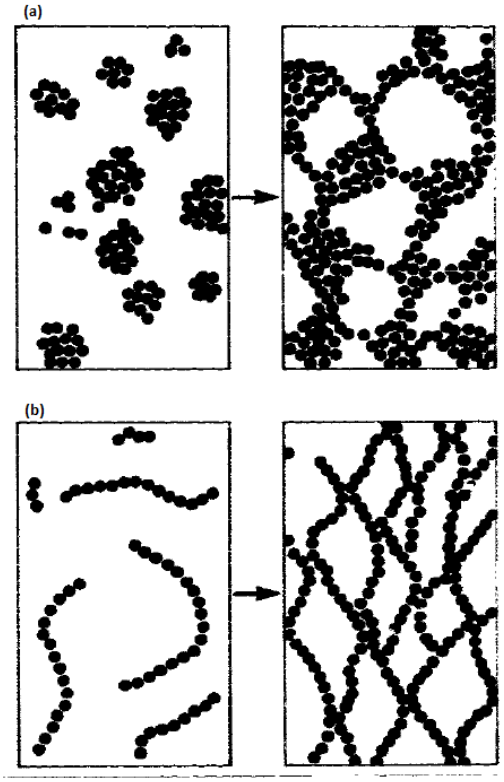


Figure 2-20: Two types of gel networks formed by the aggregation of proteins (a) particulate gels formed by random aggregation of molecules; (b) fine-stranded gels formed by aggregation of polymers (Doi, 1993).

2.6.1 Heat-induced gelation

When considering heat-induced gels, the main type of gel produced is the heat-set whey protein gel. Whey protein gels can be produced by heating the proteins to temperatures above the denaturation temperature to induce destabilisation of the native tertiary structure. Unfolding of the whey proteins exposes the hydrophobic amino acids buried deep within the native protein structure, the cysteine and cystine groups, leading to an increase in the reactivity of the whey proteins (Singh & Havea, 2003; van Vliet et al., 2004). Through the disulphide and hydrophobic interactions, the unfolded protein may associate with each other and form aggregates (Singh & Havea, 2003).

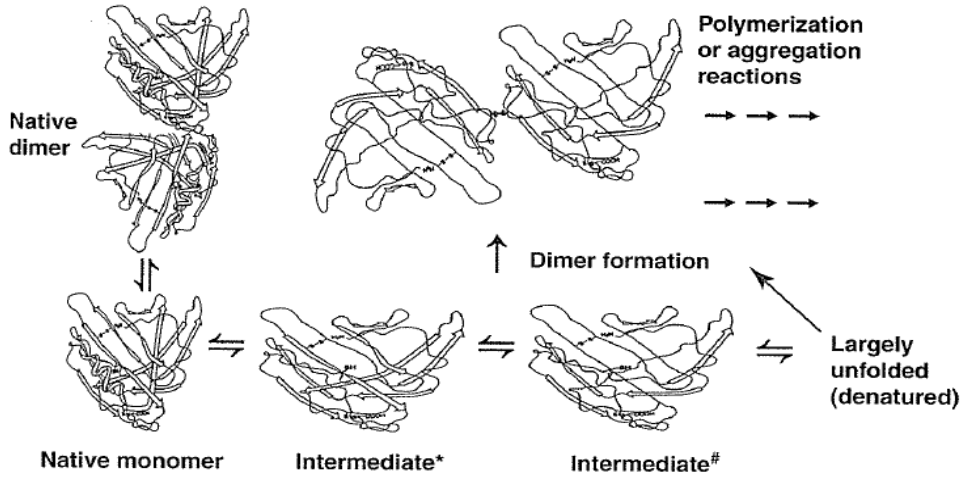


Figure 2-21: Model for the aggregation and formation of heat- induced β-lactoglobulin gels (Lucey, 2009).

β-lactoglobulin, the most abundant whey protein, has been found to greatly affect the properties of whey protein gels (Langton & Hermansson, 1992; van Vliet et al., 2004). As a result, the majority of research on whey protein gels have been focused on the impact of β-lactoglobulin. The basic steps involved in the denaturation and gelation of β-lactoglobulin are shown in Figure 2-21. The first step involves the dissociation of dimers into monomers and a conformational change which exposes and activates the sulphydryl groups, which can undergo oxidation to disulphide (S – S) or cysteic acid (-SO_3H) groups or sulphydryl–disulphide interchange reactions. The second step involves formation of aggregates where the intermediate products may associate with each other covalently through sulphydryl-disulphide interchange and non-covalently by hydrophobic interactions (Mulvihill & Kinsella, 1987; Singh & Havea, 2003). This is followed by formation of strands from the aggregates and ultimately, the alignment of the strands into a gel network if the protein concentration and other gelling conditions, such as pH and ionic strength, are favourable (Aguilera & Rademacher, 2004; Lucey, 2009; Mulvihill & Kinsella, 1987). Gelation occurs when the electrostatic repulsion

between the proteins is reduced, such as at pHs near the pI of whey proteins (pH 5 to 5.5), or at conditions of high ionic strength (Langton & Hermansson, 1992; Tang, McCarthy & Munro, 1995).

2.6.2 Cold-set whey protein gelation

Whey protein gels can also be formed by adjustment of pH or addition of salts after heating (Chung, Degne & McClements, 2013). In heat-set gelation, denaturation, aggregation and gelation occurs simultaneously in the system during a one-step heating process. On the other hand, in cold- set gelation, denaturation and aggregation are separated from the gelation step (Alting, Hamer, De Kruif & Visschers, 2003). The heat treatment in the first step of cold-set gels is usually carried out in solutions with low ionic strength and/ or far from the isoelectric point to produce aggregates that remain as a stable dispersion after cooling (Lucey, 2009; van Vliet et al., 2004). Gelation occurs in the second step where screening of the electrostatic repulsion between the aggregates is achieved to induce their self-association. This can be achieved by either pH adjustment or through the addition of salts (Chung et al., 2013).

2.6.3 Acid-induced gelation

Acid- induced milk gels are examples of casein gels which involves the aggregation of casein on acidification of milk to its isoelectric point (van Vliet et al., 2004). Due to the absence of charge on the casein micelles at its isoelectric point, increasing hydrophobic interactions allows aggregation of the casein particles (Lucey, 2009). Milk can be acidified by bacterial cultures, which ferment lactose to lactic acid during the production of yoghurts, or by addition of chemical acids such as HCl or glucono delta-lactone (GDL) (Lucey & Singh, 1998). In unheated milk, gelation occurs at around pH 4.6, which is the isoelectric point (pI) of casein. A summary of the changes in casein micelles with decreasing pH is classified into three regions by Lucey (2009):

a) pH from 6.7 to 6.0 – The decrease in pH causes a reduction in the net negative charge on casein micelles, thereby reducing electrostatic repulsion. As a relatively small amount of CCP is dissolved above pH 6.0, the structural features of the micelle remain relatively unchanged.

b) pH from 6.0 to 5.0 – As the κ-casein "hairs" on the micelle surface are charged, these "hairs" may shrink/collapse as the pH decreases. The net result is a decrease in both electrostatic repulsion and steric stabilization. The CCP in the micelle is dissolved completely by approximately pH 5.0.

c) pH ≤ 5.0 – The net negative charge of the casein micelles declines with the approach of isoelectric point (pH 4.6), and the casein particles aggregate as a result of charge neutralization. The reduction in electrostatic repulsion also allows increase hydrophobic interactions (Horne, 1998).

2.6.4 Rennet-induced gelation

Rennet-induced milk gels are formed by the addition of a milk clotting enzymes (e.g. chymosin in rennet) to hydrolyse the C-terminal part of the κ-casein, thereby reducing the electrostatic and steric stabilisation of the casein micelles and causing them to aggregate (Hyslop, 2003; van Vliet et al., 2004). It is the first step in the cheese-making process which involves coagulation of the milk. As discussed earlier in Section 2.2, the casein micelle is composed of α_s- and β- casein which are bound within the casein micelle by hydrophobic interactions and CCP, while the κ-casein lies on the surface of the micelles and stabilises the micelle by electrostatic and steric repulsion via the hydrophilic "hairy layer". The role of rennet is to remove the "hairy layer" so that there is a reduction in the net negative charge and steric repulsion of the micelle (Hyslop, 2003; Lucey, 2002). This exposes the hydrophobic groups within the casein micelles such that the micelles become susceptible to aggregation.

The process of gelation in rennet-induced milk gels can be divided into two stages: (1) the primary stage where the enzymes are activated and hydrolysis of the C-terminal of κ-casein occur; (2) the secondary stage where the casein micelles are sufficiently destabilised and begin to aggregate. During the primary stage of rennet coagulation, κ-casein is divided into *para*-κ-casein, which is relatively hydrophobic (residues 1 to 105), and caseinomacropeptide (CMP) (residues 106 to 169), which is the hydrolysed C-terminal that is very hydrophilic (Farrell et al., 2004; Hyslop, 2003; Lucey, 2009). Aggregation of the κ-casein depleted casein micelles occurs in the secondary stage where the particles approaches closer to each other due to reduction in electrostatic and steric stability. The released CMP diffuses away from the micelles into the serum phase which leads to a decrease in zeta potential by about 5 to 7 mV (about 50%), thus reducing the electrostatic repulsion between rennet-altered micelles (Hyslop, 2003; Lucey, 2009). The primary and secondary stage of rennet coagulation overlaps as aggregation begins before enzymatic reaction is complete (Dalgleish & Corredig, 2012; Hyslop, 2003; Lucey, 2009). A possible tertiary stage where structural rearrangement occurs in the milk gels has also been proposed (Lucey & Fox, 1993). It should be noted that the destabilised micelles will only aggregate in the presence of calcium ions, possibly due to neutralisation of the negative charges on the casein micelles, thereby facilitating hydrophobic interactions (Deeth & Lewis, 2015; Lucey, 2009).

The initial phase of renneting results in the reduction in viscosity due to a decrease in the hydrodynamic diameter of the casein micelles (about 5 nm). Viscosity increases after at least 60% of κ- casein has been hydrolysed, while coagulation occurs only after about 85% of the hydrolysis is complete (Dalgleish & Corredig, 2012; Lucey, 2002, 2009). The effect of renneting and aggregation processes of the casein micelles are shown in Figure 2-22.

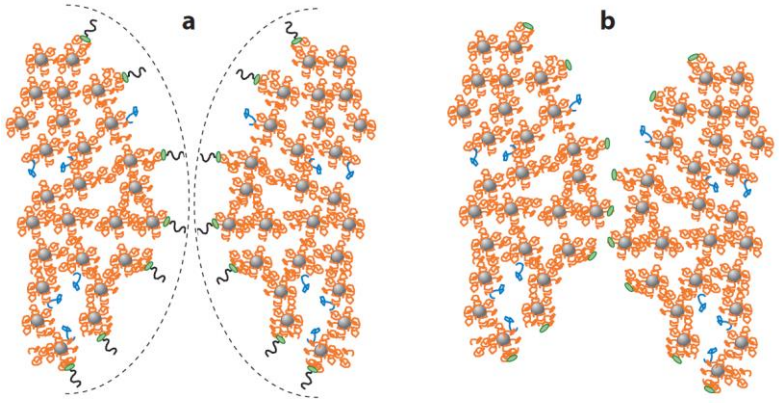


Figure 2-22: Diagram of interacting surface of micelles (a) Native micelles sterically stabilised by CMP. The zone of steric effect is indicated by the dashed lines. (b) Renneted micelles where the hairs have been removed by chymosin, allowing close approach of the micellar surfaces. *Para-* casein is green , the caseinomacropeptide chains are black (Dalgleish & Corredig, 2012).

Rheological properties of the rennet-induced milk gels are typically monitored with time after addition of rennet. A typical plot of the changes in storage modulus, *G′*, against time is shown in Figure 2-23.

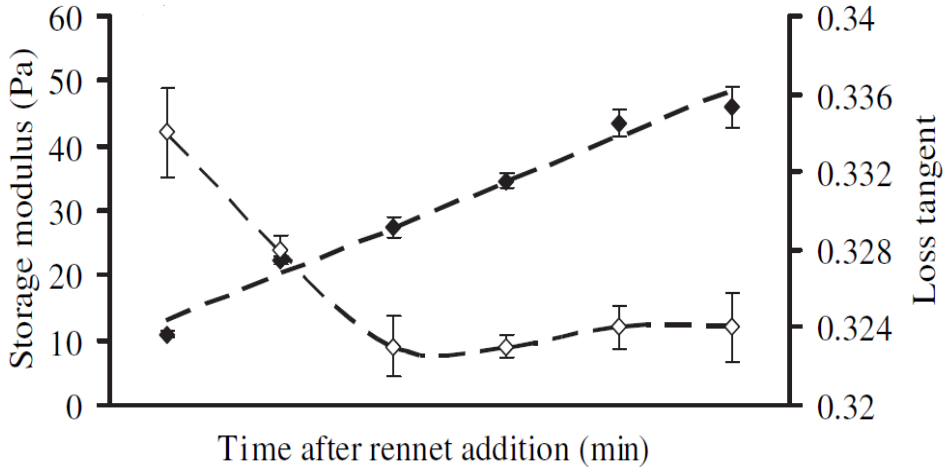


Figure 2-23: The rheological properties of rennet-induced milk gels made with milk (9% solids non- fat) and 0.1 mg mL^{-1} calcium chloride. The gelation pH was 6.56 and gelation temperature was 32.2°C. Storage modulus, *G′* (◆) and loss tangent (◇) (Mishra, Govindasamy-Lucey & Lucey, 2005).

2.7 Factors influencing the gelation of milk

The gelation of milk is dependent on the interactions between the milk proteins, which are in turn influenced by the stability of the milk proteins and the environmental conditions (Totosaus et al., 2002). As discussed in Section 2.3 and 2.5, the casein and whey proteins are stable to aggregation in native milk. However, protein stability is sensitive to changes in the milk environment. Some factors that are manipulated to alter the stability of milk proteins and effect changes in gelation of milk are discussed in this section.

2.7.1 pH of milk

As mentioned in Section 2.5.2, the pH of the protein system has great influence on the stability of the milk proteins as it determines the charge of the protein, which in turn influences the attractive and repulsive forces (Aguilera & Rademacher, 2004). In whey protein gels, pH determines the type of gel network formed (fine-stranded or particulate) (Doi, 1993). In casein gels, the effect of pH is the basis of the formation of acid-induced milk gels. Although the main mechanism in rennet-induced milk gels is through the action of enzymatic hydrolysis of κ- casein, pH has also been reported to influence the formation of rennet-induced milk gels (Roefs, van Vliet, van den Bijgaart, de Groot-Mostert & Walstra, 1990).

Whey proteins form fine-stranded gels under conditions where there is a strong electrostatic repulsion between the proteins (Langton & Hermansson, 1992). At pH away from the pI of whey protein (pH below 4 or above 7) and in a solution of low ionic strength, the repulsive forces between the protein molecules are strong (Mulvihill & Kinsella, 1988; Phan-Xuan et al., 2013; Tang et al., 1995). As such, upon heating, the exposed hydrophobic sites of the denatured proteins interact and aggregate in a more orderly manner, leading to the formation of fine-stranded gels (Lucey, 2009).

On the other hand, whey proteins form particulate gels when the intermolecular repulsion is reduced, such as at pHs near the pI (Langton & Hermansson, 1992; Tang et al., 1995). Langton & Hermansson (1992) showed that in the absence of salt at pH 5.5, β-lactoglobulin (12% *w/w*) forms particulate gels made up of mostly spherical aggregates linked together forming the strands of the gel network (Figure 2-24a). Langton & Hermansson (1992) also reported that on adjusting the pH away from the pI to pH 4 (which is the lower end of the pH range where particulate gels are formed), a mixture of particulate and fine- stranded gel, where the particles were embedded in a fine-stranded network, was observed (Figure 2-24b). This indicated the shift from particulate to fine-stranded gels as electrostatic repulsion becomes stronger at pH further from the pI. However, on increasing ionic strength, particulate gels can be formed from pH 2 to 9 due to neutralisation of the net charge and increased attraction between the proteins (Ako, Nicolai, Durand & Brotons, 2009).

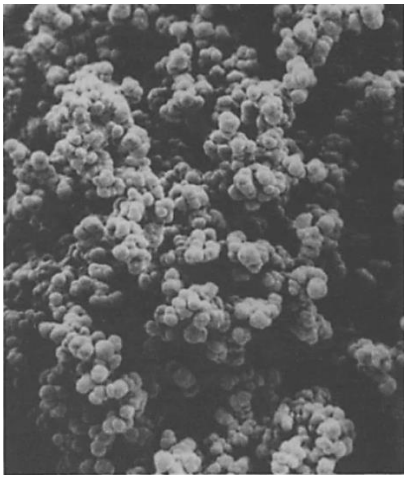
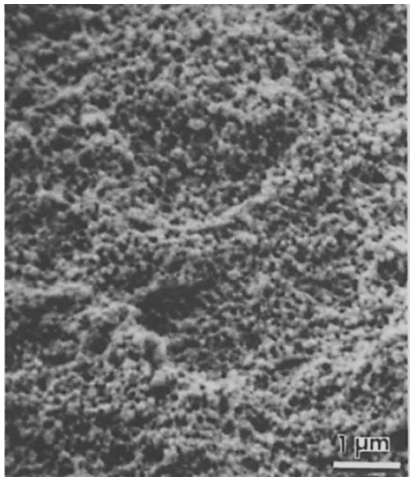
(a)  (b) 

Figure 2-24: SEM micrographs at high magnification of β- lactoglobulin gels formed at (a) pH 5.5 and (b) pH 4 (Langton & Hermansson, 1992).

Chandrapala, Augustin, McKinnon & Udabage (2010) reported a decrease in the total protein present in the supernatant after heating skim milk at pH < 6.65. This was attributed to the sedimentation of the aggregated proteins in the heated skim milk after

centrifugation. On the other hand, when skim milk was heated at pH > 6.65, the total proteins in the supernatant was higher after heating than prior to heating, possibly due to the dissociation of casein from micelles and/ or formation of soluble aggregates (Chandrapala, Augustin, et al., 2010). Similar results were reported by Anema (1998) where increasing amounts of casein were found in the supernatant with increasing milk pH before heating. These publications showed that pH influences the aggregation behaviour of proteins in heated milk, and thus affecting milk gelation.

In rennet-induced milk gels, decreasing pH has been reported to increase the rate of the coagulation, decrease rennet coagulation time (RCT) and increase gel firmness (Hyslop, 2003; Lucey, Tamehana, Singh & Munro, 2000; Shalabi & Fox, 1982). The effect of pH on rennet-induced milk gels can be attributed to a decrease in electrostatic repulsion, thus increasing electrostatic and hydrophobic attraction (Mishra et al., 2005), and the dissolution of CCP from the casein micelles with decreasing pH (Dalgleish & Corredig, 2012; Ong, Dagastine, Kentish & Gras, 2012). Dalgleish & Corredig (2012) stated that the gelation pH of rennet gels was dependent on the rate of pH change relative to the release of CCP. For instance, if the rennet action is slow relative to acidification, gelation will occur at a lower pH, albeit at a pH higher than a simple acid gelation. In contrast, if acidification occurs at a slower rate, the effect of rennet is dominating and gel formation occurs at a higher pH. As the dissolution of CCP with pH reduction results in an increase in ionic calcium, the positively charge calcium ions may also associate with the surface of *para*-κ-casein, or form calcium bridges between casein micelles (Ong et al., 2012). It has also been proposed that the permeability of rennet gels increases at lower pH, thus resulting in higher contact area between the micelles

where the micelles could fuse to a larger extent, leading to increased strength of the gel (Mishra et al., 2005).

2.7.2 Preheat treatment

For acid-induced gels, milk is commonly preheated before acidification to improve the gelation properties (Vasbinder, van de Velde & De Kruif, 2004). Unlike unheated acid-induced milk gels where only casein is involved in the gelation, preheated acid milk gels involve both casein and whey proteins due to heat-induced denaturation of whey proteins (Donato, Alexander & Dalgleish, 2007; van Vliet et al., 2004). The surface properties of the casein micelles have been found to be the determining factor for the interactions between denatured whey proteins and casein micelles during heating (Dalgleish & Corredig, 2012). Therefore, the conditions which influences the surface properties of the casein micelles, such as pH and temperature, have been found to greatly influence the effect of preheat treatment on the gelation of the milk proteins (Dalgleish & Law, 1988; O'Connell & Fox, 2003).

Preheating milk at its natural pH increases the gelation pH of acid milk gels (Anema, 2009b; Donato et al., 2007; Lucey, Tamehana, Singh & Munro, 1998). In unheated milk, whey proteins remain in their stable native forms and do not participate in the gelation. Therefore, gelation only occurs when the pH approaches the pI of casein at about 4.6 (Anema et al., 2004b). On the other hand, when milk is preheated at its natural pH, the denatured whey proteins undergo complexation with the micellar κ-casein via hydrophobic interactions and intermolecular disulphide bonds (Anema, 2009b; Donato et al., 2007; Mottar & Bassier, 1989; Schorsch, Wilkins, Jones & Norton, 2001). The interaction results in the whey proteins coating the casein micelle, thus changing the isoelectric pH at the surface of the casein micelle from 4.6 to about 5.2, which is the pI

of β- lactoglobulin (Anema et al., 2004b; Vasbinder, Alting & De Kruif, 2003). Hence, upon acidification, the casein micelles gel at a higher pH (Donato et al., 2007).

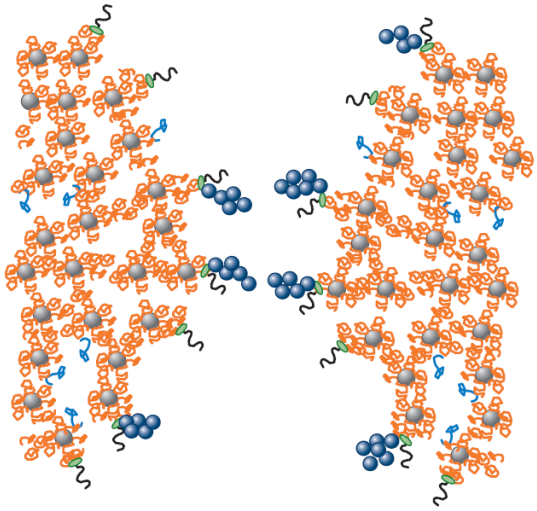


Figure 2-25: Diagram of interacting surface of micelles in heated milk with attached whey protein/ κ- casein complexes (blue spheres) (Dalgleish & Corredig, 2012).

The complex formed between whey proteins and casein micelles has been reported to contribute to the cross-linking of the casein particles during acidification, thus resulting in the increased G' (Dalgleish & Corredig, 2012; Donato et al., 2007; Lucey, 2002; Vasbinder et al., 2003). Upon acidification, electrostatic repulsion is reduced and the whey protein-κ-casein complexes act as points of attachment for the casein micelles by forming bridges between the protein particles due to the higher surface hydrophobicity of whey proteins (Figure 2-25) (Andoyo, Guyomarc'h, Cauty & Famelart, 2014; Dalgleish & Corredig, 2012; Morand, Dekkari, Guyomarc'h & Famelart, 2012). The complexes formed appear in TEM images as superficial appendages on the surface of casein micelles which form flocculates by particle to particle arrangement (Figure 2-26) (Mottar & Bassier, 1989). Using confocal laser scanning microscopy (CLSM) with separate stains for casein and whey proteins, Vasbinder et al. (2004) showed that when milk was heated at pH 6.7, the whey protein fraction was structured as an overlay of the

casein phase, suggesting that both fractions interacted in the formation of the gel network (Figure 2-27).

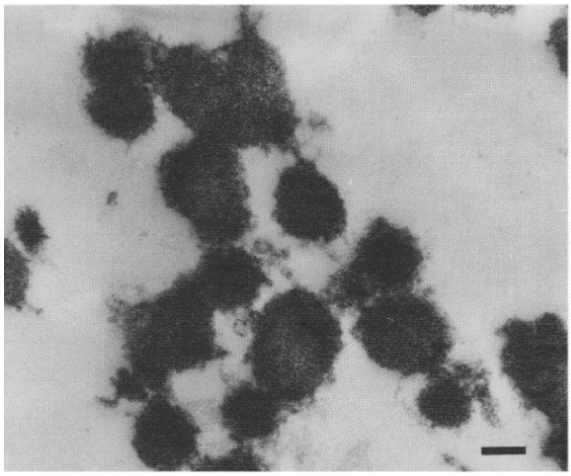


Figure 2-26: Transmission electron micrograph of casein micelles from yogurt made from milk heated for 10 min at 90°C (Magnification x 75,000) Bar = 1 μm (Mottar & Bassier, 1989).

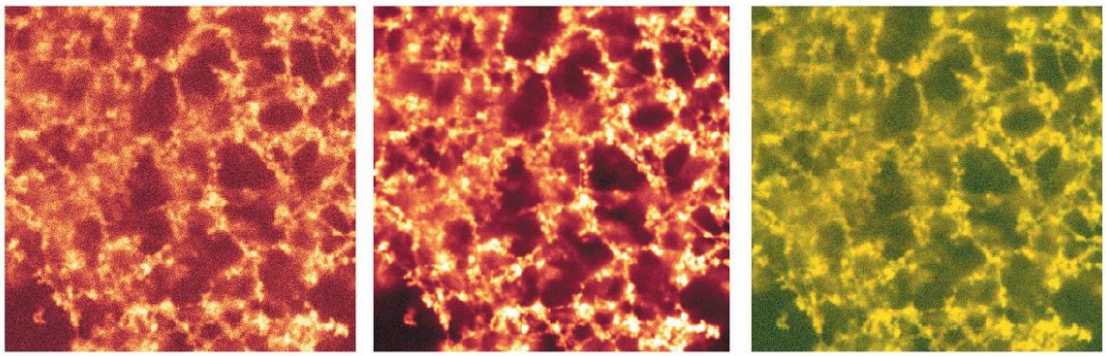


Figure 2-27: Confocal laser scanning microscopy (CLSM) images of preheated acidified protein gels. The left image shows the whey protein fraction covalently labelled with Oregon Green; the middle image shows the casein fraction covalently labelled with Texas Red; the right image shows the overlay of the two separately labelled proteins. Image size 40 × 40 μm (Vasbinder et al., 2004).

The pH at heating has been reported to influence the interactions between denatured whey proteins and casein micelles due to the effect of pH on heat-induced κ-casein dissociation (Anema et al., 2004b; Lakemond & van Vliet, 2008; van Vliet et al., 2004). Numerous studies have found that a higher amount of κ-casein is dissociated from the

casein micelles with increased pH at heating (Anema & Klostermeyer, 1997; Kudo, 1980; Singh, 2004; Singh & Fox, 1985). Anema et al. (2004b) attributed the lower levels of denatured whey proteins that are associated with casein micelles at higher preheating pH to the increased level of κ-casein dissociation. This was supported by a study by Lakemond & van Vliet (2008) where increased pH at heating resulted in lower levels of β-lactoglobulin attaching to the casein micelles (Figure 2-28). Concurrently, this resulted in the denatured β-lactoglobulin aggregating in the serum phase, thus resulting in increased level of soluble aggregated β-lactoglobulin.

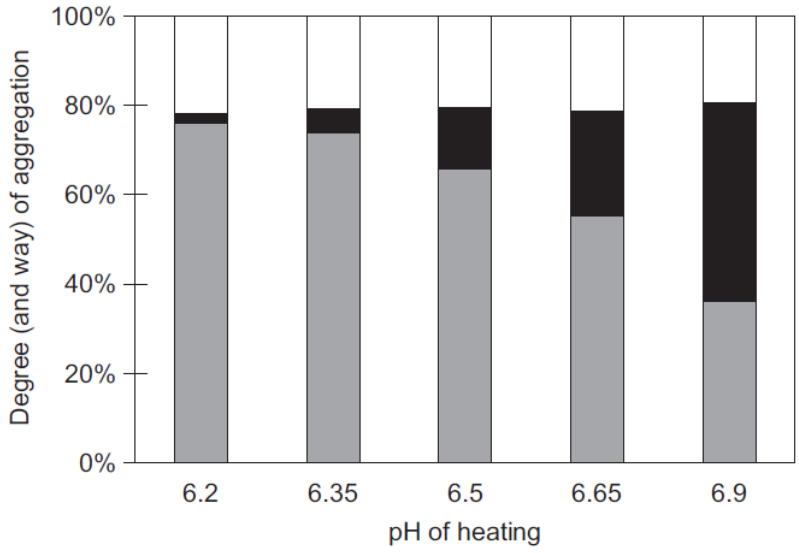


Figure 2-28: Degree (and way) of aggregation of β-lactoglobulin after heat treatment for 10 min at 80°C as a function of heating pH. The β-lactoglobulin attached to casein micelles is represented by the gray bars, the soluble β-lactoglobulin aggregates by the black bars and the non- aggregated (native) β-lactoglobulin by the white bars (Lakemond & van Vliet, 2008).

Increasing pH at heating, and denatured whey protein aggregates in the serum phase, has been reported to result in higher gelation pH and *G'* of the acid milk gels (Dalgleish & Corredig, 2012; Donato et al., 2007; Lakemond & van Vliet, 2008; Lucey et al., 1998). The increase in gelation pH could be attributed to increased dissociation of κ-casein, thus reducing the "hairy" layer of casein micelle which promotes hydrophobic

interactions. This leads to gelation of the aggregated whey proteins at their isoelectric point (pH 5 to 5.5) in the serum phase (Anema et al., 2004b; Vasbinder et al., 2004).

The increased gel strength due to higher number of aggregating particles in the serum phase (aggregated denatured whey proteins and heat-induced dissociated κ-casein) was attributed to higher number of contact points for interactions and a more complex gel formation compared to milk systems with denatured whey proteins attached to the casein micelles surfaces (Anema, 2008). Dalgleish & Corredig (2012) also proposed that the aggregated whey proteins were able to rearrange and fill the voids between the casein micelles after formation of the gel network, hence increasing gel strength. Another possible mechanism is the involvement of disulphide linkages in interconnecting the colloidal particles. Schorsch et al. (2001) proposed that a second heating step (i.e. acidification followed by second heating) was required for the thermal reduction of the disulphide links within the whey aggregates to allow subsequent interactions with casein micelles. However, Vasbinder et al. (2004) reported that even though the reactivity of disulphide linkages is very low at low pH and ambient temperature, the contribution was still significant. Anema (2008) agreed that in acid gels heated at higher pH and therefore containing a higher amount of aggregated whey proteins in the serum, a greater number of disulphide bonds interconnecting the colloidal particles may explain the higher breaking stress.

2.7.3 Temperature during gelation

The temperature dependence of milk protein gelation is well documented (Dalgleish, 1983; Horne & Lucey, 2014; Peng, Horne & Lucey, 2010). In general, increased temperature results in more rapid movement of molecules, thus a higher frequency of collision (McClements & Keogh, 1995). However, even if interactions occur when the particles collide, the attractive forces must be strong enough to ensure permanent

attachment for aggregation and gelation (Dalgleish, 1983). Therefore, changes in the attractive forces at different temperatures are crucial and may explain the changes in the interactions between the colloidal particles and therefore the gelation of the proteins.

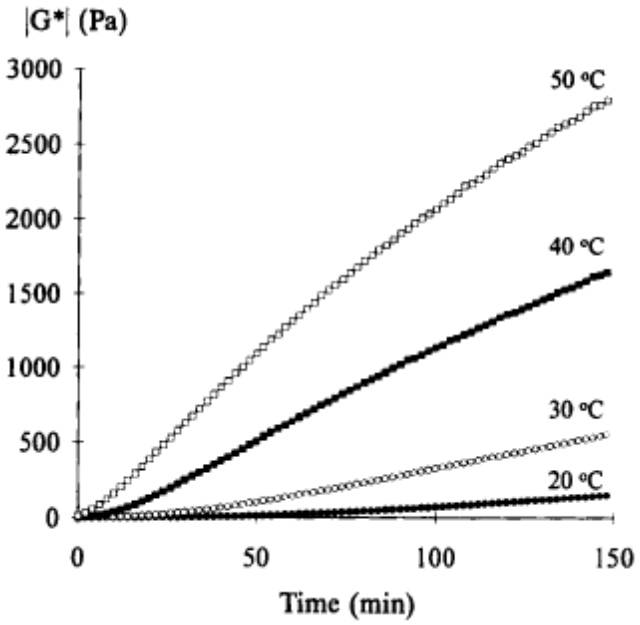


Figure 2-29: Time dependence of the complex modulus, G^*, of 90 g kg^{-1} heat-denatured WPI solutions (pH 7.0, 0.2 mol/ kg NaCI) stored at different temperatures (Frequency = 1 Hz, Strain = 0.01) (McClements & Keogh, 1995).

Hydrophobic interactions have been reported to increase with increasing temperature (Baldwin, 1986; Hummer, Garde, García, Paulitis & Pratt, 1998). Therefore, increasing temperature during gelation has been reported to increase gel strengths in both whey protein and casein gels (Figure 2-29) (Dalgleish, 1983; Harwalkar & Kalab, 1980; Horne, 1998; McClements & Keogh, 1995). McClements & Keogh (1995) showed that the complex modulus (G*) of cold-set whey protein gels increased more rapidly with time as temperature at gelation increased, indicating a greater rate of gelation at higher temperatures (Figure 2-29). This observation could be attributed to both increased magnitude of hydrophobic interactions and more rapid movement of the molecules at higher temperatures (McClements & Keogh, 1995).

The dependence of gel strength on temperature in rennet gels cannot be attributed only to increased hydrophobic interactions as the relative increase in hydrophobic attraction with increased temperatures is only very slight (Horne & Lucey, 2014). Dalgleish (1983) and Horne (1998) proposed that the effect of calcium binding to the caseins at varying temperatures may also play a role. The binding of calcium ions to casein has been found to increase with increasing temperature (Dalgleish & Parker, 1980; Pappas & Rothwell, 1991). A possible explanation for increased binding of calcium at higher temperatures is the unfolding of the proteins at higher temperatures, which exposes more calcium- binding sites (Pappas & Rothwell, 1991). Therefore, with increasing temperature, the increases in hydrophobic interactions and the reduction in electrostatic repulsion due to increased binding of calcium may swing the energy balance in favour of attraction and lead to greater bond strength (Horne & Lucey, 2014).

2.8 Effect of salt addition

The effect of salts is important in the gelation of milk proteins as the number of ions available for screening of charged groups on the protein influences the balance between the attractive and repulsive forces of the proteins (Lucey, 2009; Mulvihill & Kinsella, 1988). At the native pH of milk, both whey proteins and casein micelles are negatively charged. Therefore, the addition of positively-charged ions such as Ca^{2+} and Na^{+} screens the charge of the proteins, reducing the electrostatic repulsion and hence promoting hydrophobic interactions and aggregation. The addition of salts also changes the ionic strength of the solution which may induce changes in the calcium equilibrium in milk and alter the stability of the proteins (van Boekel, 2008b). The effect of the addition of salts such as calcium chloride and sodium chloride on whey protein gels, acid- and rennet-induced milk gels has been studied and reviewed extensively in literature (Awad, 2007; Kinsella & Morr, 1984; Lucey, Van Vliet, Grolle, Geurts &

Walstra, 1997; McClements & Keogh, 1995). More recently, the addition of calcium salts to milk or casein systems (micellar casein and caseinates) to induce gelation on heating has also been reported. This section will therefore cover the effect of salt addition on the types of milk gels described in Section 2.6, as well as review the gelation in milk or casein systems induced by calcium salt addition. The effect of salts will be discussed with emphasis on calcium salts. The effect of sodium chloride will also be briefly discussed to determine the effect of changes in the ionic strength with a monovalent ion in comparison to divalent Ca^{2+} ions.

2.8.1 Effect of salt addition on whey proteins

The effect of calcium and sodium ions on whey proteins was found to be dependent on the charge of the protein, which in turn is determined by the pH of the system (Kinsella & Morr, 1984). For example, in heat-set whey protein gels, Tang et al. (1995) showed that when the initial charge of the proteins was net positive (pH 4), increasing addition of salts (sodium chloride and calcium chloride) had little effect on the gel strength (as measured by the storage modulus, G'). On the other hand, at higher pH (pH 8) where the net charge of the proteins was negative, increasing salt concentration was needed to neutralise the repulsive forces and achieve higher gel strength (Figure 2-30). However, when the concentration of added salt was increased further, the G' decreased as a result of random aggregation due to excessive attractive forces (Tang et al., 1995).

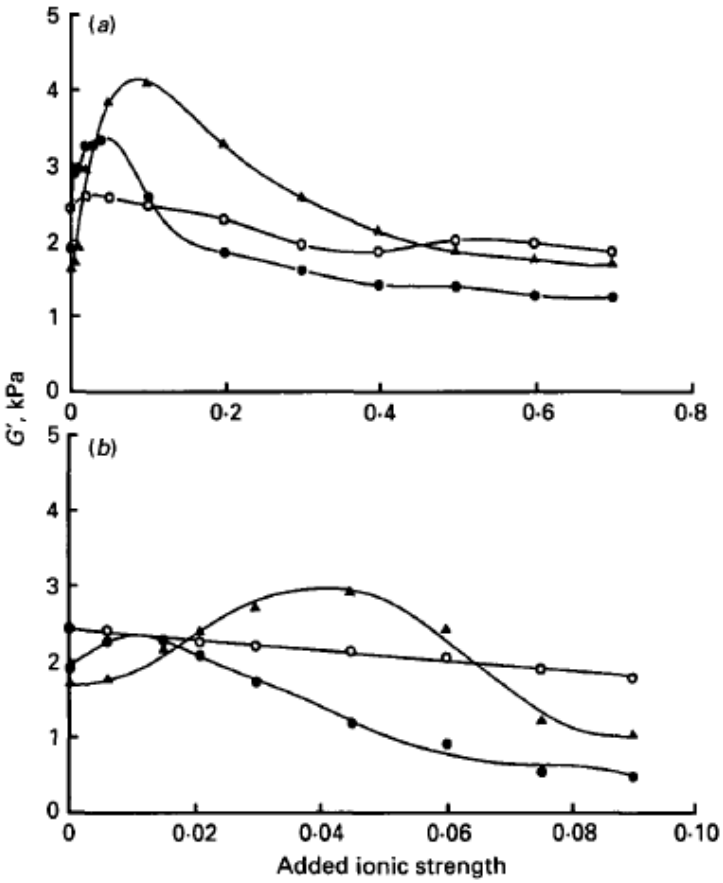


Figure 2-30: Effects of (a) sodium chloride concentration and (b) calcium chloride concentration, expressed as added ionic strength, on the storage modulus *G'* of whey protein concentrate gels containing 150 g total solids kg^{-1} at 80 °C after 44 min heating at 80 °C and pH values of, 4 (○); 7 (●); 8 (▲) (Tang et al., 1995).

Both sodium chloride and calcium chloride addition to whey protein solutions were able to induce gelation on heating due to charge screening effect, but a lower concentration of calcium chloride was required to induce gelation compared to sodium chloride (Mulvihill & Kinsella, 1988; Tang et al., 1995). Mulvihill & Kinsella (1988) proposed that the lower concentration of calcium chloride needed for gelation compared to sodium chloride suggested that Ca^{2+} ions acted as cross-links between the negatively charged proteins to form the gel matrix. The lower concentration of Ca^{2+} ions required to induce gelation compared to Na^{+} ions also suggested that Ca^{2+} could be participating in an ion-specific interactions with the β-lactoglobulin (Croguennec, O'Kennedy & Mehrab, 2004; Jeyarajah & Allen, 1994). For example, Ca^{2+} was suggested to promote

conformational changes during heating of whey proteins (Croguennec et al., 2004; Jeyarajah & Allen, 1994; Riou, Havea, McCarthy, Watkinson & Singh, 2011). Jeyarajah & Allen (1994) studied the conformational changes in β-lactoglobulin with intrinsic fluorescence and found significant increases of the intrinsic fluorescence with heat treatment and the addition of calcium chloride (1 to 15 mmol L^{-1}). The reactive sulphydryl group content also increased with addition of calcium chloride. The results suggested that calcium binding to β-lactoglobulin induced structural changes in β-lactoglobulin which may have led to increase in hydrophobic interaction, thus promoting aggregation (Jeyarajah & Allen, 1994). Riou et al. (2011) suggested that binding of Ca^{2+} to whey proteins may prevent the unfolded regions from refolding into the native state, hence promoting aggregation during heating.

The order of the salt addition to whey proteins also determines the type of whey protein gels formed. As discussed in Section 2.6.1 and Section 2.6.2, addition of salts to heated whey protein solutions produces cold-set whey protein gels. Similar to heat-set whey protein gels, gelation in cold-set whey protein gels is induced by addition of salts to screen the electrostatic repulsion between the denatured protein molecules, and/or to form calcium bridges between protein filaments (Chung et al., 2013; Ju & Kilara, 1998; McClements & Keogh, 1995). However, the structures and properties of the two types of gels induced by salt addition appeared to be different. Barbut & Foegeding (1993) reported that cold-set gels induced by addition of 10 mmol L^{-1} of calcium chloride after heating produced a fine-stranded and more transparent gel than the heat-set gels (addition of 10 mmol L^{-1} of calcium chloride before heating). This was supported by Chung et al. (2013) who showed that cold-set gels had a fine and uniform structure while heat-set gels had a coarse heterogeneous structure containing large particulates of about 10 μm (20 mmol L^{-1} calcium chloride addition before or after heating) (Figure

2-31). Chung et al. (2013) proposed that the difference was because in heat-set gels, the proteins undergo non-specific aggregation leading to the formation of random, large spherical aggregates. Conversely, cold-set gels formed fine-stranded aggregates when heated without salts, and were only brought together upon an increase in ionic strength during gelation (Ako, Nicolai & Durand, 2010). The fine-stranded gel network (i.e., more transparent gel) was usually the result of less aggregation and more order in the gel structure (Hongsprabhas & Barbut, 1997).

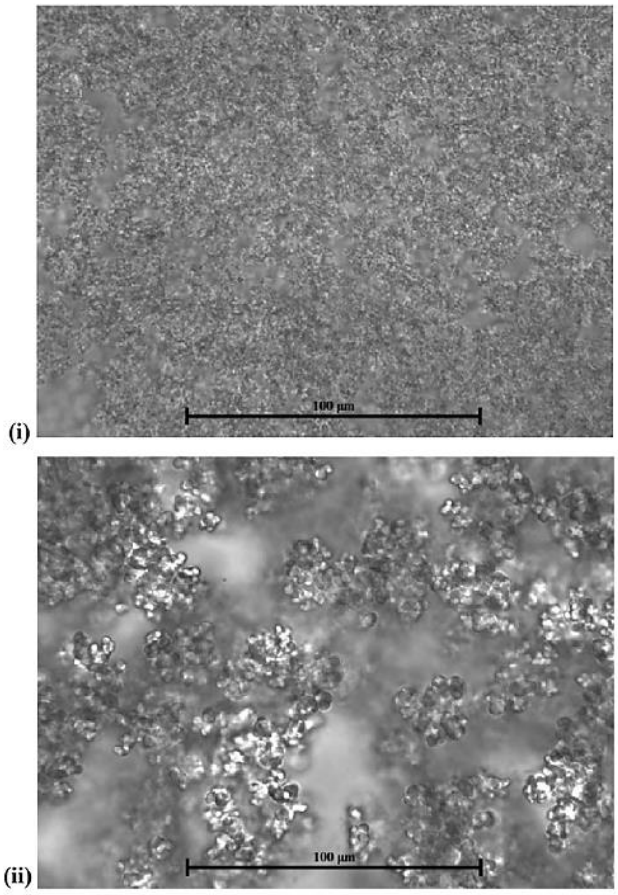


Figure 2-31: Optical micrographs (60X magnification) of (i) 20 mmol L^{-1} of added calcium chloride cold-set whey protein gel; (ii) 20 mmol L^{-1} of added calcium chloride heat-set whey protein gel (Chung et al., 2013).

2.8.2 Effect of salt addition on rennet-induced milk gels

In rennet-induced milk gels, addition of calcium decreases the rennet coagulation time (RCT) and increases gel firmness (Bohlin, Hegg & Ljusberg-Wahren, 1983; Kowalchyk & Olson, 1979; Lucey & Fox, 1993; Malacarne et al., 2014; Sandra, Ho, Alexander & Corredig, 2012; Tsioulpas et al., 2007; Udabage, McKinnon & Augustin, 2001). It is proposed that the role of ionic calcium in rennet-induced milk gels is to reduce the zeta-potential of the casein micelles by binding either to the charged phosphoserine groups or carboxylic acid groups of the α_s- and β- caseins, thereby reducing electrostatic repulsion and facilitating hydrophobic interactions between the micelles and formation of a coagulum (Deeth & Lewis, 2015; Lucey & Fox, 1993; Udabage et al., 2001). Ionic calcium may also form calcium bridges between the micelles and enhance cross-linking in the gel structure (Sandra et al., 2012). A study by Sandra et al. (2012) illustrated that the addition of calcium increased the viscoelastic properties of the renneted milk gels, where G' at 45 minutes after gelation was 13 Pa for skim milk, but addition of 1 mmol L^{-1} of calcium doubled the G' to about 30 Pa (Figure 2-32).

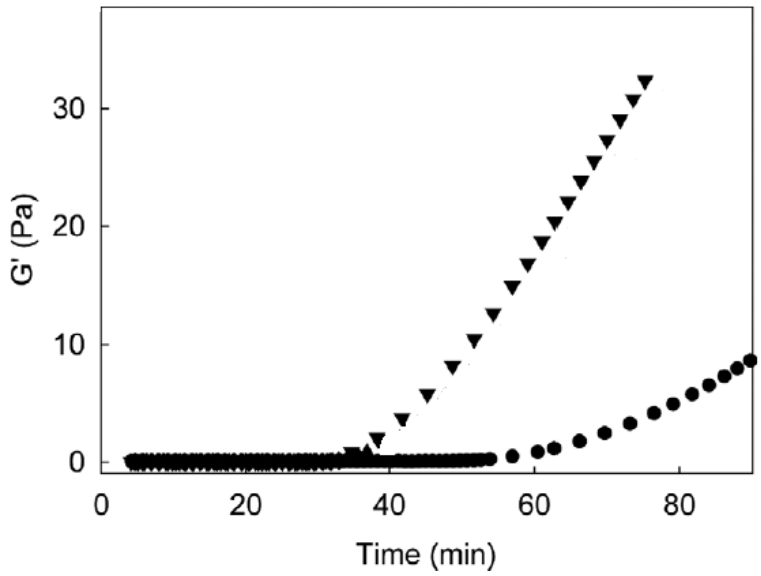


Figure 2-32: Development of storage modulus, G' for control skim milk (●) and skim milk with 1 mmol L^{-1} of added calcium chloride (▼) (Sandra et al., 2012).

The difference between the influence of calcium salt on the formation of whey protein gels versus milk gel is that the addition of calcium to milk involves not only the neutralisation of charges by the ionic calcium, but also its changes to the calcium equilibrium in milk and the level of CCP in the casein. CCP has been suggested to play a more dominant role in inducing gelation of the casein micelles than ionic calcium in rennet gels (Malacarne et al., 2014; Shalabi & Fox, 1982; Udabage et al., 2001). Shalabi & Fox (1982) reported that no coagulation occurred when the concentration of CCP was reduced by 30%. Malacarne et al. (2014) also reported that milks with optimal rennet coagulation time and gel firmness had the highest colloidal calcium, with no difference observed in the amount of soluble calcium. Increased amount of CCP increases the cross-linking of casein molecules (including soluble casein in the serum phase) and facilitates salt bridging between the casein micelles, thereby promoting gelation and increasing gel strength (Choi, Horne & Lucey, 2007; Lee & Lucey, 2010; McMahon & Brown, 1984; Udabage et al., 2001).

2.8.3 Effect of salt addition on acid-induced milk gels

Contrary to whey protein and rennet-induced milk gels, addition of calcium chloride to acidified milk has been reported to decrease gel strength. Bringe & Kinsella (1991) reported that increasing calcium chloride from 10 to 50 mmol L^{-1} decreased the maximum rate of casein aggregation and decreased the pH at which aggregation was initiated. Similar results were reported by Goddard & Augustin (1995) where the addition of 10 to 70 mmol L^{-1} of calcium chloride decreased the gel strength of acidified milk, especially at pH < 5.5. The effect of pH on gel strength also became less pronounced with addition of calcium (Figure 2-33) (Goddard & Augustin, 1995). However, Ramasubramanian, Restuccia & Deeth (2008) reported that addition of ionic

calcium up to 2 mmol L^{-1} increased firmness of stirred yogurt, although gel strength decreased with further addition up to 13.5 mmol L^{-1}.

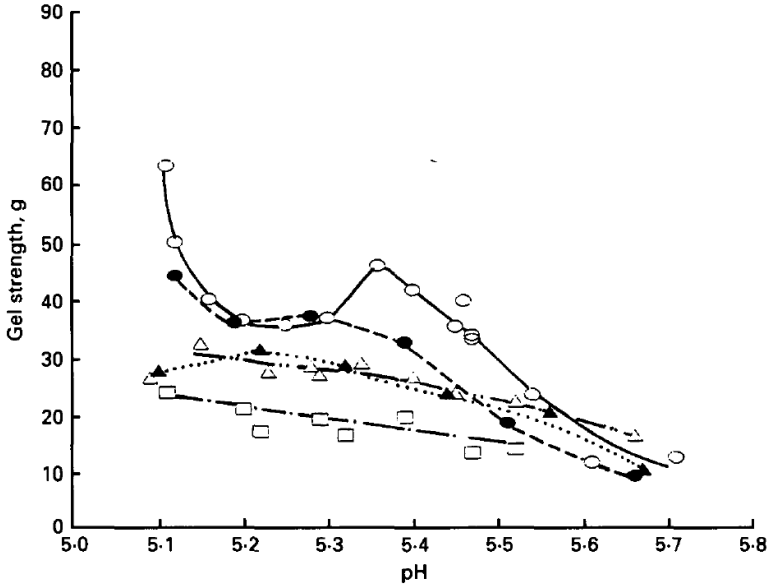


Figure 2-33: Effect of pH and calcium chloride addition on the strength of reconstituted high heat milk gels (200 g solids/L) made with no added salt (○); and 10 mmol L^{-1} (●); 30 mmol L^{-1} (△); 50 mmol L^{-1} (▲) and 70 mmol L^{-1} (□) of calcium chloride (Goddard & Augustin, 1995).

It appears that while higher salt concentration is generally known to destabilise colloidal systems, it may not be necessarily true for acidified milk protein dispersions (Auty, O'Kennedy, Allan-Wojtasb & Mulvihill, 2005). The increase in firmness of yoghurt up to 2 mmol L^{-1} of ionic calcium addition as observed by Ramasubramanian et al. (2008) could be explained by the increase in CCP and thus enhanced aggregation. However, at higher concentrations of calcium addition, the gel strength decreased (Bringe & Kinsella, 1991; Goddard & Augustin, 1995; Ramasubramanian et al., 2008). Bringe & Kinsella (1991) proposed that calcium alters the protein-protein interactions that occur between casein micelles by affecting the repulsive hydration forces between the protein surfaces on approaching contact. Hydration repulsion forces are short range forces that are caused by the water structure in the ionic hydration shell (Goddard & Augustin,

1995). Protein-protein interactions are favoured by binding of H^+ ions, as hydrogen bonding is possible. However, if ionic calcium is bound to the protein, hydration repulsion forces may reduce the interaction between the caseins. While H^+ ions are able to displace the calcium ions bound to casein, increased concentration of bound calcium would require a higher concentration of H^+ to displace the calcium from the proteins and induce coagulation (Bringe & Kinsella, 1991).

2.8.4 Effect of salt addition to casein systems and milk

Addition of calcium salts to milk was reported to reduce the heat stability of the milk proteins (Dalgleish et al., 1987; Vyas & Tong, 2004). The heat stability of milk is characterised by its ability to resist coagulation, thickening or gelation upon heating (Faka et al., 2009). At the native pH of milk, micellar caseins are stable to heating up to 100°C, but addition of calcium chloride was reported to induce coagulation and gelation in milk and micellar casein systems when heated to between 70°C and 100°C (Balakrishnan et al., 2018; On-Nom, Grandison & Lewis, 2012; Ramasubramanian et al., 2014).

As casein micelles are generally stable to heat, a minimum concentration of added calcium salt is required for gelation to occur. Balakrishnan et al. (2018) reported that in 6% micellar casein solutions at pH 6.7, gelation was observed only at > 7.0 mmol L^{-1} of added calcium chloride on heating of the micellar casein solutions. In a whole milk system, Ramasubramanian et al. (2014) showed that with no added calcium chloride, heating whole milk did not induce gelation (Figure 2-34). The critical calcium concentration required for gelation was 10 mmol L^{-1}, with G' increasing with increasing added calcium chloride thereafter (Figure 2-34).

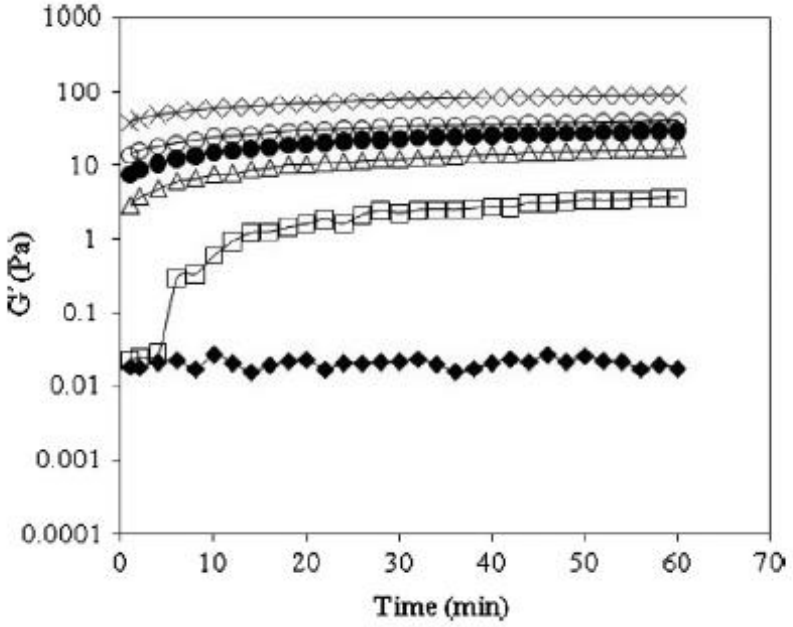


Figure 2-34: Storage modulus, *G′*, of calcium- added milk samples as a function of holding time at 70°C. Milk samples were preheated at 90°C for 10 min: 0 mmol L^{-1} (◆); 10 mmol L^{-1} (□); 12.5 mmol L^{-1} (△); 15 mmol L^{-1} (●); 17.5 mmol L^{-1} (○); and (×) 20 mmol L^{-1} calcium chloride added. The temperature- time combination at which the *G′* of the milk sample reached 1 Pa was defined as the gelation point (Ramasubramanian et al., 2014).

The effect of interactions between whey protein and casein have also been reported to influence the gelation in milk proteins induced by calcium salt addition. Addition of sodium caseinate to the whey protein isolate (WPI) solutions was reported to inhibit or promote the gelation of WPI solutions with added calcium chloride, depending on the concentration of proteins and calcium ions present (Nguyen, Balakrishnan, Jacquette, Nicolai & Chassenieux, 2016). For example, on heating a 34 g L^{-1} solution of WPI solution with 10 mmol L^{-1} of added calcium chloride at 80°C, increasing the concentration of sodium caseinate from 6 to 13 g L^{-1} decreased the storage modulus (*G′*) of the solution, but on further addition of sodium caseinate up to 100 g L^{-1}, the *G′* increased (Figure 2-35). Nguyen et al. (2016) attributed the decrease in *G′* at lower concentration of sodium caseinate to competition for calcium ion bindings, while the promotion of gelation at higher concentrations of sodium caseinate was suggested to be

due to reduction in the amount of water per unit volume, resulting in excluded volume effects which may increase the effective WPI concentration and thus induce gelation.

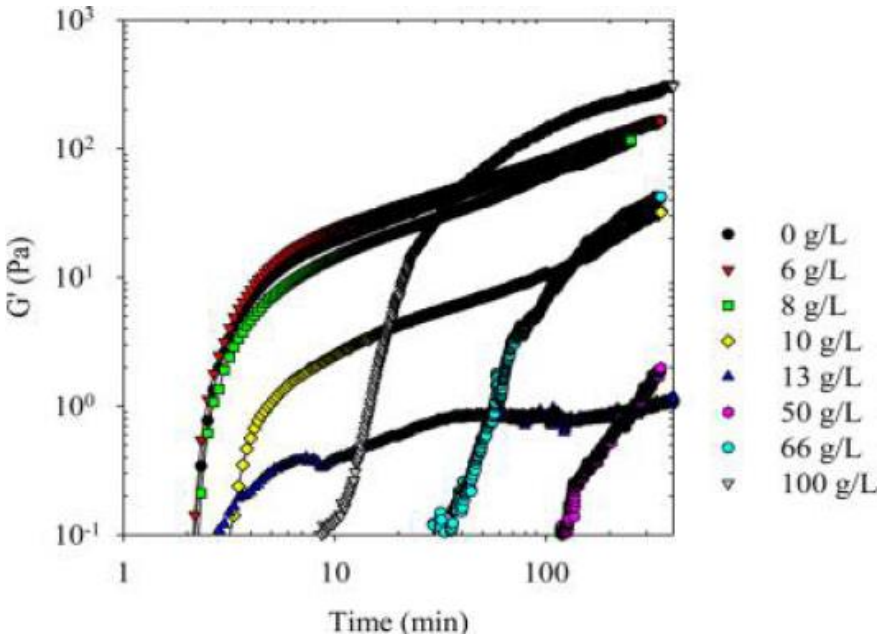


Figure 2-35: Evolution of the storage modulus, *G′*, at 0.1 Hz with heating time at 80°C for mixtures containing 34 g L^{-1} WPI and 10 mmol L^{-1} of added calcium chloride. Symbols and on the right hand side of the plot represents the concentration of sodium caseinate added to the system (Nguyen et al., 2016).

As discussed in Section 2.7.2, preheat treatment of milk influences the interactions between casein and whey proteins. Hence, the preheat treatment applied to milk prior to gelation in calcium-added milk with heat may influence the properties of the calcium-induced milk gel. Ramasubramanian et al. (2014) studied the effect of different preheating methods where milks were unheated, heated at 90°C for 10 min, or UHT treated prior to the addition of calcium. When no preheat treatment or UHT treatment was given, a higher concentration of added calcium chloride (15 mmol L^{-1}) was needed for gelation. For whole milk with no preheat treatment, the higher concentration of calcium chloride required for gelation was attributed to the lack of denatured whey protein-κ-casein complex formation on the micelle surface which helps in the cross-linking of the gel network. Ramasubramanian et al. (2014) attributed the rapid and

direct heating in UHT treated milk to the formation of more denatured whey protein-κ-casein complexes on the casein micelle, which prevented fusion of the micelles. On the other hand, slower heating as is the case for milk heated at 90°C for 10 min, favoured formation of denatured whey protein aggregates in the serum, which resulted in formation of stronger gels as discussed earlier in Section 2.7.2.

To determine the effect of charge screening on gelation, Balakrishnan et al. (2018) studied the effect of sodium chloride addition to micellar casein systems at pH 5.8. Increasing concentration of added calcium chloride decreased the critical gelation temperature, while the addition of sodium chloride increased the critical gelation temperature. This indicated that the gelation induced by calcium chloride in micellar casein systems could not be fully explained by the screening of electrostatic repulsion effect.

2.9 Kinetics of gelation

2.9.1 Collision theory

According to the collision theory, interactions between two reactants occur when the reacting particles approach each other closely on a molecular scale (Connors, 1990). The colliding particles must also possess a critical minimum energy, termed the activation energy, for a chemical reaction to occur (Tyagi, 2006). The total rate of collision and the fraction of molecules which have at least the critical energy and is temperature dependent will typically follows the Arrhenius relationship (Connors, 1990):

$$k = k_0 e^{-\frac{E_A}{RT}}$$

where k is the rate constant,

k_0 is the pre-exponential factor,

E_A is the activation energy,

R is the gas constant,

T is the absolute temperature.

The rate of reaction depends on the magnitude of the activation energy (E_A) required (Wright, 2004). At the same temperature, reactions requiring a lower E_A will proceed at a faster rate. The Arrhenius equation can be used to demonstrate the effect of temperature on gelation rates. Increasing the temperature (T) will result in a faster reaction rate (k) (Wright, 2004). The fraction of molecules which collide with enough energy to overcome the E_A increases at higher temperatures, thus resulting in faster reaction rates (Wright, 2004).

2.9.2 DLVO theory

As discussed in Section 2.5.6, the DLVO theory describes the repulsive electrostatic and attractive van der Waals forces involved in colloidal stability. Besides predicting if two approaching particles will interact and aggregate, the DLVO theory can also predict the rates in which aggregation occurs. For instance, as shown in Figure 2-36, in solutions with high salt concentrations, the DLVO theory predicts that attractive van der Waals forces will dominate and aggregation will be rapid since there is no free energy barrier. In contrast, in low salt solutions, there is strong electrostatic repulsion between the particles. Hence, there is a large energy barrier to overcome for the particles to aggregate, resulting in slow aggregation (De Young, Fink & Dill, 1993).

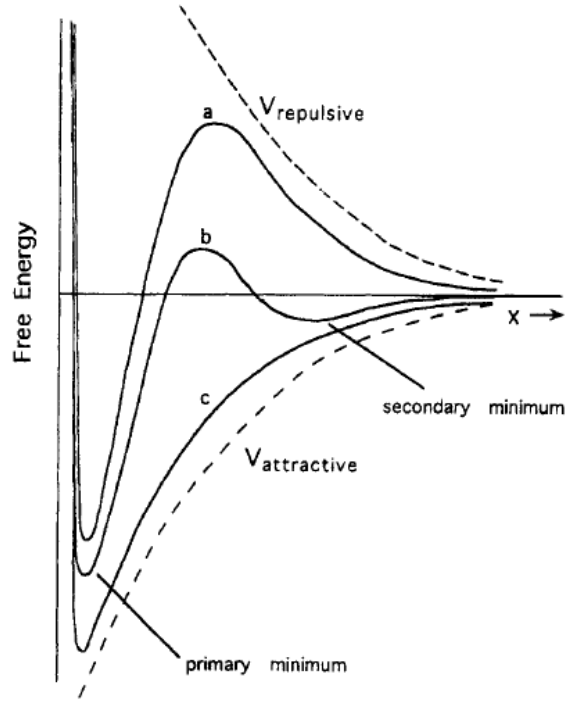


Figure 2-36: Summary of DLVO theory. Attractive van der Waals ($V_{attractive}$) and repulsive electrostatic forces ($V_{repulsive}$) plotted as a function of intermolecular separation (x). The balance of these two forces at (a) low, (b) medium, and (c) high salt concentrations determines whether a colloidal solution will be stable or will aggregate in the primary or secondary minimum (De Young et al., 1993).

2.9.3 Smoluchowski theory

The kinetics of colloidal aggregation is also often described using the Smoluchowski theory. According to the Smoluchowski theory, the rate of aggregation can be limited by diffusion, termed diffusion-limited aggregation (DLA), or by reaction, termed reaction-limited aggregation (RLA) (De Young et al., 1993). In diffusion-limited DLA, there is no primary maximum barrier (Figure 2-36). The particles attract so strongly that the rate-limiting step for aggregation is the diffusion of the particles (all collisions result in aggregation) (De Young et al., 1993; Eastman, 2005). In RLA, the association process rather than the diffusion process is rate limiting. This occurs when there is a large energy barrier that prevents the particles from coming together, hence resulting in a slower reaction rate. The interaction is weak and only a fraction of the particles collide

with sufficient energy to exceed the height of the barrier and stick (De Young et al., 1993; Eastman, 2005).

2.9.4 Kinetics of milk protein gelation

Kinetic studies on milk protein gelation is limited in literature. Nevertheless, several authors have investigated the kinetics of whey protein gelation as an effect of salt and protein concentration, size and structure of aggregates, and temperature (Ako et al., 2010; Kharlamova, Nicolai & Chassenieux, 2018; Le Bon, Nicolai & Durand, 1999). In literature, reaction orders of 1, 1.5, and 2 have been reported for the aggregation process of β-lactoglobulin (Le Bon et al., 1999).

A common measurement of aggregation kinetics is the gel time (t_g). An Arrhenius relationship has been reported for whey protein gelation, i.e. t_g is temperature dependent (Kharlamova et al., 2018; Le Bon et al., 1999). In particular, Ako et al. (2010) reported that heat-induced gelation of whey proteins is highly dependent on temperature because it involves denaturation as the first step, which is a process characterised by a large activation energy. The salt and protein concentration of was found to have no effect on the E_A, but increases in salt and protein concentrations decreased the t_g, indicating an increased gelation rate (Ako et al., 2010; Kharlamova et al., 2018).

2.10 Rheological measurements for the study of gel structures

Rheology is the study of the flow and deformation of matter (Barnes, Hutton & Walters, 1989; Ma & Barbosa-Canovas, 1995). Gels are viscoelastic materials, which mean they exhibit both solid-like and liquid-like behaviour. The viscoelastic behaviour of macromolecular gels is related to the nature and rate of configurational rearrangements of the macromolecules, and the type and number of intermolecular bonds (Auty et al., 2005). Therefore, the study of the viscoelasticity or rheological properties can elucidate useful information on the characteristics of a gel, such as its molecular structure (Rao,

2014). Rheological measurements can be divided into those that induce small or large deformations (Hermansson, 1994). Small-deformation tests provide information about the viscoelastic properties of the gel network by performing dynamic oscillatory tests. On the other hand, large-deformation tests measures the stress, strain and failure properties of a given material (Hermansson, 1994).

Dynamic oscillatory measurements allow measurements of the changes during gelation since the induced deformations are usually so small that their effect on structure is negligible (Ma & Barbosa-Canovas, 1995). Two independent parameters are obtained from the dynamic measurements: the storage modulus, G', which describes the amount of energy that is stored elastically in the structure; and loss modulus, G'' which is the measure of the energy loss or the viscous response per cycle of deformation (Lucey & Singh, 1998). For a perfectly elastic solid, all the energy is stored ($G'' = 0$), and the stress and the strain will be in phase (Figure 2-37). In contrast, for a liquid with no elastic properties, all the energy is dissipated as heat ($G' = 0$), and the stress and the strain will be out of phase by 90° (Figure 2-37). The value of a modulus is dependent on the number of junction zones (which in turn is dependent on concentration and geometry of the network), and the strength of bonds in a junction zone (Walstra et al., 2006).

The gel point is defined as the point at which the material transitions from a liquid or solution (sol) to a gel (Rao, 2014). It is the transition of connectivity of the physical or chemical bonds between the particles, from being disconnected, to linking up to a network (Rao, 2014). Several methods have been proposed to define the gelation point. The point at which the G' and G'' crosses over, at a specified frequency, have been suggested as the gelation point (Stading & Hermansson, 1990). However, the crossover of the G'/ G'' has been found to be dependent on the frequency. As the gelation point is

an intrinsic property of the material, it cannot be dependent on the frequency (Rao, 2014). Moreover, in some cases, the lowest detected value of G' may already be higher than G'' (Gosal, Clark & Ross-Murphy, 2004). Therefore, other methods of detection of gel point have been proposed. The increase in the G' to a value greater than the experimental noise level, for instance a value > 1 Pa, (Liu et al., 2014; Ramasubramanian et al., 2014), or at the point of rapid rising of the G' values (Hsieh, Regenstein & Anandha Rao, 1993) has been suggested. The sol-gel transition state can also be measured by a sudden decrease in tan δ, where the tan δ of a viscous fluid is 90°, and the tan δ of a milk gel is about 15° (Bohlin et al., 1983). It should be noted that the choice of experimental definition of gel point is somewhat arbitrary. This meant that it is possible for a given system to be considered a gel in one system and a solution in another (Eisenberg & King, 1977). For instance, a larger volume of gel has to be formed in the continuous phase in concentric cylinders than in cone-and-plate geometries for the elastic component (G') to dominate. Hence, the choice of gel point and technique used for measurement may result in different gel point values.

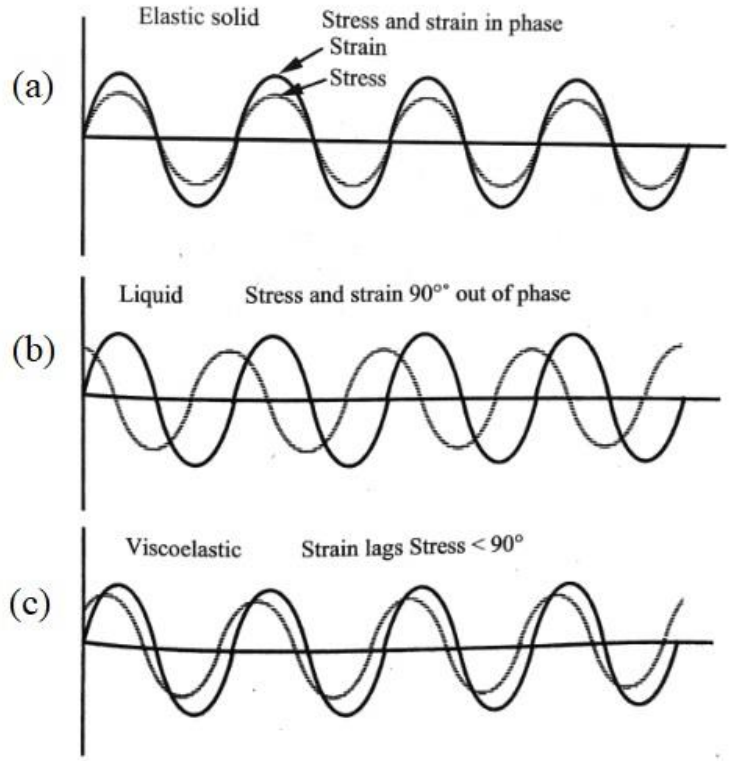


Figure 2-37: Stress versus strain response of a (a) perfectly elastic solid, (b) Newtonian liquid, and (c) viscoelastic material (Rao, 2014).

2.11 Conclusions

Investigations on the effect of added calcium salts on milk protein gelation have been focused on conventional milk protein gels, such as whey protein gels, acid- and rennet-induced milk gels. Although the effect of calcium addition on the heat stability of milk protein has also been studied extensively, the aim of most research has been to determine how the heat stability of milk proteins can be improved to prevent aggregation and coagulation of calcium-added milk during heat treatment. The addition of calcium salts to milk for specific texture modification is relatively new and not well-understood. Information on how the changes in the physicochemical properties, such as the calcium ion activity and calcium distribution between serum and colloidal phase, influence the calcium-induced gelation in milk is limited. Further, the effect of factors

that are commonly altered in milk to modify the textures and gel strength in acid- and rennet-induced milk gels have not been studied extensively in a calcium-induced milk gel. The contribution of each protein in milk, casein and whey protein, and the effect of their interactions on the gelation induced by calcium and heat have not been explored. Hence, this research will investigate the gaps in the current knowledge of calcium-induced gelation in milk.

Chapter 3 - Materials and Methods

3.1 Materials

Low-heat skim milk powder (32.7% protein, 0.9% fat, 54.5% carbohydrate, 7.9% ash, 3.93% moisture (*w/w*)) and whey protein isolate (93.91% protein, 0.3% fat, < 1% carbohydrate, 1.7% ash, 4.8% moisture (*w/w*)) were supplied from Fonterra Co-operative Group, Auckland, New Zealand. Calcium chloride dihydrate (≥ 99.0%), calcium lactate pentahydrate (> 98.0%), calcium gluconate monohydrate (> 98.0%), calcium lactobionate monohydrate (> 98.0%), calcium iodide hydrate (98%) and sodium chloride (≥ 99.0%) were purchased from Sigma-Aldrich (Auckland, New Zealand).

3.2 Skim milk sample preparation

Low heat skim milk powder (SMP) was reconstituted to 12% (*w/w*) in distilled water at 20 ± 2°C. Sodium azide (> 99.0%, SERVA Electrophoresis GmbH, Heidelberg, Germany) (0.02% *w/v*) was added as a preservative and the reconstituted skim milk was stirred for 30 min on a magnetic stirrer. The reconstituted skim milk was stored for a minimum of 10 h before use to ensure complete hydration. The same batch of reconstituted skim milk were used for analysis as long as it is within 20 h after reconstitution. As sodium azide was added to the skim milk and used within 20 h after reconstitution, the skim milk was not stored in 4°C for preservation. Preliminary experiments showed that pH values and calcium ion activity remained the same from 10- 20 h after reconstitution. and then stored at 20°C for 10 to 20 h to allow complete hydration before use.

3.3 Preparation of calcium stock solutions

Stock solutions of the various calcium salts were prepared with distilled water. The concentration of the stock solution for each calcium salt varied depending on the solubility of the calcium salt. The solubility and the concentration of the stock solutions for the various calcium salts are shown in Table 3-1. For calcium salts that were less soluble (e.g. calcium lactate and calcium gluconate), the calcium stock solutions were heated to ~ 40°C and magnetically stirred for at least 10 h to aid in solubilisation. Visual assessment of the calcium stock solutions was carried out to ensure complete solubilisation of all calcium salts. The concentrations for the stock solutions were calculated knowing the molecular weight of the calcium salt. Based on one mole of Ca^{2+} ion per mole of calcium salt (e.g. one mole $CaCl_2.2H_2O$ contains one mole Ca^{2+} ion), the molar concentration of calcium salt prepared was equivalent to the molar concentration of Ca^{2+} ion.

Table 3-1: Solubility and concentration of aqueous stock solutions of the various calcium salts.

Calcium salt	**CAS number**	**Solubility (gram of salt / 100 mL)**	**Concentration of calcium salt stock solution (mmol L^{-1})**
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	10035-04-8	75 (Mullin, 2001)	250
Calcium lactate pentahydrate ($[CH_2CH(OH)COO]_2Ca.5H_2O$)	5743-47-5	5.8 (Vavrusova, Liang & Skibsted, 2014)	200
Calcium D-gluconate monohydrate ($C_{12}H_{22}CaO_{14}.H_2O$)	66905-23-5	3.27 (Vavrusova et al., 2014)	60
Calcium lactobionate monohydrate ($C_{24}.H_{42}CaO_{24}.2H_2O$)	110638-68-1	45.2 (Vavrusova et al., 2014)	250
Calcium iodide hydrate ($CaI_2.xH_2O$) *	71626-98-7	204 (Schwartz & Myerson, 2002)	310

* x: degree of hydration = 0.9

For calcium chloride dihydrate, calcium chloride lactate pentahydrate, calcium gluconate monohydrate and calcium lactobionate monohydrate, the degree of hydration used for calculation of the calcium concentrations were based on the manufacturer's specification. For calcium iodide hydrate, a target concentration of 250 mmol L^{-1} was desired. The degree of hydration provided by the manufacturer's specification was 4 to 6. Thus, a degree of hydration of 5 was assumed and used for calculation of the calcium iodide concentration. However, calcium analysis by EDTA titration (Section 3.9) indicated that the actual degree of hydration was 0.9 for the calcium iodide hydrate used. The actual calcium concentration for the calcium iodide stock solution was 310 mmol L^{-1} (Table 3-1). Calculations to determine the actual degree of hydration can be found in Appendix 1.

3.4 Preheat treatment of skim milk sample

The reconstituted skim milk was preheated from 20 to 90°C in 5 min in a water bath (GD100, Grant Instruments Ltd, Cambridge, England) set at 93.5°C, then held at 90 ± 2°C for 10 min. After the 10 min holding period, the skim milk was cooled to 20 ± 2 °C within 5 min in a 4 ± 2°C water bath. The calcium salt stock solutions were added after the preheated skim milk had been cooled to 20 ± 2°C.

3.5 Addition of calcium salt stock solutions to skim milk

Calcium salt solutions prepared according to Section 3.3 were added to prepare skim milk samples with final calcium concentrations between 5 and 40 mmol L^{-1}. Distilled water was added to the samples to adjust the final skim milk concentration to 9.6% total solids (*w/w*) in all samples (3.3% total proteins *w/w*). As the calcium stock solutions were prepared at varying concentrations, the volume of calcium stock solutions added to the skim milk for different calcium salts varied. The amount of each calcium stock solution and distilled water added to make up 100 mL of calcium-added skim milk is shown in Table 3-2 to Table 3-5. The calcium stock solutions were added to the skim milk under magnetic stirring. Following the methods of Koutina, Christensen, Bakman, Anderson & Skibsted (2015a), where an equilibration time of 10 min was given between calcium salt addition and pH measurements, the calcium-added skim milk was stirred for 1 min, and left to equilibrate for another 10 min before measurement of calcium ion activity and pH. The measurement of calcium ion activity and pH takes approximately 5 min. Hence, the time from addition of the calcium salts to commencement of the rheological measurements of the skim milk was approximately 15 min.

Table 3-2: Solutions containing calcium chloride and calcium lactobionate. The volume of skim milk, calcium stock solutions (250 mmol L^{-1}) and distilled water added to make up 100 mL of calcium-added skim milk with final concentration of 9.6% total solids (*w/w*).

Concentration of added calcium salt (mmol L^{-1})	**Volume of 12% (*w/w*) skim milk (mL)**	**Volume of calcium stock solution (mL)**	**Volume of distilled water (mL)**
0	83.3	0	16.7
5	83.3	2	14.7
10	83.3	4	12.7
12.5	83.3	5	11.7
15	83.3	6	10.7
20	83.3	8	8.7
30	83.3	12	4.7
40	83.3	16	0.7

Table 3-3: Solutions containing calcium lactate. The volume of skim milk, calcium stock solution (200 mmol L^{-1}) and distilled water added to make up 100 mL of calcium-added skim milk with final concentration of 9.6% total solids (*w/w*).

Concentration of added calcium salt (mmol L^{-1})	**Volume of 12% (*w/w*) skim milk (mL)**	**Volume of calcium stock solution (mL)**	**Volume of distilled water (mL)**
0	83.3	0	16.7
5	83.3	2.5	14.2
10	83.3	5	11.7
12.5	83.3	6.3	10.4
15	83.3	7.5	9.2
20	83.3	10	6.7

Table 3-4: Solutions containing calcium gluconate. The volume of skim milk, calcium stock solution (60 mmol L^{-1}) and distilled water added to make up 100 mL of calcium-added skim milk with final concentration of 9.6% total solids (*w/w*).

Concentration of added calcium salt (mmol L^{-1})	**Volume of 13.5% (*w/w*)$^{+}$ skim milk (mL)**	**Volume of calcium stock solution (mL)**	**Volume of distilled water (mL)**
0	74.1	0	25.9
5	74.1	8.3	17.6
10	74.1	16.7	9.2
12.5	74.1	20.8	5.1
15	74.1	25	0.9

$^{+}$ Skim milk was reconstituted at 13.5% (*w/w*) for calcium gluconate-added skim milk samples to achieve up to 15 mmol L^{-1} of calcium gluconate added and with a final skim milk concentration of 9.6% total solids (*w/w*).

Table 3-5: Solutions containing calcium iodide. The volume of skim milk, calcium stock solution (310 mmol L^{-1}) and distilled water added to make up 100 mL of calcium-added skim milk with final concentration of 9.6% total solids (*w/w*).

Concentration of added calcium salt (mmol L^{-1})	**Volume of 12% (*w/w*) skim milk (mL)**	**Volume of calcium stock solution (ml)**	**Volume of distilled water (ml)**
0	83.3	0	16.7
6.2	83.3	2	14.7
12.4	83.3	4	12.7
15.5	83.3	5	11.7
18.6	83.3	6	10.7
24.8	83.3	8	8.7
37.2	83.3	12	4.7
49.6	83.3	16	0.7

3.6 pH and calcium ion activity measurement in skim milk

The pH of the skim milk samples was determined using a pH meter (PB-20, Sartorius, New Zealand) with a pH electrode (InLab® Expert Pro-ISM, Mettler-Toledo, New Zealand). Three readings were taken for each sample. All measurements were taken at 20 ± 2°C.

Calcium ion activity was determined using a calcium-selective electrode (Sentek, Essex, England) connected to a pH/ ion meter (S220 SevenCompact™, Mettler-Toledo, Switzerland). Three readings were taken for each sample. All measurements were taken at 20 ± 2°C.

The calcium ion activity in a solution, $a_{Ca^{2+}}$, is related to its calcium ion concentration and can be calculated from Equation 3-1 (van Boekel, 2008a).

$$a_{Ca^{2+}} = c_{Ca^{2+}} \cdot \gamma_{Ca^{2+}}$$

Equation 3-1

where $c_{Ca^{2+}}$ is the calcium ion concentration (mmol L^{-1}) and
$\gamma_{Ca^{2+}}$ is the activity coefficient

The activity coefficient, $\gamma_{Ca^{2+}}$, was derived from Equation 3-2, the Davies equation (Vavrusova et al., 2014):

$$\log \gamma_{Ca^{2+}} = -A_{DH} z^2 \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I \right)$$

Equation 3-2

where A_{DH} is the Debye-Hückel constant with a numerical value of 0.506 at 20°C (Vavrusova et al., 2014),

z is the charge of calcium ion (= +2),

I is the ionic strength of the solution.

The ionic strength of the solution (I) was determined from Equation 3-3:

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

Equation 3-3

Where c is the molar concentration of ion i (mol L^{-1}),

z is the charge of ion i

Table 3-6: Maximum calcium chloride concentration, calcium ion activity and range of calcium chloride concentration studied at each ionic strength of calcium standards.

Ionic strength of calcium standard (mmol L^{-1})	Maximum calcium chloride concentration (mmol L^{-1})	Calcium ion activity coefficient ($\gamma_{Ca^{2+}}$)	Range of calcium chloride concentration studied (mmol L^{-1})
40	13	0.49	0.5 to 13
80	26	0.40	1 to 26
160	53	0.33	1 to 53
250	80	0.30	1 to 80

The total ionic strength of natural cow's milk is approximately 80 mmol L^{-1} (van Boekel, 2008a). Therefore, calcium standards used for calibrating the calcium ion electrode were standardised at ionic strength of 80 mmol L^{-1} by other authors (Crowley et al., 2014; Ramasubramanian et al., 2014). The research presented in this thesis required the addition of calcium salt up to concentrations of 40 mmol L^{-1}. Skim milk contains approximately 30 mmol L^{-1} of total calcium. This equated to the total calcium concentration of 70 mmol L^{-1} when the calcium salts were added to the skim milk. However, based on Equation 3-3, if the calcium standards were standardised at an ionic strength of 80 mmol L^{-1}, the maximum concentration of calcium chloride in the calcium standards would only be 26 mmol L^{-1} (Table 3-6). For the calcium standards to cover the concentration range of up to 70 mmol L^{-1}, the ionic strength of the calcium standards had to be increased.

The effect of the ionic strength of the calcium standards on the measured a_{Ca}^{2+} was investigated. At increasing values of ionic strength, the maximum calcium chloride concentration, the activity coefficient, and the concentration range for the calcium standards prepared was calculated from Equation 3-3 and shown in Table 3-6. Sample calculations for ionic strength, activity coefficient and a_{Ca}^{2+} can be found in Appendix 2.

The calcium standards were prepared using the calcium chloride stock solutions described in Section 3.3, a stock solution of 800 mmol L^{-1} potassium chloride (KCl), and distilled water to achieve calcium standards with the set calcium chloride concentrations and ionic strengths.

The measured voltage of the calcium standards at different calcium concentrations and ionic strengths was determined using the calcium selective electrode and ion meter. As the calcium concentration and ionic strength of the solutions made with the calcium chloride were known, the calcium ion activity of the calcium standards was calculated using Equation 3-1 and 2 for each concentration. Hence, it was possible to plot the log of a_{Ca}^{2+} vs the measured voltage from the ion meter as shown in Figure 3-1. Plots of the voltage reading vs a_{Ca}^{2+} were generated for the different ionic strengths (Figure 3-1). Statistical analysis using ANOVA showed that the regression models at the different ionic strengths were not significantly different ($p > 0.05$). The slope of the regression equation for plots of all four ionic strengths were within the manufacturer's specification (29 to 36 mV). Therefore, to achieve calcium chloride concentrations of up to 80 mmol L^{-1} in the calcium standards, the ionic strength for the calcium standards at 250 mmol L^{-1} was selected as the standardised ionic strength for all calcium standards in

this work. The calcium chloride concentrations used for the calcium standard plot ranged from 1 to 80 mmol L^{-1}.

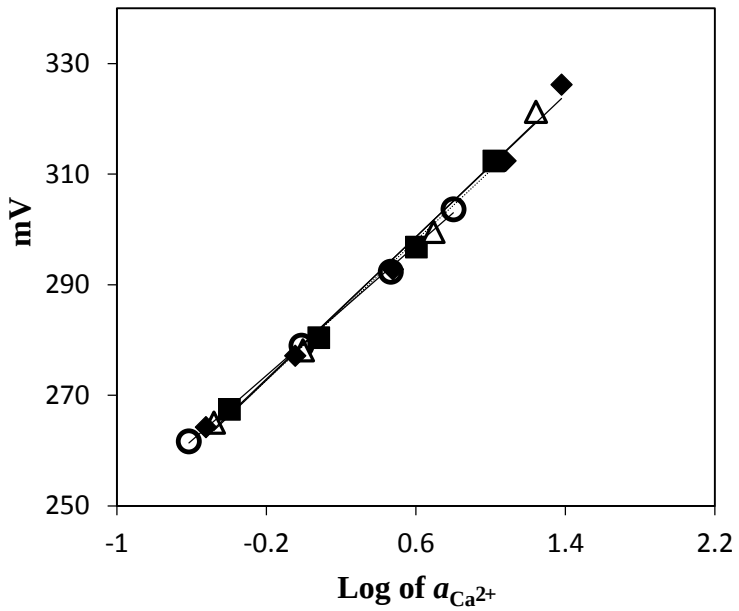


Figure 3-1: Typical plots showing the relationship between voltage readings and log of calcium activity, a_{Ca}^{2+}, of the calcium standards at 0.04 mol L^{-1} (○), 0.08 mol L^{-1} (■), 0.16 mol L^{-1} (△) and 0.25 mol L^{-1} (◆).

To determine the a_{Ca}^{2+} in the skim milk samples, the voltage of the skim milk samples was measured with the calcium-selective electrode. The calcium ion activity (a_{Ca}^{2+}) was then interpolated from the standard curves of voltage values vs log (a_{Ca}^{2+}) obtained from the calcium standards at standardised ionic strength of 250 mmol L^{-1} (Figure 3-1). At least three readings were taken for each sample. All measurements were taken at 20 ± 2°C.

3.7 Centrifugation of skim milk samples to separate serum and sediment phases

Skim milk samples (1.3 g) were weighed into 1.5 mL Eppendorf tubes and centrifuged at 21,500 *g* for 90 min at 20°C in a high speed micro-centrifuge (Himac CT15RE, Hitachi, Tokyo, Japan). According to Anema et al. (2004b), 21,000 g provided almost

identical separation between colloidal and soluble phases to ultracentrifugation method. Hence this lower centrifugation speed was chosen for this study.

The clear supernatant (serum phase) was carefully pipetted off to separate it from the sediment. The weight of the sediment was determined after centrifugation and the weight of separated serum was calculated by subtracting the mass of wet sediment from the original mass of skim milk.

3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and laser densitometry

The skim milk and serum samples were analysed for the distribution of proteins present, based on molecular weight, by SDS-PAGE according to the methods of Anema (2009a) and Nguyen, Wong, Havea, Guyomarc'h & Anema (2013) with modification, using a Bio-Rad Mini-PROTEAN Tetra Cell electrophoresis unit (Bio-Rad Laboratories Inc, California, USA). Sodium dodecyl sulphate (SDS) (≥ 85.0%), Tris-hydrochloride (Tris-HCl) (≥ 99.0%), and Amido black 10 B Dye (≥ 80.0%) were purchased from Merck KGaA (Darmstadt, Germany). Glycine (≥ 99.0%) and β-mercaptoethanol (≥ 99.0%) were purchased from Sigma Aldrich (Auckland, New Zealand). Tris-base (≥ 99.8%) and bromophenol blue dye powder were purchased from Bio-Rad Laboratories (California, USA).

3.8.1 Preparation of sample buffer

The sample buffer was prepared by combining the solutions according to Table 3-7. The 0.5 M Tris-HCl buffer was adjusted to pH 6.8 using 3 M sodium hydroxide solution (NaOH). The 0.4% bromophenol blue solution was prepared by dissolving 1.6 g of bromophenol blue in approximately 10 mL of 0.1 M NaOH and made up to 400 mL with distilled water in a measuring cylinder.

Table 3-7: Composition of sample buffer.

Chemical	Volume (ml)
10% SDS solution	200
0.5 M Tris-HCl buffer (pH 6.8)	125
Glycerol	100
0.4% bromophenol blue solution	25
Distilled water	500

3.8.2 Preparation of running buffer

The stock running buffer at five times concentration was prepared by weighing the chemicals according to Table 3-8 and made up to 1 L with distilled water in a volumetric flask. For each electrophoresis run, 200 ml of the stock running buffer was diluted to 1 L with distilled water.

Table 3-8: Composition of one litre of stock running buffer (five times concentration).

Chemical	Weight (g)
Tris-base	15.0
Glycine	72.0
SDS	100.0

3.8.3 Sample treatment

The skim milk samples were diluted 1:40 (*v/v*), and the serum samples were diluted 1:20 (*v/v*) with the sample buffer. β-mercaptoethanol (20 µL) was added to 1 mL of the diluted sample. A broad range protein standard (6.5 to 200 kDa, Bio-Rad Laboratories Inc, Richmond, California, USA) was used to determine the molecular weight of the bands. The protein standards were diluted at 1:40 (*v/v*) with the sample buffer. β-mercaptoethanol (25 µL) was then added to 475 µL of the diluted protein standard, according to the manufacturer's instructions. All samples were placed in Eppendorf tubes and heated in a 95 ± 2°C water bath for 10 min prior to gel electrophoresis.

3.8.4 SDS gel electrophoresis running conditions

Twenty microliters of the prepared skim milk and serum samples were pipetted into the wells of the precast gels (Bio-Rad Mini-PROTEAN® TGX^{TM}). According to the manufacturer's instructions, only 10 μL of the protein standard was pipetted into the wells. A skim milk sample with known protein concentration was used in every gel as a control. The gel electrophoresis was performed at a constant current of 30 mA and allowed to run for approximately 1 h.

3.8.5 Staining and destaining of gels

After the electrophoresis runs were completed, the gels were placed in a solution containing 0.1% (*w/v*) Amido black, 25% (*v/v*) isopropanol and 10% (*v/v*) acetic acid for 14 h on a rocking table for staining. After staining, the gels were destained in 10% (*v/v*) acetic acid solution. The destaining solution was replaced every 30 min for the first 2 h, then every hour subsequently for another 2 h.

3.8.6 Analysis of gels

The destained gels were scanned using a densitometer (Bio-Rad ChemiDoc^{TM} XRS System, California, USA). The band intensities were analysed with Image Lab^{TM} Software 6.0 (Bio-Rad Laboratories, Inc., California, USA). Following the methods of Anema & Klostermeyer (1997), the total intensities from the protein bands in the skim milk (control) was determined. The intensities of the major protein bands of the samples were determined and compared with the intensities of the protein bands of the skim milk (control). The quantity of each protein band in the samples was determined with reference to the known protein concentration in the skim milk sample. For the serum samples, corrections were applied for the mass of serum per gram of milk. Each skim milk and serum sample was analysed in at least two replicates, with the two replicates

on two separate gels. Sample calculations for determination of the protein concentration can be found in the Appendix 3.

3.9 Determination of calcium concentration in skim milk and serum samples by EDTA titration

The calcium concentration was determined in the calcium-added skim milk and serum phase using the ethylenediaminetetraacetic acid (EDTA) titration method described by Patton & Reeder (1959) and Pearce (1977) with modifications. Calcium determination by EDTA titration measures the total calcium in the sample. In the serum samples, measurements would include the total soluble calcium, i.e. concentration of calcium ions as well as calcium from the soluble calcium salts (calcium phosphate, calcium citrate). For the skim milk samples, measurements would include the serum calcium as well as the colloidal calcium present in the sediment. Standard curves were prepared with a series of aqueous calcium chloride standards ranging from 0 to 3.2 mg g^{-1} calcium chloride before each analysis (Figure 3-2). One mL of the sample to be analysed was dispensed into a conical flask and the weight of the sample per mL was recorded. The average densities of the calcium standards and serum samples were found to be 1.00 ± 0.01 g, and the density of the skim milk samples was found to be 1.02 ± 0.01 g. The sample was then diluted with 50 mL of distilled water. Three millilitres of 8 mol L^{-1} NaOH was then added to raise the pH of the sample. The pH of the solution was determined by a pH meter and found to be pH 13.4 ± 0.2. The samples were allowed to stand for 5 min with occasional swirling to allow any magnesium present to precipitate as hydroxide, such that it is not determined along with the calcium (Pearce, 1977). A few drops of 0.5% (*w/v*) Patton-Reeder indicator (Sigma Aldrich, Auckland, New Zealand), in 1 mol L^{-1} NaOH, was added to the sample prior to titration. The sample was then titrated with 0.01 mol L^{-1} EDTA until the sample colour changed from pink to

a persistent blue. The mass of calcium per gram of milk or serum sample was determined using the standard curves (Figure 3-2). For the serum samples, corrections were applied for the mass of serum per gram of milk. The mass of calcium in the sediment was determined as the difference between the total calcium in milk and serum calcium. At least three titrations were performed for each sample.

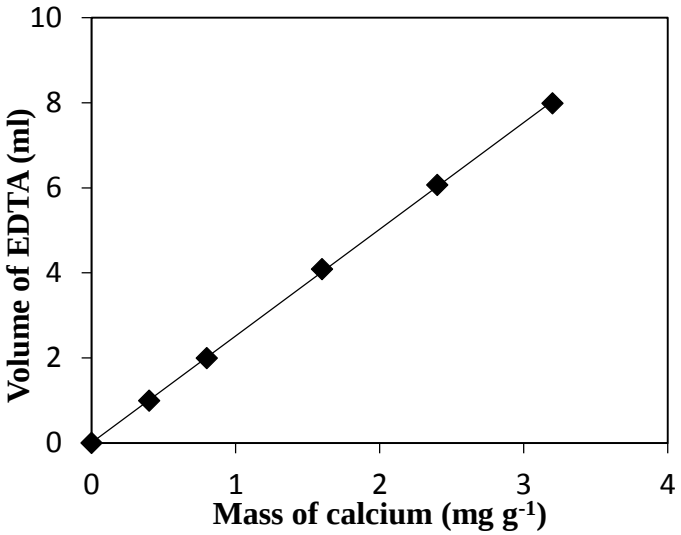


Figure 3-2: An example of a typical standard curve showing the relationship between the volume of EDTA used for titration and mass of calcium in one gram of calcium standard.

The determination of calcium concentration by EDTA titration was validated against the atomic absorption spectrometric (AAS) method (Institute of Fundamental Sciences, Massey University, Palmerston North). The results showed that both methods were in good agreement up to 30 mmol L^{-1} of added calcium chloride in skim milk. Based on calculations of the expected concentration of calcium in skim milk with 40 mmol L^{-1} of added calcium chloride, the AAS method gave a higher calcium concentration. As only one sample at each concentration was sent for external analysis using the AAS method, the results gave an indication that the EDTA titration method was reliable. The comparison for the EDTA titration and AAS method can be found in Appendix 4.

3.10 Particle size and zeta-potential measurement

3.10.1 Preparation of skim milk permeate for particle size dilution

Calcium-added skim milk was prepared according to methods described in Sections 3.2, 3.4 and 3.5 at between 5 to 40 mmol L^{-1} of added calcium chloride and calcium lactobionate. The skim milk permeate was obtained by filtering 72 mL of calcium-added skim milk at 20 ± 2°C through a crossflow polyethersulfone (PES) membrane cassette with a molecular weight cut-off of 10 kDa (Vivaflow 50, Sartorius Stedim Lab Ltd, Gloucestershire, UK). The retentate was recirculated through the membrane unit using a peristaltic pump (Masterflex® L/S® Variable-speed economy modular drives, Cole-Parmer Instrument Company, Illinois, USA) at a flow rate of 190 ± 10 mL min^{-1}. The ultrafiltration process ceased when the volume concentration factor (VCF) was approximately 3. The permeate was analysed by SDS-PAGE and found to contain no casein or whey proteins. The permeate was collected and used as the diluent for the calcium-added skim milk for particle size analysis. The permeate collected were stored at 4°C until required but for no longer than 3 days.

3.10.2 Sample preparation and measurement of particle size and zeta-potential

Samples for particle size and zeta-potential measurement were prepared by diluting calcium-added skim milk 1:200 (*v/v*) with skim milk permeate. The permeate used for the dilution of each sample was prepared with skim milk permeate containing the same concentration of calcium salt added to the skim milk. The diluted sample was filled into a disposable plastic cuvettes for particle size measurement, or a disposable folded capillary cell (Zetasizer nano series DTS1070, Malvern, Australia) for zeta-potential measurements. All samples were analysed with temperature set at 20 °C in the zetasizer (Zetasizer Nano ZS, Malvern, Australia), with the material index at 1.39 and the dispersant refractive index at 1.33 (Crowley et al., 2014). Zeta-potential was measured

at 20V using the monomodal analysis method. Each sample was analysed in at least duplicate, with three readings taken by the instrument for each replicate.

3.11 Rheological measurements of the calcium-induced skim milk gels in the rheometer

Rheological measurements were carried out using a rheometer (Discovery HR-3, TA Instrument, USA) fitted with a vane geometry (28 mm diameter, 42 mm length). A hard-anodised (HA) aluminium single gap cylinder cup (34 mm diameter) or a stainless steel (SS) single gap cylinder cup (30 mm diameter) was used for holding the sample for analysis. The high calcium concentrations in the calcium-added skim milk were found to result in calcium deposits in the HA cups that were difficult to clean and remove. A SS cup with appropriate cleaning agents suitable for removing calcium deposit was recommended by the manufacturer. Results presented in this thesis will identify which cup was used. As the SS cup heated up and cooled down at a slower rate compared to the HA cup, the temperature procedure for the SS cup was modified to ensure the sample temperature achieved the desired temperature. Temperature profiles of the rheometer set temperature and the sample temperature for the HA and SS cups can be found in Figure 3-3 and Figure 3-4, respectively. The sample temperatures were checked using a thermocouple probe (Traceable®, Fisher Scientific, United Kingdom) and the temperature profiles shown in Figure 3-3 and Figure 3-4 are the average temperatures of two runs. The samples underwent a temperature sweep from 20°C to the holding temperature, followed by a time sweep at the holding temperature for 60 min, and finally another temperature sweep where the sample was cooled from the holding temperature to 20°C. The changes in the storage modulus (G') of the skim milk were monitored throughout the heating, holding and cooling phase. Sunflower oil (viscosity: 0.06 Pa.s at 20°C, Countdown Home brand, New Zealand) was carefully

layered on the surface of the sample and a solvent trap cover was used to minimise sample evaporation during measurement. The measurements were carried out under constant strain of 0.05% and frequency at 0.1 Hz.

After the skim milk was cooled to 20°C, a frequency sweep was performed on the sample from 0.01 to 10 Hz at a strain of 0.05%. The frequency sweeps were carried out at rheometer set temperature of 20°C. Frequency sweeps were performed only on samples that had formed gels after cooling to 20°C (G' > 1 Pa). An amplitude sweep from 0.01% to 100% was performed on the weakest gel (formed at 12.5 mmol L^{-1} of added calcium gluconate) after cooling to 20°C in order to confirm the strain of 0.05% used for the temperature, time and frequency sweeps was within the linear viscoelastic region of the gel (Figure 3-5). The amplitude sweep was performed at 0.1 Hz and 20°C.

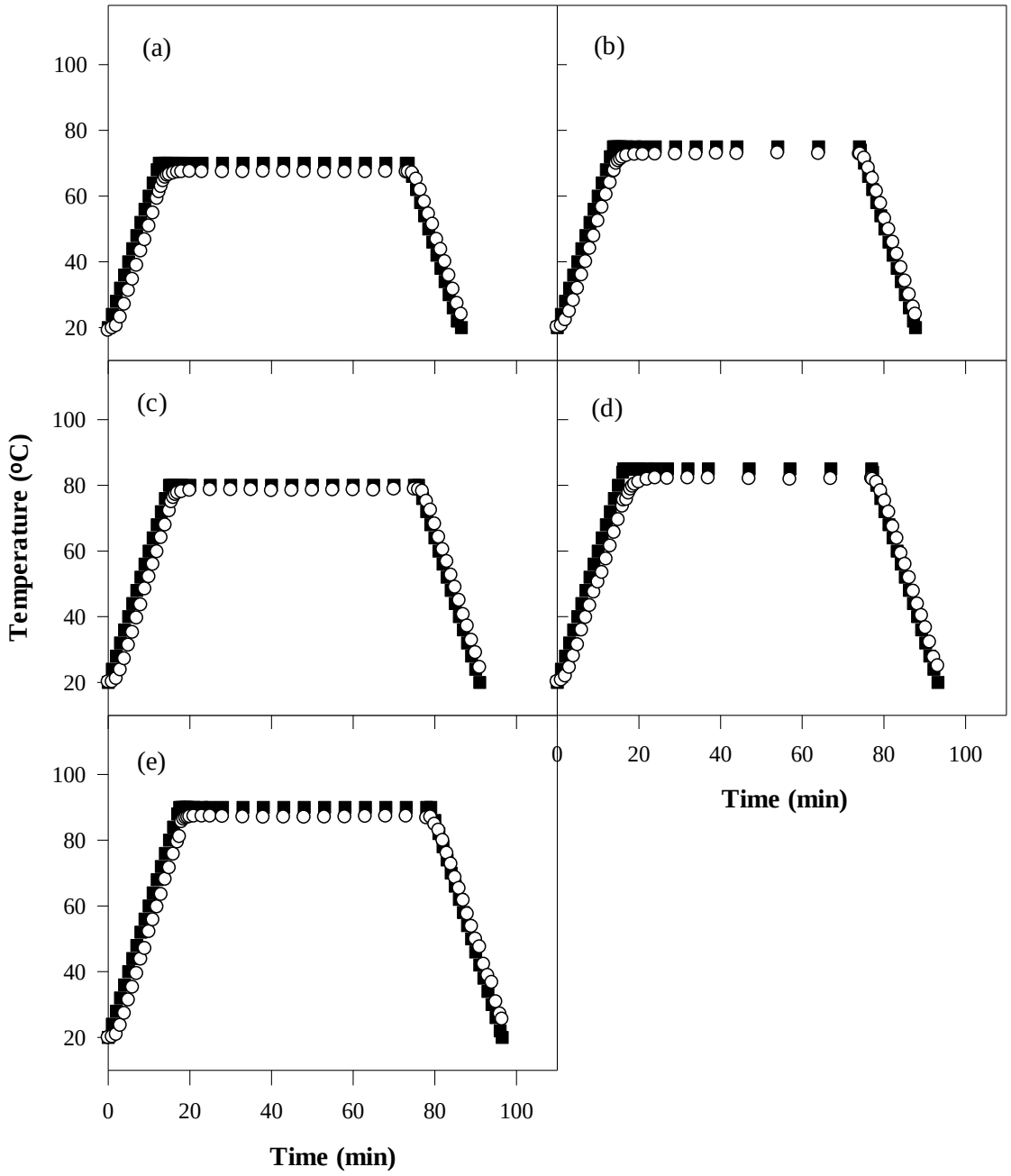


Figure 3-3: Temperature profile of rheometer set temperature (■) and sample temperature (○) using hard-anodised aluminium (HA) cup. Set holding temperatures for rheometer was (a) 70°C, (b) 75°C, (c) 80°C, (d) 85°C and (e) 90°C.

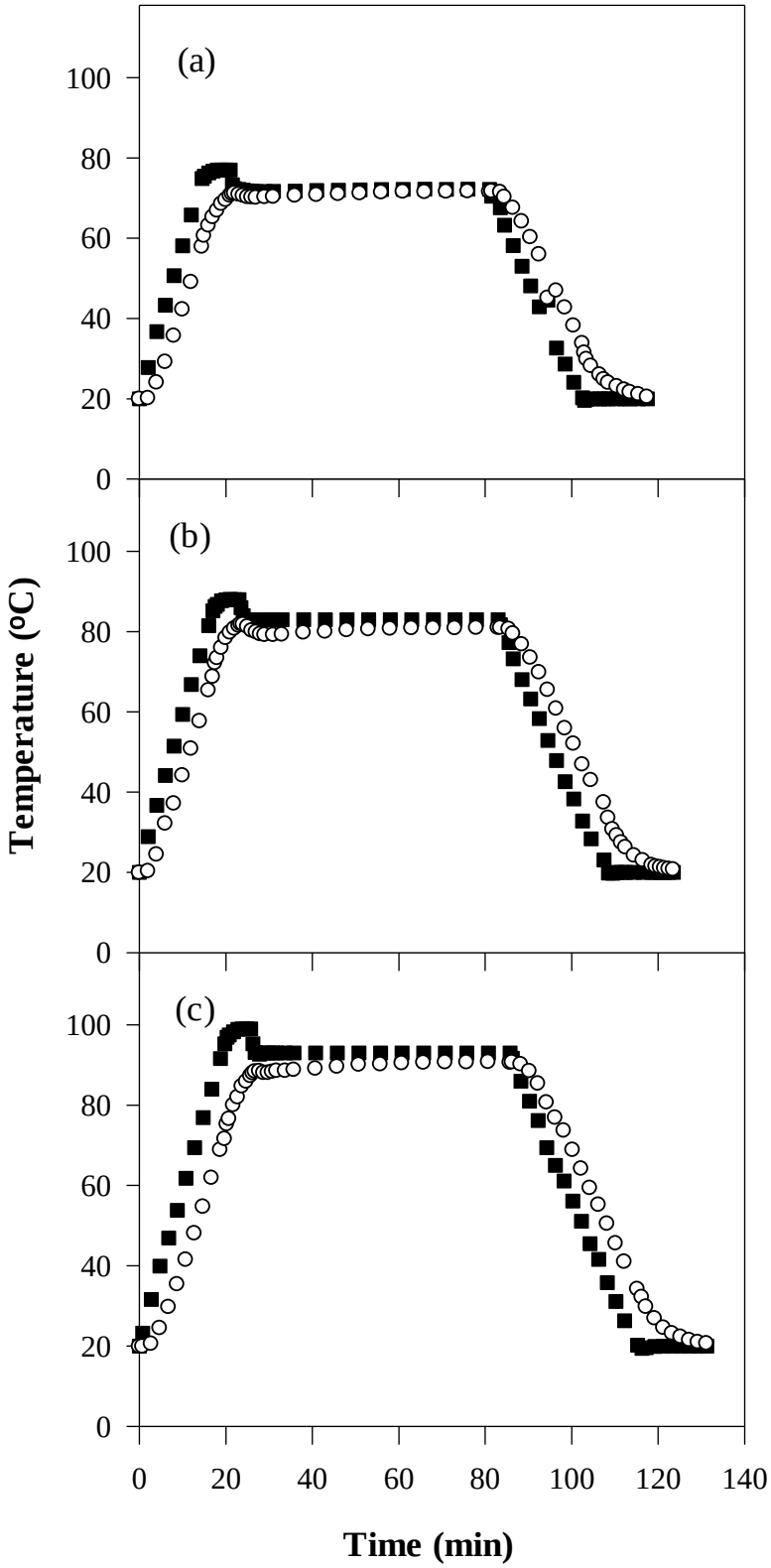


Figure 3-4: Temperature profile of rheometer set temperature (■) and sample temperature (○) using stainless steel (SS) cup. Set holding temperatures for rheometer was (a) 70°C, (b) 80°C and (c) 90°C.

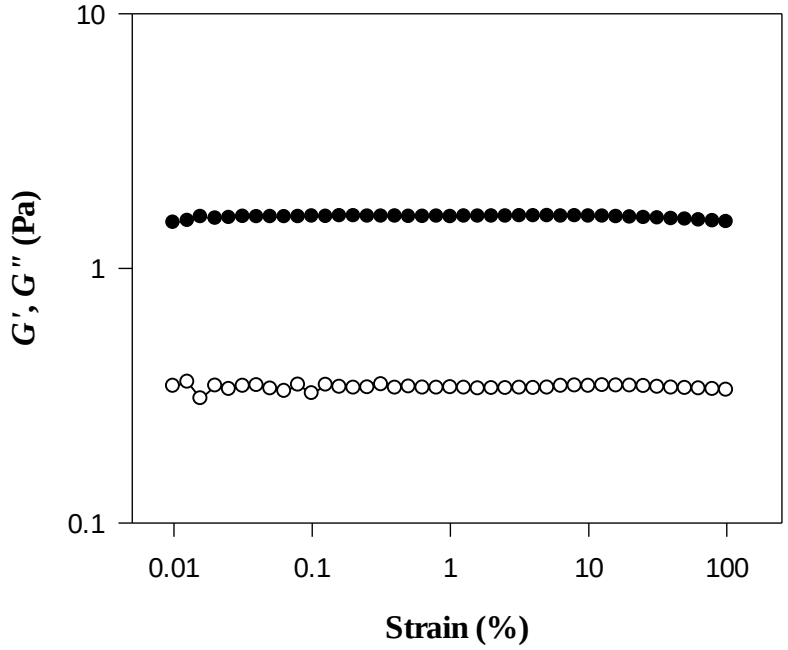


Figure 3-5: Amplitude sweep of calcium-induced skim milk gels formed by addition of 12.5 mmol L^{-1} of calcium gluconate measured at 0.1 Hz and 20°C. The close symbols (●) represent the storage modulus, *G′*, and the open symbols (○) represent the loss modulus, *G″*.

3.12 Preparation of skim milk permeate for whey protein isolate dispersion

Skim milk was prepared by reconstituting SMP as described in Section 3.2, without the addition of sodium azide. The reconstituted skim milk was stored at 4°C for at least 10 h before filtration.

The skim milk permeate was obtained by filtering 1.6 kg of reconstituted skim milk through a polyethersulfone (PES) membrane (Sartocon slice cassette, molecular weight cut off: 10 kDa, Sartorius Stedim Biotech, Göttingen, Germany) operating at an feed (inlet) pressure of 2.0 ± 0.1 bar and retentate (outlet) pressure of 0.5 ± 0.1 bar using a gear pump (MOT TE80, WEG Electric Corporation, USA). The temperature of the skim milk during filtration was 20 ± 2°C. The retentate was recirculated back to the feed tank and the permeate was collected. The ultrafiltration process ceased when the volume concentration factor (VCF) was approximately 3. Sodium azide (0.02% *w/v*) was added

to the permeate as a preservative and stored at 4°C until required but for no longer than 10 days.

The pH and calcium ion activity of the skim milk permeate was 6.64 ± 0.02 and 0.6 ± 0.1, respectively. The skim milk permeate obtained was analysed using SDS-PAGE as described in Section 3.8 and found to contain no casein or whey proteins.

3.13 Preparation of simulated milk ultrafiltrate (SMUF)

SMUF was prepared following the methods of Jenness & Koops (1962) and Dumpler, Kieferle, Wohlschläger & Kulozik (2017) with modifications. All salts except calcium chloride dihydrate and 1 M KOH were weighed according to Table 3-9 into a 5 L volumetric flask. After distilled water was added to approximately 75% of the flask, the flask was swirled to solubilise the salts. Lactose powder (Fonterra Co-operative Group, Palmerston North, New Zealand) was then added to the volumetric flask according to Table 3-9. Two millilitres of 1 M KOH was added to the solution and distilled water was filled up to the 5 L mark. The calcium-free SMUF was kept at 4°C for up to three days.

The calcium-free SMUF was warmed up to 20°C and filled into a 1 L or 5 L volumetric flask. Calcium chloride dihydrate was added according to Table 3-9. The change in the volume after addition of the calcium chloride dihydrate was assumed to be negligible. The pH was adjusted to 6.64 ± 0.02 by addition of 1 M KOH to mimic the pH of the skim milk permeate (Section 3.12). The final calcium ion activity after addition of calcium chloride dihydrate was measured by the calcium-selective electrode and found to be 0.6 ± 0.1.

Table 3-9: Composition and quantities of salt used for preparation of SMUF.

Salt	Chemical formula	Weight (g L^{-1})
Monopotassium phosphate	KH_2PO_4	0.933
Dipotassium phosphate	K_2HPO_4	0.867
Potassium citrate monohydrate	$K_3C_6H_5O_7.H_2O$	0.7
Sodium citrate dihydrate	$Na_3C_6H_5O_7.2H_2O$	1.2
Magnesium citrate nonahydrate	$Mg_3(C_6H_5O_7).9H_2O$	0.6
Citric acid monohydrate	$C_6H_8O_7.H_2O$	0.057
Potassium chloride	KCl	0.633
Sodium chloride	NaCl	0.167
Potassium sulphate	K_2SO_4	0.2
1 M potassium hydroxide	KOH	0.2 (mL)
Lactose powder		54.5
Calcium chloride dihydrate	$CaCl_2.2H_2O$	1.1

3.14 Preparation of whey-depleted skim milk (WDSM)

Skim milk was prepared by reconstituting SMP as described in Section 3.2, without the addition of sodium azide. The reconstituted skim milk was stored at 4°C for at least 10 h before use. The reconstituted skim milk was warmed up to 20 ± 2°C before filtration.

The WDSM was obtained by filtering 3.0 kg of reconstituted skim milk through a polyethersulfone (PES) membrane (Sartocon slice cassette, pore size: 0.1 µm, Sartorius Stedim Biotech, Göttingen, Germany) operating at a feed (inlet) pressure of 2.0 ± 0.1 bar and retentate (outlet) pressure of 0.5 ± 0.1 bar, at 22 ± 2°C with a gear pump (MOT TE80, WEG Electric Corporation, USA). The retentate, which retained casein, was recirculated back to the feed tank and the permeate, which contained the whey proteins, was collected and the volume of the permeate collected was measured at four min intervals. Diafiltration was initiated by addition of SMUF to the feed at the same volume of the permeate removed at four min intervals. The total volume of SMUF added was approximately 15 L. The retentate obtained at the end of the diafiltration was

designated as the WDSM. Sodium azide (0.02% *w/v*) was added to the WDSM as a preservative and stored at 4°C until required but for no longer than 10 days.

3.15 Statistical analysis

All experiments were carried out in at least duplicates for both sample treatment and sample analyses. Standard deviations and pooled standard deviations were used where appropriate to indicate the variability between repeated experiments or measurements. Significant difference between the means was analysed using one-way ANOVA (Tukey multiple comparison test) in the Minitab 18 Statistical software (Minitab Inc, Pennsylvania, USA).

Chapter 4 - Effect of the addition of different soluble calcium salts to skim milk

4.1 Introduction

The addition of soluble calcium salts, such as calcium chloride, to milk is known to reduce the stability of dairy proteins, particularly when milk is heated (Deeth & Lewis, 2015). The addition of soluble calcium salts results in a decrease in pH and an increase in calcium ions in solution (Crowley et al., 2014; On-Nom et al., 2012). The formation of a protein gel network is dependent on the destabilisation of milk proteins (De Kruif et al., 1995). Depending on the type of soluble calcium salt added, the stability of milk can be altered differently (Crowley et al., 2014). Hence, the type of soluble calcium salt added to milk could influence the gelation properties of the calcium-induced milk gels through their different destabilising effects.

The effects of the addition of inorganic and organic calcium salts to milk have been studied (Crowley et al., 2014; Koutina, Knudsen & Skibsted, 2015; Philippe et al., 2003; Philippe, Gaucheron & Le Graet, 2004). The Hofmeister series is an ordering of the efficiency (measured by the concentration of salt) of different anions with a fixed cation, or cations with a fixed anion, in precipitating suspensions of egg white lecithin (Parsons, Bostrom, Lo Nostro & Ninham, 2011). For anions, the following order was found: $F^- > CH_3COO^- > Cl^- > NO_3^- > Br^- > I^- > SCN^-$ (Lund, Heyda & Jungwirth, 2010). Based on the Hofmeister effect, different anions of calcium salts may affect the stability of the milk proteins and therefore gelation of the skim milk differently. Calcium chloride and calcium iodide were selected as the two calcium salts to investigate the Hofmeister effect.

Organic calcium salts, such as calcium lactate, calcium gluconate and calcium lactobionate have received increasing attention as calcium fortifying agents due to the

potential for higher calcium bioavailability compared to calcium chloride (Singh et al., 2007; Skibsted, 2016). Complex binding between the hydroxycarboxylate anion group of lactate, gluconate and lactobionate and calcium may prevent precipitation of the calcium ions in the intestines, thus potentially increasing the calcium bioavailability (Skibsted, 2016). The different calcium salts have also been shown to affect the pH, calcium ion activity and heat stability of milk differently (Crowley et al., 2014; Omoarukhe et al., 2010). However, there have been no studies relating the effect of the different calcium salts on the calcium ion activity, pH and rheological properties of skim milk. Therefore, the aim of this study was to evaluate the effects of various soluble calcium salts (calcium chloride, calcium lactate, calcium gluconate, calcium lactobionate, calcium iodide) on the physico-chemical and rheological properties of skim milk.

4.2 Materials and Methods

4.2.1 Skim milk sample preparation

The skim milk samples were prepared according to Section 3.2 and 3.4. As discussed in Section 3.3, the concentration of the calcium stock solution depended on the solubility of the calcium salt. For instance, stock solutions for calcium chloride were prepared at 250 mmol L^{-1} while calcium gluconate solutions were prepared at 60 mmol L^{-1} as calcium gluconate are less soluble. In turn, a larger amount of the calcium gluconate stock solution was needed to achieve the same added calcium salt concentration compared to calcium chloride. To ensure a final total solids of 9.6% (*w/w*) in the skim milk, the maximum amount of calcium stock solution that could be added was therefore limited by the concentration of the calcium stock solutions. The concentration of calcium salt added to the skim milk for the different calcium salts is shown in Table 4-1.

Table 4-1: Concentrations of the different calcium salts added to skim milk.

Calcium salt	Chemical formula	Molecular weight (g mol^{-1})	Concentration added (mmol L^{-1})
Calcium chloride dihydrate	$CaCl_2.H_2O$	147.01	0, 5, 10, 12.5, 15, 20, 30, 40
Calcium lactate pentahydrate	$[CH_2CH(OH)COO]_2Ca.5H_2O$	790.68	0, 5, 10, 12.5, 15, 20
Calcium gluconate monohydrate	$C_{12}H_{22}CaO_{14}.H_2O$	308.29	0, 5, 10, 12.5, 15
Calcium lactobionate monohydrate	$C_{24}.H_{42}CaO_{24}.2H_2O$	443.39	0, 5, 10, 12.5, 15, 20, 30, 40
Calcium iodide hydrate	$CaI_2.xH_2O$	310.08	0, 6.2, 12.4, 15.5, 18.6, 24.8, 37.2, 49.6

4.2.2 Analysis of calcium-added skim milks

This chapter reports the effect of adding different calcium salts on the physicochemical and rheological properties of skim milk. The assays and techniques used in this chapter can be found in Section 3.6, to 3.11. For the rheological measurement in Section 3.11, the hard-anodised aluminium cup was used in this study. All samples were heated to and held at 60 min in the rheometer at the set temperature of 70°C. The frequency sweeps were performed only on samples that formed gels after cooling to 20°C (final G' > 1 Pa).

4.3 Results and Discussion

4.3.1 Calcium ion activity and pH in skim milk

The calcium ion activity (a_{Ca}^{2+}) increased as more calcium salt was added to the preheated skim milk, regardless of the salt type (Figure 4-1a). The maximum amount of

salt added to milk was limited by the solubility of each salt. In terms of solubility in water at 20°C, calcium iodide was the most soluble (204 g / 100 mL), followed by calcium chloride (75 g/ 100 mL), calcium lactobionate (45.2 g/ 100 mL), calcium lactate (5.8 g/ 100 mL) and calcium gluconate (3.27 g/ 100 mL) (Mullin, 2001; Schwartz & Myerson, 2002; Vavrusova et al., 2014).

At the same molar concentration of added calcium salt, the order of a_{Ca}^{2+} from the highest to the lowest was calcium chloride > calcium lactate > calcium gluconate > calcium lactobionate > calcium iodide (Figure 4-1a). With the exception of calcium iodide, the observed difference in a_{Ca}^{2+} between the different calcium salts could be due to the dissolution behaviour of the calcium salts in water. Calcium chloride dissociates into Ca^{2+} and Cl^- ions in a single step:

$$CaCl_2 \longrightarrow Ca^{2+} + 2Cl^-$$

Equation 4-1

The dissolution of calcium lactate, calcium gluconate and calcium lactobionate occurs in two steps (Vavrusova et al., 2014):

$$CaL_2 \rightleftharpoons CaL^+ + L^-$$

Equation 4-2

$$CaL^+ \rightleftharpoons Ca^{2+} + L^-$$

Equation 4-3

where CaL_2 is the calcium salt,

L^- is the lactate, gluconate or lactobionate group

and CaL^+ is the intermediate complex of each of the anionic groups with Ca^{2+}

The association constant values, which indicate the affinity of the anion for the Ca^{2+}, was the lowest for calcium chloride (3.98) (Johnson & Pytkowicz, 1978), followed by

calcium lactate (40 ± 3), calcium gluconate (66 ± 1), and the highest for calcium lactobionate (120 ± 2) (Vavrusova & Skibsted, 2014). Therefore, calcium lactobionate releases the least Ca^{2+} ions and calcium chloride releases the most Ca^{2+} ions at the same molar concentration of added salt. As a_{Ca}^{2+} is related to the concentration of Ca^{2+} ions in solution, calcium salts that released more Ca^{2+} would therefore exhibit higher calcium ion activities (a_{Ca}^{2+}).

The dissociation behaviour of calcium iodide was expected to be similar to that of calcium chloride, with complete dissociation into Ca^{2+} and I^- ions. However, the a_{Ca}^{2+} of calcium iodide was the lowest amongst the five calcium salts and did not increase even with addition up to 50.0 mmol L^{-1} of calcium iodide (Figure 4-1a). The results for the a_{Ca}^{2+} of calcium iodide may have been due to interference between iodide ions and the electrode membrane, which hindered the measurement of the true a_{Ca}^{2+} in the calcium iodide added milk samples. This postulation is related to the bond dissociation energy, for Ca-Cl, it is 409 ± 8.7 kJ mol^{-1}, while for Ca-I, it is 284.7 ± 8.4 kJ mol^{-1} (Luo, 2016-2017). The bond dissociation energy is also referred to as bond disruption energy, bond strength, or binding energy (Luo, 2016-2017). The lower bond strength for Ca-I suggests a lower affinity for I^- ions to Ca^{2+} ions compared to Cl^- ions. Thus, it is likely that calcium iodide dissociated into Ca^{2+} and I^- ions, but the released Ca^{2+} ions could not be detected by the calcium-selective electrode due to the interference from the I^- ions. Addition of calcium iodide to water also showed similar trend in the a_{Ca}^{2+} where a low a_{Ca}^{2+} was measured throughout the concentration of added calcium iodide studied (Table 4-2). Further, addition of potassium iodide to 20 mmol L^{-1} and 40 mmol L^{-1} of calcium chloride in water decreased the a_{Ca}^{2+}, indicating the interfering effects of I^- ions

on a_{Ca}^{2+} measurement (Table 4-2). Addition of potassium chloride to a calcium chloride in water solution did not significantly reduce the a_{Ca}^{2+} (Table 4-2), indicating that the reduction in a_{Ca}^{2+} on addition of potassium iodide was not due the K^+ ions.

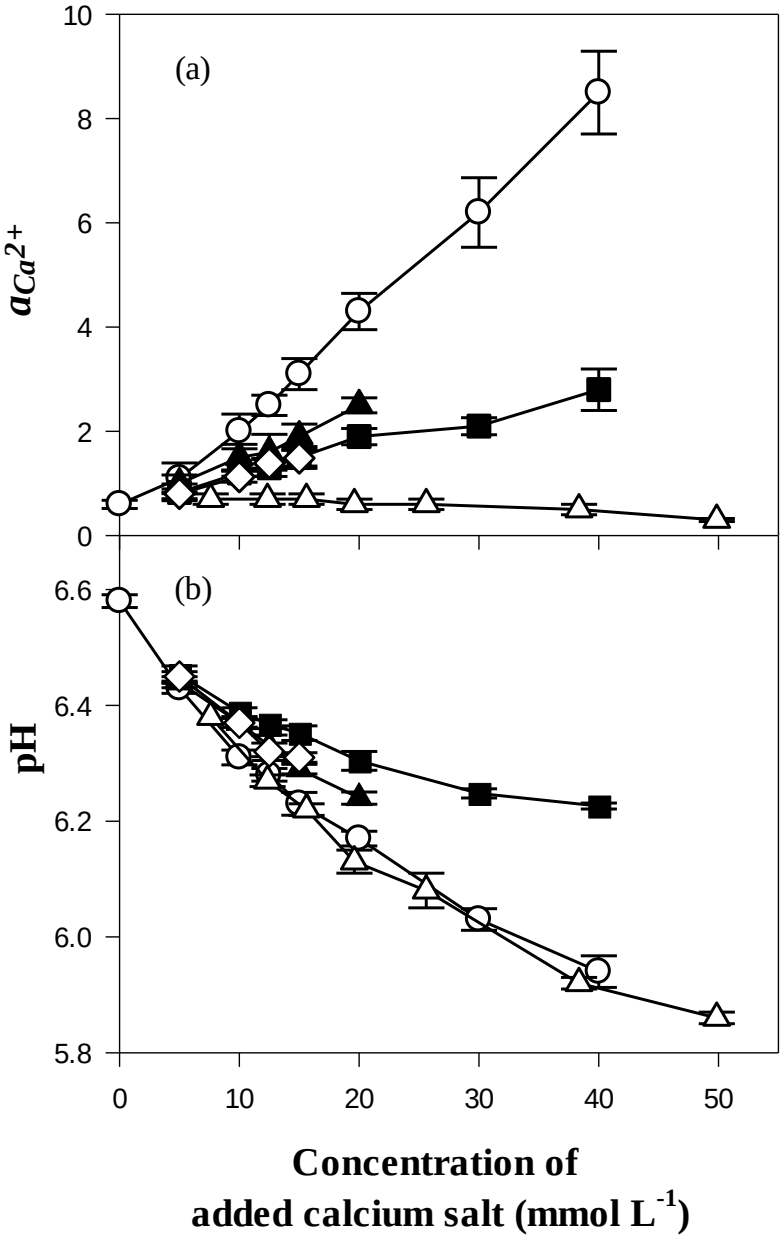


Figure 4-1: The effect of adding calcium chloride (○); calcium lactate (▲); calcium gluconate (◇), calcium lactobionate (■) and calcium iodide (△) to skim milk after preheat treatment at 90°C for 10 min on (a) $a_{Ca^{2+}}$ and (b) pH. Data points are mean values ± standard deviation (n= 9).

Table 4-2: The effect of adding calcium chloride ($CaCl_2$), calcium iodide (CaI_2), and mixtures of calcium chloride with potassium iodide (KI) or potassium chloride (KCl) at various concentrations on the $a_{Ca^{2+}}$ in water (n=6).

Concentration of added salt	$a_{Ca^{2+}}$
$CaCl_2$ (mmol L^{-1})	
5	2.7
10	4.9
15	7.1
20	8.8
30	12.1
40	14.9
CaI_2 (mmol L^{-1})	
5	1.3
10	1.1
15	0.9
20	0.7
30	0.5
40	0.4
20 mmol L^{-1} $CaCl_2$ + 20 mmol L^{-1} KI	2.0
20 mmol L^{-1} $CaCl_2$ + 40 mmol L^{-1} KI	0.8
40 mmol L^{-1} $CaCl_2$ + 20 mmol L^{-1} KI	2.7
40 mmol L^{-1} $CaCl_2$ + 40 mmol L^{-1} KI	1.2
20 mmol L^{-1} $CaCl_2$ + 20 mmol L^{-1} KCl	8.1
20 mmol L^{-1} $CaCl_2$ + 40 mmol L^{-1} KCl	7.8

Pooled standard deviation: ± 0.4

The addition of calcium salts resulted in a reduction in the skim milk pH after addition of all five salts (Figure 4-1b). The decrease in pH with the addition of calcium salt to milk has been reported previously (Crowley et al., 2014; Sievanen et al., 2008). The disruption of the equilibrium of the ionic species in milk causes the decrease in pH. Added calcium salt released calcium ions, some of which may form calcium citrate and calcium phosphate with the available citrate and phosphate ions in the serum phase. This in turn disrupts the equilibrium between the citrate ions ($C_6H_6O_7^{2-}$ and $C_6H_5O_7^{3-}$) and that between the phosphate ions (HPO_4^{2-} and $H_2PO_4^-$) in solution. An example of

the change in calcium phosphate equilibrium is shown in Equation 4-4 and Equation 4-5.

$$HPO_4^{2-} + Ca^{2+} \rightleftharpoons CaHPO_4$$

Equation 4-4

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$$

Equation 4-5

When calcium salts were added to skim milk, the increase in Ca^{2+} ions forms $CaHPO_4$ with the HPO_4^{2-} ions (Equation 4-4). The reduction in HPO_4^{2-} ions in turn leads to the conversion of $H_2PO_4^-$ to H^+ and HPO_4^{2-} to restore the equilibrium (Equation 4-5). The concomitant release of H^+ ions due to the shift in equilibrium results in the reduction of the pH of milk (Croguennec et al., 2016; Gaucheron, 2005). Therefore, calcium salts with higher a_{Ca}^{2+} will result in lower pH in milk.

While the a_{Ca}^{2+} of calcium iodide added to skim milk could not be confirmed due to the interference of I^- ions with the electrode, the pH of the skim milk with added calcium iodide was similar to that of the milk with added calcium chloride at the same molar concentration of calcium salt added (Figure 4-1b). The result suggests that calcium ions were released from the calcium iodide salts, which altered the calcium equilibrium and resulted in the decrease in the milk pH. This also confirms that there was interference with the I^- ions with the calcium ion electrode. The reason for the interference between the I^- ions and the calcium ion electrode is beyond the scope of this thesis to identify. The calcium ion electrode manufacturer was also unable to provide an explanation for the interference observed.

4.3.2 Protein composition in the skim milk serum

Milk serum samples were analysed by SDS-PAGE to determine if the addition of calcium salts affected the distribution of proteins between the serum and sediment phases. It was found that ~ 15% of the total casein and ~ 70% of the total β-lactoglobulin was in the serum phase of the preheated skim milk (with no calcium salt added), suggesting that ~ 30% of the β-lactoglobulin is in the sediment after centrifugation at 21, 500 *g*, which is in line with the values reported by other researchers for the natural pH of milk (Table 4-3) (Dalgleish & Corredig, 2012). A possibility for the sedimentation of β-lactoglobulin could be due to interactions with the casein micelles during preheat treatment, which in turn led to its sedimentation with the casein micelles on centrifugation (Dalgleish & Corredig, 2012).

Adding 5 mmol L^{-1} of any calcium salt to the skim milk resulted in a significant decrease ($p < 0.05$) in serum casein, however a further addition of calcium salt did not lead to a significant change in the amount of serum casein (Table 4-3). A reduction in serum casein was also reported by Le Ray et al. (1998) with the addition of calcium chloride to milk, and by Koutina et al. (2015), with the addition of calcium lactobionate to milk. A reduction in the intensity of the casein band in the serum samples was also observed on the SDS-PAGE gel when calcium salt was added to skim milk (Figure 4-2). Casein aggregation may occur through binding of calcium ions with the phosphate and carboxylate residues of casein molecules which reduces the intermolecular electrostatic repulsion, hence resulting in sedimentation after centrifugation (Holt, 1992; Swaisgood, 2003). The amount of β-lactoglobulin and α-lactalbumin in the milk serum did not change significantly ($p < 0.05$) with added calcium salts.

Table 4-3: Concentration of casein, β-lactoglobulin, α-lactalbumin per gram of skim milk or serum phase of skim milk at various concentrations of calcium salt added determined by SDS-PAGE. Results presented are means ± standard deviation (n = 2 to 4). For each type of protein, different superscript letters (a, b) indicate significant difference between the calcium concentrations for each type of added calcium salt (at 95% confidence level).

	Concentration of added calcium salt (mmol L^{-1})	**Casein (mg g^{-1})**	**β-lactoglobulin (mg g^{-1})**	**α-lactalbumin (mg g^{-1})**
Preheated skim milk (PSM)	0	27.0 ± 0.3	5.1 ± 0.2	1.3 ± 0.3
PSM serum	0	4.1 ± 0.5 [a]	3.6 ± 0.6 [a]	1.1 ± 0.2 [a]
PSM + calcium chloride serum	5	2.6 ± 0.1 [b]	3.5 ± 0.2 [a]	1.1 ± 0.1 [a]
	10	2.4 ± 0.1 [b]	3.2 ± 0.1 [a]	1.0 ± 0.1 [a]
	15	2.0 ± 0.1 [b]	3.1 ± 0.2 [a]	0.9 ± 0.1 [a]
	20	2.3 ± 0.2 [b]	3.2 ± 0.2 [a]	1.1 ± 0.1 [a]
	30	2.0 ± 0.2 [b]	3.0 ± 0.1 [a]	1.0 ± 0.1 [a]
	40	2.2 ± 0.1 [b]	3.0 ± 0.2 [a]	1.0 ± 0.1 [a]
PSM + calcium lactobionate serum	5	2.6 ± 0.3 [b]	3.1 ± 0.1 [a]	1.1 ± 0.2 [a]
	10	2.2 ± 0.1 [b]	3.2 ± 0.2 [a]	1.2 ± 0.2 [a]
	15	2.1 ± 0.1 [b]	3.0 ± 0.1 [a]	1.1 ± 0.1 [a]
	20	2.0 ± 0.1 [b]	3.3 ± 0.4 [a]	1.0 ± 0.1 [a]
	30	2.0 ± 0.2 [b]	3.1 ± 0.3 [a]	1.0 ± 0.1 [a]
	40	2.0 ± 0.1 [b]	3.3 ± 0.4 [a]	1.1 ± 0.1 [a]
PSM + calcium lactate serum	5	2.4 ± 0.6 [b]	3.3 ± 0.4 [a]	1.0 ± 0.2 [a]
	10	2.2 ± 0.1 [b]	3.4 ± 0.3 [a]	1.1 ± 0.1 [a]
	15	1.9 ± 0.2 [b]	3.1 ± 0.3 [a]	0.9 ± 0.1 [a]
	20	2.0 ± 0.2 [b]	3.1 ± 0.2 [a]	0.9 ± 0.1 [a]
PSM + calcium gluconate serum	5	2.5 ± 0.1 [b]	3.1 ± 0.2 [a]	0.9 ± 0.1 [a]
	10	2.5 ± 0.1 [b]	2.8 ± 0.1 [a]	0.7 ± 0.1 [a]
	15	2.2 ± 0.1 [b]	2.9 ± 0.1 [a]	0.8 ± 0.2 [a]
PSM + calcium iodide serum	7.5	2.5 ± 0.2 [b]	3.0 ± 0.1 [a]	0.7 ± 0.2 [a]
	12.5	2.2 ± 0.2 [b]	2.9 ± 0.1 [a]	0.8 ± 0.1 [a]
	19.5	2.2 ± 0.1 [b]	3.0 ± 0.3 [a]	0.9 ± 0.3 [a]
	25.5	2.2 ± 0.1 [b]	2.9 ± 0.1 [a]	0.8 ± 0.2 [a]
	38.5	1.7 ± 0.1 [b]	2.1 ± 0.3 [a]	0.4 ± 0.3 [a]
	50.0	2.2 ± 0.3 [b]	2.4 ± 0.1 [a]	0.8 ± 0.1 [a]

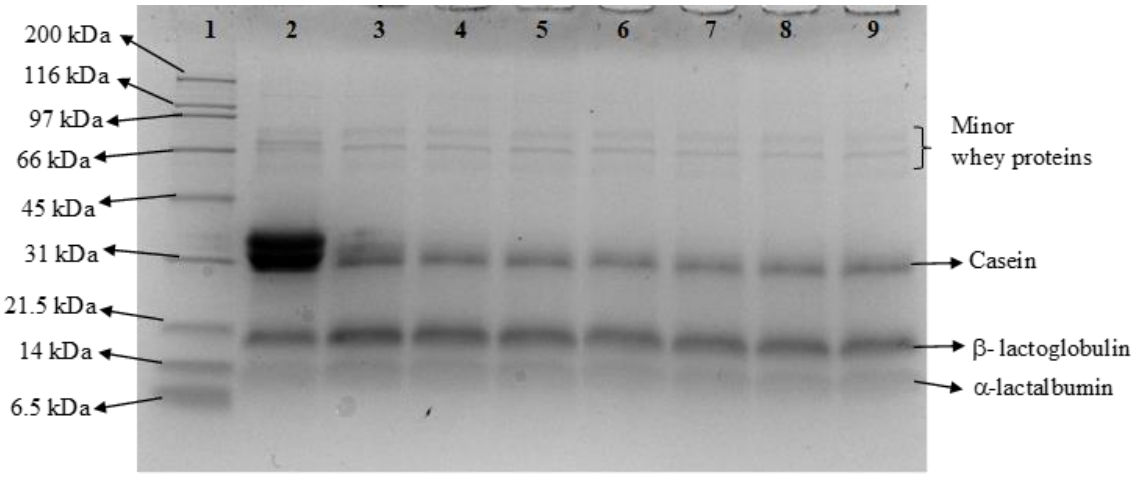


Figure 4-2: SDS-PAGE gel pattern of preheated skim milk (PSM) and serum phase of PSM milk with added calcium chloride. Lane 1: Marker; Lane 2: PSM; Lane 3: Serum of PSM; Lane 4: Serum of PSM + 5 mmol L^{-1} calcium chloride; Lane 5: PSM + 10 mmol L^{-1} calcium chloride; Lane 6: PSM + 15 mmol L^{-1} calcium chloride; Lane 7: PSM + 20 mmol L^{-1} calcium chloride; Lane 8: PSM + 30 mmol L^{-1} calcium chloride; Lane 9: PSM + 5 mmol L^{-1} calcium chloride.

4.3.3 Quantification of calcium in the sediment and serum phases of skim milk

The quantification of calcium in the serum phase of the calcium-added skim milk after centrifugation at 21,500 *g* was carried out to provide insights into how the calcium equilibrium in the milk was influenced by addition of the different calcium salts. The mass of calcium in the sediment was determined by mass balance.

Both serum and sediment calcium increased significantly ($p < 0.05$) as the amount of added calcium was increased from 5 to 40 mmol L^{-1} (Table 4-4) for all five calcium salts. The results revealed the dynamic nature of calcium equilibrium in milk. It was noted when 5 mmol L^{-1} of any calcium salt was added, the increase in mass of calcium in the sediment was higher than in the serum. However, further addition of calcium salt resulted in smaller increases in mass of calcium in the sediment than in the serum. For instance, when 5 mmol L^{-1} of calcium chloride (equivalent to 0.2 mg g^{-1} calcium) was added to milk, the ratio of serum to sediment calcium was 0.38. In contrast, when 40

mmol L^{-1} of calcium chloride (equivalent to 1.6 mg g^{-1} of calcium) was added, the ratio of serum to sediment increased to 1.09, indicating a higher amount of added calcium remaining in the serum phase at higher concentrations of added calcium chloride. The findings that the majority of added calcium is not ending up in the sediment with increasing calcium salt added were previously reported by other authors (Sievanen et al., 2008; Udabage et al., 2000). As milk is supersaturated with respect to calcium phosphate (Walstra et al., 1999), an increase in calcium ions through the addition of calcium salts will result in the ion activity product of calcium phosphate exceeding its solubility product, leading to the precipitation of calcium phosphates. Philippe et al. (2003) reported that on increasing addition of calcium chloride, the concentration of calcium in the serum increased while the concentration of phosphates in the serum decreased. This may also be occurring in the calcium-added skim milk in this study as increasing concentration of calcium in the serum may have led to more precipitation of calcium phosphate and hence a decrease in phosphates in the serum. The precipitation of serum calcium phosphate into the sediment would then allow the serum to accommodate more calcium ions before the ion activity product exceeds the solubility product. This may explain why at higher concentrations of calcium salt addition, the amount of added calcium remaining in the serum phase was much greater relative to the increase in the sediment. The solubility product behaviour may also be the reason why the addition of calcium salts that release less calcium ions, such as calcium lactobionate, resulted in lower sediment calcium as there are less available calcium ions to exceed the solubility product and result in precipitation. It should be noted that addition of calcium iodide to skim milk displayed the same trend as the other calcium salts, where increase in calcium salt added increased both serum and sediment calcium significantly (Table

4-3). However, statistical comparisons between calcium iodide and the other salts was not possible due to the difference in the actual concentrations of the calcium salt added.

The increased amount of calcium in the sediment as concentration of added calcium salt increased may be a result of the interactions between Ca^{2+} and milk proteins found in the sediment (Table 4-4). Studies have suggested that Ca^{2+} may bind to the negatively charged surface of the casein micelle (Philippe et al., 2003), or by forming calcium bridges between the phosphate and carboxyl groups of casein (Bringe & Kinsella, 1993; McMahon & Brown, 1984). It is also suggested that adding calcium salts to milk results in the formation of calcium phosphates and calcium citrates in the serum phase, which shifts the calcium equilibrium towards the calcium salts entering the casein micelles (Croguennec et al., 2016; Gaucheron, 2005).

Table 4-4: Concentration of calcium (mg g^{-1}) in skim milk, serum and sediment of skim milk at various concentrations of added calcium salts (n = 3 to 6). For each type of sample (serum or sediment), different superscript letters (a, b, c, d, e, f, g) indicate significant difference across the added calcium concentrations for each type of calcium salt. For each type of sample, (serum or sediment), different superscript Greek letters (χ, γ) indicate significant difference across the different types of calcium salt at each calcium concentration (95% confidence level).

	Concentration of added calcium salt (mmol L^{-1})	Mass of calcium added (mg)	Total calcium in skim milk^{+} (mg g^{-1})	Serum calcium^{+} (mg g^{-1})	Sediment calcium* (mg g^{-1})	Ratio of serum to sediment calcium
Preheated skim milk (PSM)	0	0	1.18	0.30^{a}	0.88^{a}	0.38
PSM + calcium chloride	5	0.20	1.38	$0.40^{b, \chi}$	$1.00^{b, \chi}$	0.38
	10	0.40	1.60	$0.52^{c, \chi}$	$1.08^{c, \chi\gamma}$	0.48
	15	0.60	1.78	$0.66^{d, \chi}$	$1.12^{c, \chi\gamma}$	0.59
	20	0.80	2.00	$0.82^{e, \chi}$	$1.18^{d, \chi}$	0.69
	30	1.20	2.38	$1.14^{f, \chi}$	$1.24^{e, \chi}$	0.92
	40	1.60	2.76	$1.44^{g, \chi}$	$1.32^{f, \chi}$	1.09
PSM + calcium lactobionate	5	0.20	1.38	$0.36^{b, \gamma}$	$1.00^{b, \chi}$	0.38
	10	0.40	1.58	$0.52^{c, \chi}$	$1.06^{c, \chi\gamma}$	0.49
	15	0.60	1.76	$0.66^{d, \chi}$	$1.10^{c, d, \chi}$	0.60
	20	0.80	1.98	$0.84^{e, \chi}$	$1.14^{d, \gamma}$	0.74
	30	1.20	2.38	$1.18^{f, \chi}$	$1.20^{e, \gamma}$	0.98
	40	1.60	2.72	$1.50^{g, \chi}$	$1.22^{e, \gamma}$	1.23
PSM + calcium lactate	5	0.20	1.40	$0.38^{b, \chi\gamma}$	$1.02^{b, \gamma}$	0.37
	10	0.40	1.58	$0.53^{c, \chi}$	$1.05^{c, \chi}$	0.50
	15	0.60	1.78	$0.68^{d, \chi}$	$1.10^{d, \chi}$	0.62
	20	0.80	2.02	$0.86^{e, \chi}$	$1.16^{e, \chi\gamma}$	0.74
PSM + calcium gluconate	5	0.20	1.40	$0.38^{b, \chi\gamma}$	$1.02^{b, \gamma}$	0.37
	10	0.40	1.60	$0.52^{c, \chi}$	$1.09^{c, \gamma}$	0.48
	15	0.60	1.80	$0.66^{d, \chi}$	$1.14^{d, \gamma}$	0.58
PSM + calcium iodide	6.2	0.26	1.48	0.40^{b}	1.08^{b}	0.37
	12.4	0.50	1.68	0.55^{c}	1.13^{c}	0.49
	18.6	0.74	1.96	0.78^{d}	1.18^{d}	0.66
	24.8	1.00	2.20	0.94^{e}	1.26^{e}	0.75
	37.2	1.48	2.70	1.32^{f}	1.38^{f}	0.96
	49.6	1.98	3.18	1.74^{g}	1.44^{g}	1.21

Pooled standard deviation: ± 0.02 mg g^{-1}

$^{+}$ Concentration determined by EDTA titration

* Concentration determined by difference between milk and serum calcium

4.3.4 Particle size and zeta-potential analysis in skim milk

The particle size analysis was carried out to determine the effect of adding various calcium salts on the size of the particles in the skim milk. The z-average, defined as the harmonic intensity averaged particle diameter, is a value that describes the mean diameter of the particles in a dispersion or solution derived by intensity-based calculations (Malvern Instruments, 2011). Horne (2003) reported that the diameter of casein micelles ranged from 50 to 600 nm, with an average diameter of approximately 200 nm. The z-average of skim milk with no calcium added was found to be 240.5 ± 0.5 nm in this study. As the particle size distribution of the skim milk was found to be monomodal and within the range of the particle sizes of casein micelles (Figure 4-3), the particles analysed were likely to be the casein micelles in the skim milk. The larger particle size in the present study compared to those previously reported (Crowley et al., 2014; Holt, 1992; Huppertz et al., 2017) could be due to the preheat treatment applied in this study. Anema & Li (2003) measured the change in the size of casein micelles on heating skim milk to 90°C for up to 45 min and found an overall increase in 30 to 35 nm in the casein micelle size. The complexation of whey proteins with casein micelles (Dalgleish & Corredig, 2012) was likely the cause for the larger casein micelle size in the preheated skim milk (Anema & Li, 2003).

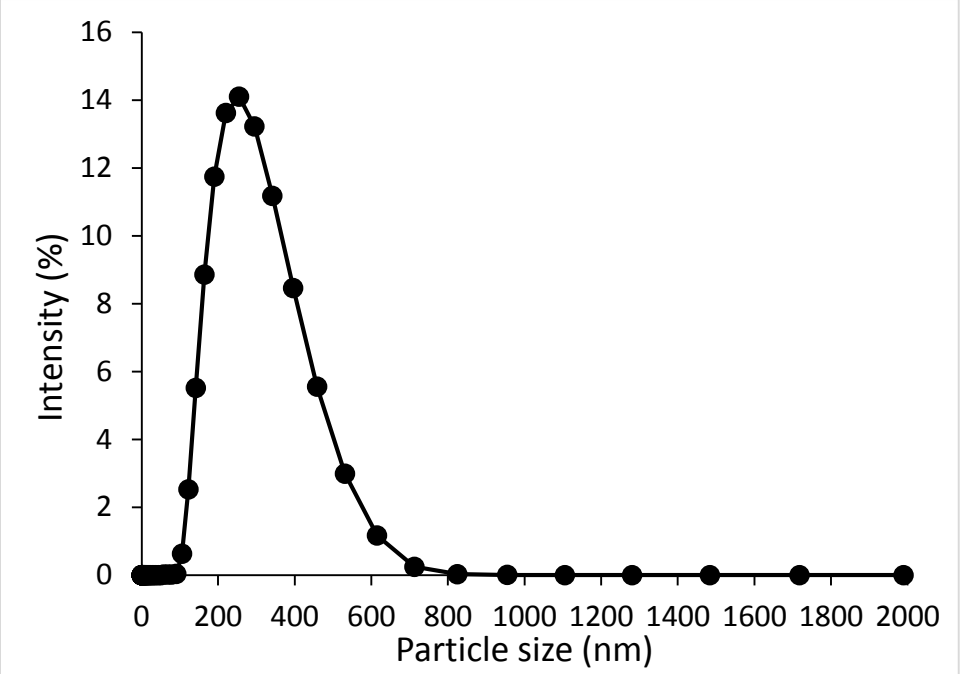


Figure 4-3: Typical plot of intensity-weighted particle size distribution of skim milk with no calcium added.

A significant decrease in the particle size was observed when calcium chloride was added at a concentration of 5 mmol L^{-1} ($p < 0.05$), although no significant difference in particle size was observed on further increase in added calcium chloride from 5 to 40 mmol L^{-1} (Table 4-5). Crowley et al. (2014) reported that adding 12.5 mmol L^{-1} of calcium chloride did not significantly change the size of casein micelles. However, Crowley et al. (2014) adjusted the pH of milk to 6.8 to compensate for the decrease in pH due to calcium chloride addition. The pH was not adjusted in this study which may explain the difference in the observations. Further, as preheat treatment was applied in this study, the complexation of β-lactoglobulin with casein micelles in preheated milks (Dalgleish & Corredig, 2012) could have also affected the interactions between calcium and the casein micelles, leading to differences in the particle size observed in this study and those previously looked at by Crowley et al. (2014).

Table 4-5: Particle size expressed as z-average and zeta-potential of casein micelles at various concentrations of added calcium salt. Results presented are mean ± standard deviation (n = 6 to 9). Different letters (a, b, c, d, e, f) indicate significant difference across the added calcium concentrations for each type of calcium salt. Different superscript Greek letters (χ, γ) indicate significant difference across the different types of calcium salt at each calcium concentration (95% confidence level).

	Calcium concentration added (mmol L^{-1})	**z-average of casein micelles (nm)**	**Zeta-potential of casein micelles (mV)**
Preheated skim milk (PSM)	0	240.4 ± 3.5 ^{a}	-13.25 ± 0.37 ^{a}
PSM + calcium chloride	5	236.2 ± 2.7 $^{b, \chi}$	-11.23 ± 0.98 $^{b, \chi}$
	10	235.8 ± 1.5 $^{b, \chi}$	-10.00 ± 0.93 $^{c, \chi}$
	15	235.8 ± 1.5 $^{b, \chi}$	-8.61 ± 0.58 $^{d, \chi}$
	20	233.6 ± 2.1 $^{b, \chi}$	-8.00 ± 0.80 $^{de, \chi}$
	30	234.5 ± 3.3 $^{b, \chi}$	-7.15 ± 0.76 $^{e, \chi}$
	40	235.5 ± 3.0 $^{b, \chi}$	-6.27 ± 0.57 $^{e, \chi}$
PSM + calcium lactobionate	5	241.8 ± 1.6 $^{ab, \gamma}$	-11.46 ± 0.79 $^{b, \chi}$
	10	240.7 ± 1.9 $^{ab, \gamma}$	-10.86 ± 0.76 $^{bc, \chi}$
	15	243.9 ± 3.1 $^{abc, \gamma}$	-10.03 ± 0.55 $^{cd, \chi}$
	20	244.9 ± 3.8 $^{bc, \gamma}$	-9.77 ± 1.49 $^{cd, \gamma}$
	30	245.0 ± 3.2 $^{bc, \gamma}$	-9.17 ± 0.54 $^{d, \gamma}$
	40	247.0 ± 1.8 $^{c, \gamma}$	-7.62 ± 0.88 $^{e, \gamma}$
PSM + calcium lactate	5	235.6 ± 2.7 $^{b, \chi}$	-12.08 ± 1.81 $^{a, \chi}$
	10	236.0 ± 2.4 $^{b, \chi\gamma}$	-10.09 ± 0.91 $^{b, \chi}$
	15	239.0 ± 0.8 $^{ab, \chi}$	-9.78 ± 1.31b $^{c, \chi}$
	20	237.8 ± 1.8 $^{ab, \chi}$	-8.67 ± 0.90 $^{c, \chi\gamma}$
PSM + calcium gluconate	5	239.9 ± 1.6 $^{a, \gamma}$	-12.05 ± 0.72 $^{ab, \chi}$
	10	238.6 ± 4.7 $^{a, \chi\gamma}$	-11.02 ± 0.94 $^{b, \chi}$
	15	240.9 ± 3.9 $^{a, \chi\gamma}$	-9.62 ± 1.26 $^{c, \chi}$
PSM + calcium iodide	7.5	230.1 ± 2.0 ^{b}	-11.03 ± 0.70 ^{b}
	12.5	226.1 ± 4.9 ^{b}	-9.78 ± 0.55 ^{c}
	19.5	227.0 ± 2.7 ^{b}	-8.59 ± 0.40 ^{d}
	25.5	227.3 ± 3.0 ^{b}	-8.03 ± 0.60 ^{de}
	38.5	228.1 ± 2.6 ^{b}	-7.51 ± 0.88 ^{e}
	50	230.7 ± 2.0 ^{b}	-6.05 ± 0.61 ^{f}

Addition of calcium lactobionate increased the particle size of the casein micelles, although significant increase in particle size was only observed at > 20 mmol L^{-1} (Table 4-5). At lower concentrations of added calcium salt (5 to 20 mmol L^{-1}), the particle sizes of the casein micelles in skim milk with added calcium lactobionate and calcium gluconate were significantly larger than calcium chloride and calcium lactate-added skim milk. It appears that calcium salts which resulted in higher a_{Ca}^{2+} may lead to smaller particle size. Addition of calcium increases the CCP which facilitates crosslinking of the casein molecules within the casein micelles, possibly reducing electrostatic repulsion between the casein molecules (Horne, 2002; van Boekel et al., 1989). Beliciu & Moraru (2009) reported an increase in casein micelle particle size when CCP dissolved into the serum phase, and attributed it to the micelle structure becoming looser and more porous. As CCP is likely to increase with the addition of calcium salts to the skim milk, the opposite to what was observed by Beliciu & Moraru (2009) could be happening to the calcium-added skim milk in this study. Native casein micelles have an open and porous internal structure. The increase in crosslinking within the casein micelle may result in a more compact structure, and hence decreasing the overall particle size. A possibility for the increase in particle size on addition of calcium lactobionate and calcium gluconate could be the presence of the intermediate cation complexes, CaL^+. The positively-charged CaL^+ may be attracted to the negatively-charged sites on the casein micelles surface. The hydroxycarboxylate groups of calcium lactobionate and calcium gluconate are higher in molecular weight than Cl^- ions (Table 4-1). Hence, attachment of these groups on casein micelles may contribute to the overall size of the casein micelles.

As mentioned in Section 4.3.3, statistical comparison of calcium iodide with the other calcium salts could not be carried out due to differences in the actual concentration of

calcium salt added. However, the overall trend shows that the particle size of calcium iodide was lower than the particle size of the other four calcium salts. It appeared that the presence of I^- may result in a lower casein micelle size compared to Cl^- ions or the hydroxycarboxylate groups. According to the Hofmeister series, I^- ions are less efficient in inducing protein-protein aggregation than Cl^- ions (Kunz & Neueder, 2010). As such, skim milk with added calcium iodide may be less likely to associate with other casein in the serum, thus leading to the smaller particle size observed in this study.

The zeta-potential of the skim milk decreased in negativity with increase in concentration of all five calcium salts (Table 4-5). This was likely due to the charge neutralisation effect of the positively charged ions, Ca^{2+} and H^+, on calcium salt addition (Philippe et al., 2003). In agreement with the results of calcium ion activity and pH, calcium chloride, which had the highest calcium ion activity and lowest pH, had the strongest effect on the decrease in negativity of the zeta-potential (Table 4-5). Conversely, calcium lactobionate, which had the lowest calcium ion activity and highest pH, had the weakest effect.

4.3.5 Rheological properties

The changes in G' of the calcium-added skim milk while heating the skim milk samples from 20°C to 70°C (rheometer set temperature) is shown in Figure 4-4. The gelation point was arbitrarily defined as the point when the G' reaches ≥ 1 Pa. Gelation during heating up was only observed at higher concentrations of calcium salt added; with 30 and 40 mmol L^{-1} of added calcium chloride or calcium iodide (Figure 4-4). Development of the gel network in skim milk samples with added calcium salts occurred mainly during the holding phase (60 min) regardless of the type of salt added as seen by the increase in G' in Figure 4-5. The G' increased rapidly for the first 20 min during holding, indicating the formation of a gel network. After 20 min, the G' began to

plateau. As the samples were cooled from 70°C to 20°C (rheometer set temperature), the G' values increased approximately two times (Figure 4-6). Bikker, Anema, Li & Hill (2000) reported similar increase in G' during cooling of acid milk gels, where the G' values at 5°C were approximately twice that at 30°C. Lucey (2009) attributed the increase in G' on cooling of acid-induced milk gels to the swelling of the casein particles resulting from weaker hydrophobic interactions as temperature decreases. Similarly, van Vliet, Roefs, Zoon & Walstra (1989) also reported an increase in G' during cooling of rennet gels and attributed this to the decreased hydrophobic interactions which resulted in decreased intramolecular bonds. This increased the size of the casein particles, resulting in increased intermolecular bonds, leading to increased G'. This could be one of the causes for the observed increase in G' of the calcium-induced skim milk gels on cooling.

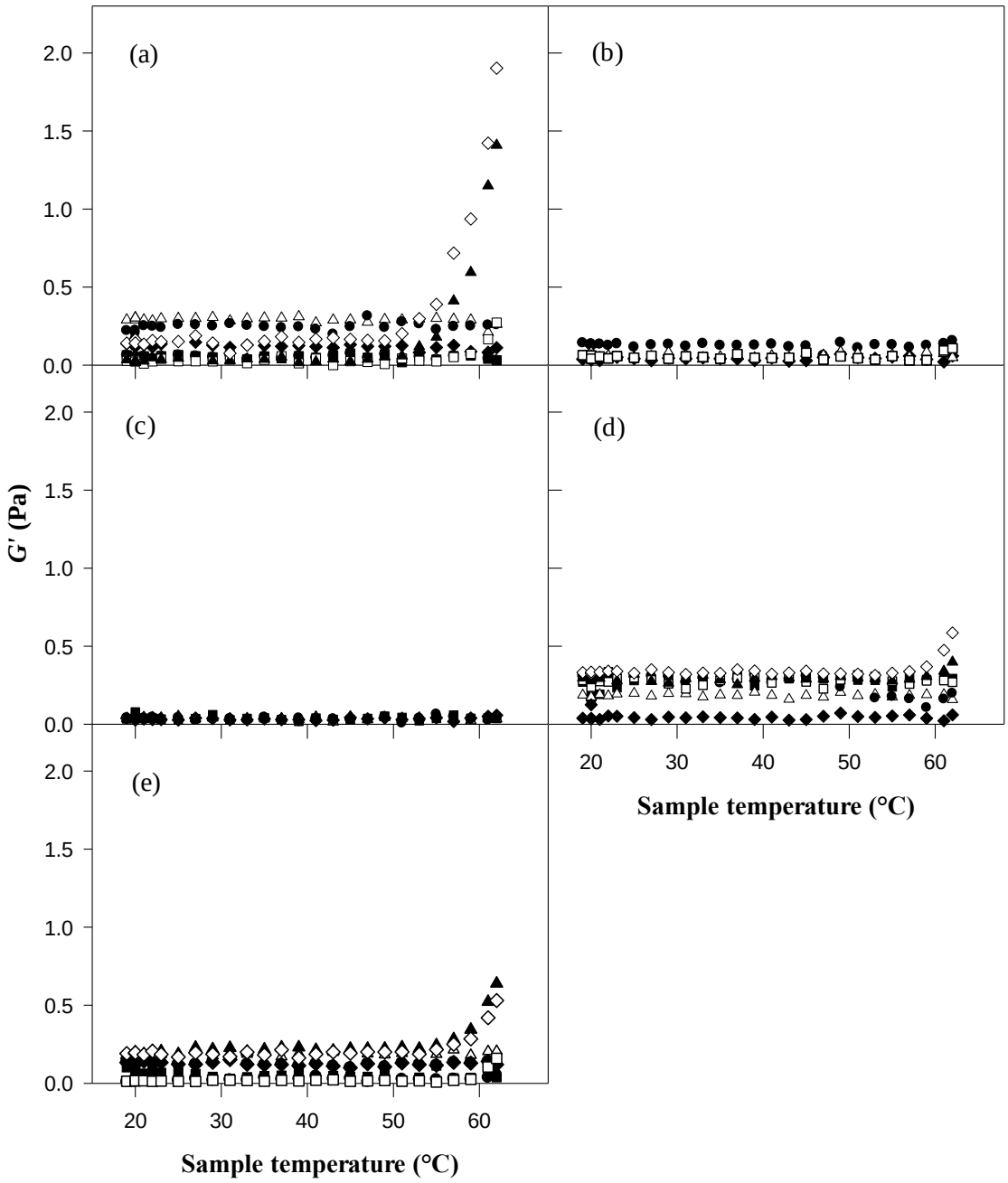


Figure 4-4: Typical plots showing the change in G' during heating with rheometer set temperature from 20°C to 70°C for skim milk with added (a) calcium chloride; (b) calcium lactate; (c) calcium gluconate; (d) calcium lactobionate and (e) calcium iodide: concentration of added calcium salt was 0 mmol L^{-1} (○); 5 mmol L^{-1} (■); 10 mmol L^{-1} (△); 12.5 mmol L^{-1} (◆); 15 mmol L^{-1} (●); 20 mmol L^{-1} (□); 30 mmol L^{-1} (▲) and 40 mmol L^{-1} (◇).

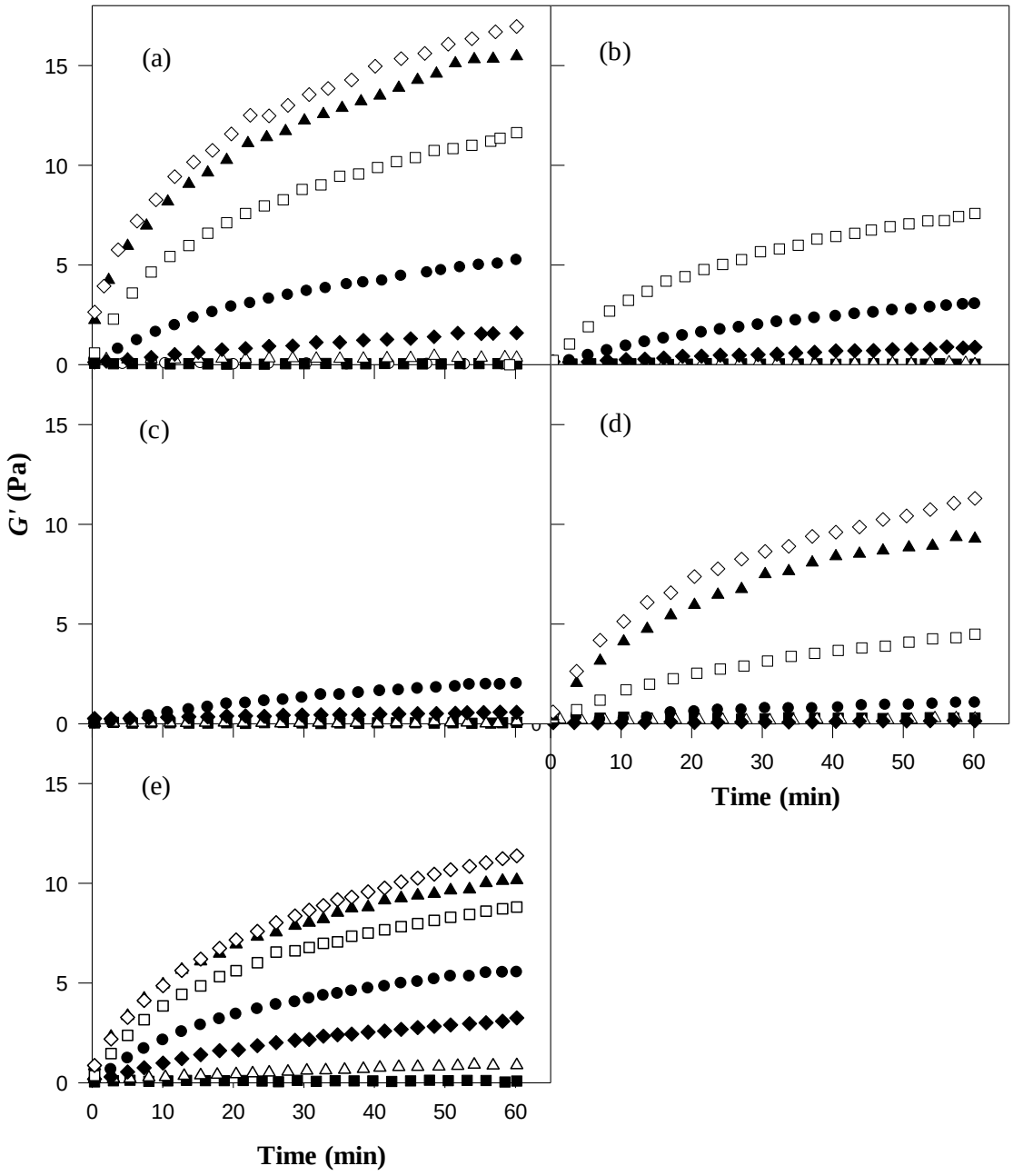


Figure 4-5: Typical plots showing the change in G' during holding with sample temperature at rheometer set temperature of 70°C 60 minutes for skim milk with added (a) calcium chloride; (b) calcium lactate; (c) calcium gluconate; (d) calcium lactobionate and (e) calcium iodide: concentration of added calcium salt was 0 mmol L^{-1} (○); 5 mmol L^{-1} (■); 10 mmol L^{-1} (△); 12.5 mmol L^{-1} (◆); 15 mmol L^{-1} (●); 20 mmol L^{-1} (□); 30 mmol L^{-1} (▲) and 40 mmol L^{-1} (◇).

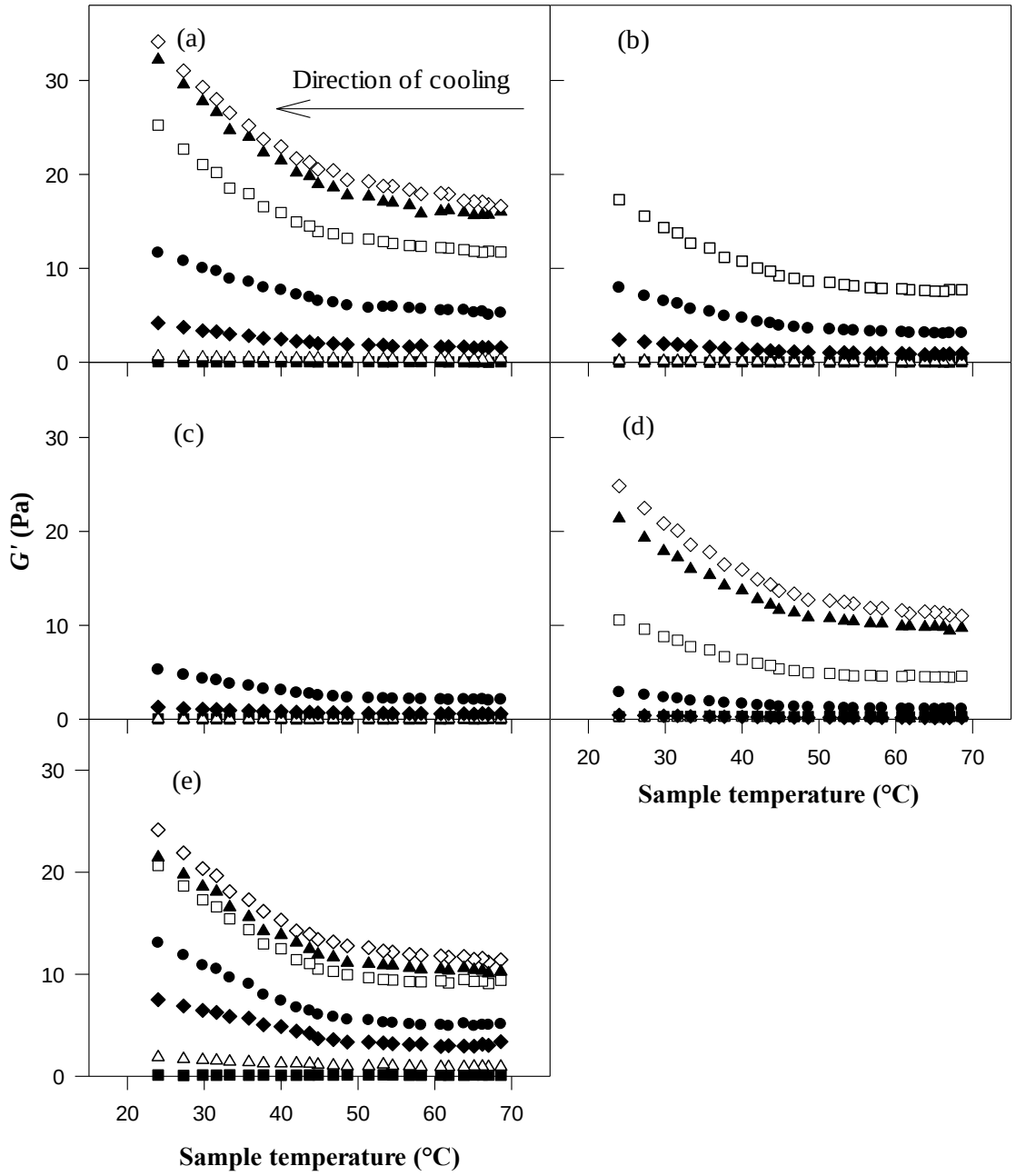


Figure 4-6: Typical plots showing the change in G' during cooling with rheometer set temperature from 70°C to 20°C for skim milk with added (a) calcium chloride; (b) calcium lactate; (c) calcium gluconate; (d) calcium lactobionate and (e) calcium iodide: concentration of added calcium salt was 0 mmol L^{-1} (○); 5 mmol L^{-1} (■); 10 mmol L^{-1} (△); 12.5 mmol L^{-1} (◆); 15 mmol L^{-1} (●); 20 mmol L^{-1} (□); 30 mmol L^{-1} (▲) and 40 mmol L^{-1} (◇).

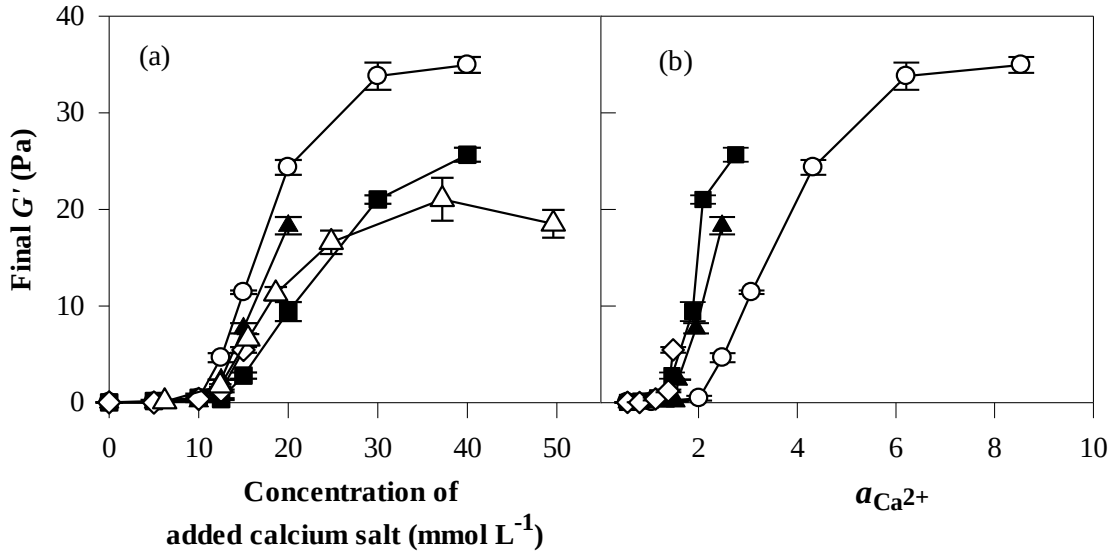


Figure 4-7: Relationship between final G' of calcium-induced skim milk gels after cooling to rheometer set temperature at 20°C, as a function of (a) concentration of added calcium salt and (b) calcium ion activity ($a_{Ca^{2+}}$) in skim milk with calcium chloride (○); calcium lactate (▲); calcium gluconate (◇) and calcium lactobionate (■); and calcium iodide (△). Data points are mean values ± standard deviation (n= 3).

The final G' of the gels after cooling to 20°C increased with increasing concentration of added calcium salt (Figure 4-7a). For skim milk with added calcium chloride, calcium iodide, calcium lactate and calcium gluconate, gelation occurred after the addition of 12.5 mmol L^{-1} of calcium salt, while addition of 15 mmol L^{-1} of calcium lactobionate to skim milk was required for gelation to occur (Figure 4-7a). Between 15 to 20 mmol L^{-1} of added calcium salt, the final G' was the highest with added calcium chloride, followed by calcium lactate, calcium iodide, calcium gluconate and calcium lactobionate. However, on increasing calcium iodide addition from 25 to 50 mmol L^{-1}, the final G' started to plateau such that the final G' values of the calcium iodide samples were lower than calcium lactobionate after 30 mmol L^{-1} calcium salt added (Figure 4-7a). In contrast, when the final G' is considered as a function of $a_{Ca^{2+}}$ (Figure 4-7b), skim milk samples with added calcium gluconate had the highest final G' at $a_{Ca^{2+}} < 2$, followed by calcium lactobionate, calcium lactate then calcium chloride. For instance,

when the a_{Ca}^{2+} in milk was approximately 2, addition of calcium lactobionate and calcium lactate produced gels with G' of 10 and 8 Pa, respectively. On the other hand, calcium chloride addition at the same a_{Ca}^{2+} did not induce gelation in the milk. As the a_{Ca}^{2+} was unable to be determined for calcium iodide-added skim milk, results with calcium iodide were not included in Figure 4-7b.

The results from the frequency sweep test showed that the viscoelastic properties of the calcium-induced skim milk gels varied depending on the type and concentration of added calcium salt (Figure 4-8). At lower frequencies (0.01 to 0.1 Hz), the storage modulus, G', of all gels was greater than the loss modulus, G'', indicating a more solid-like behaviour of the gels. However, the difference in the G' and G'' values was less than one log, indicating that the gels were probably held together by weak interactions (Ramasubramanian et al., 2014). For the gels with lower final G' values after cooling to 20°C, such as the samples with 12.5 mmol L^{-1} of added calcium salts, transitioning of a solid-like to liquid-like behaviour was observed at higher frequencies (0.1 to 10 Hz) as the G'' crosses over the G', possibly due to a loss of structure of the gel at higher frequencies (Figure 4-8). For samples with > 20 mmol L^{-1} of added calcium salts, the structure of the gel can be interpreted to be stronger than the samples with 12.5 to 15 mmol L^{-1} of added calcium salts as the G' values were higher throughout the range of frequencies studied, and the G', G'' values were almost independent of frequency (Rao, 2014). In essence, calcium-induced skim milk gels that achieved a higher final G' after cooling to 20°C would display the characteristics of a stronger gel in the frequency sweep test than the gels with a lower final G' after cooling to 20°C.

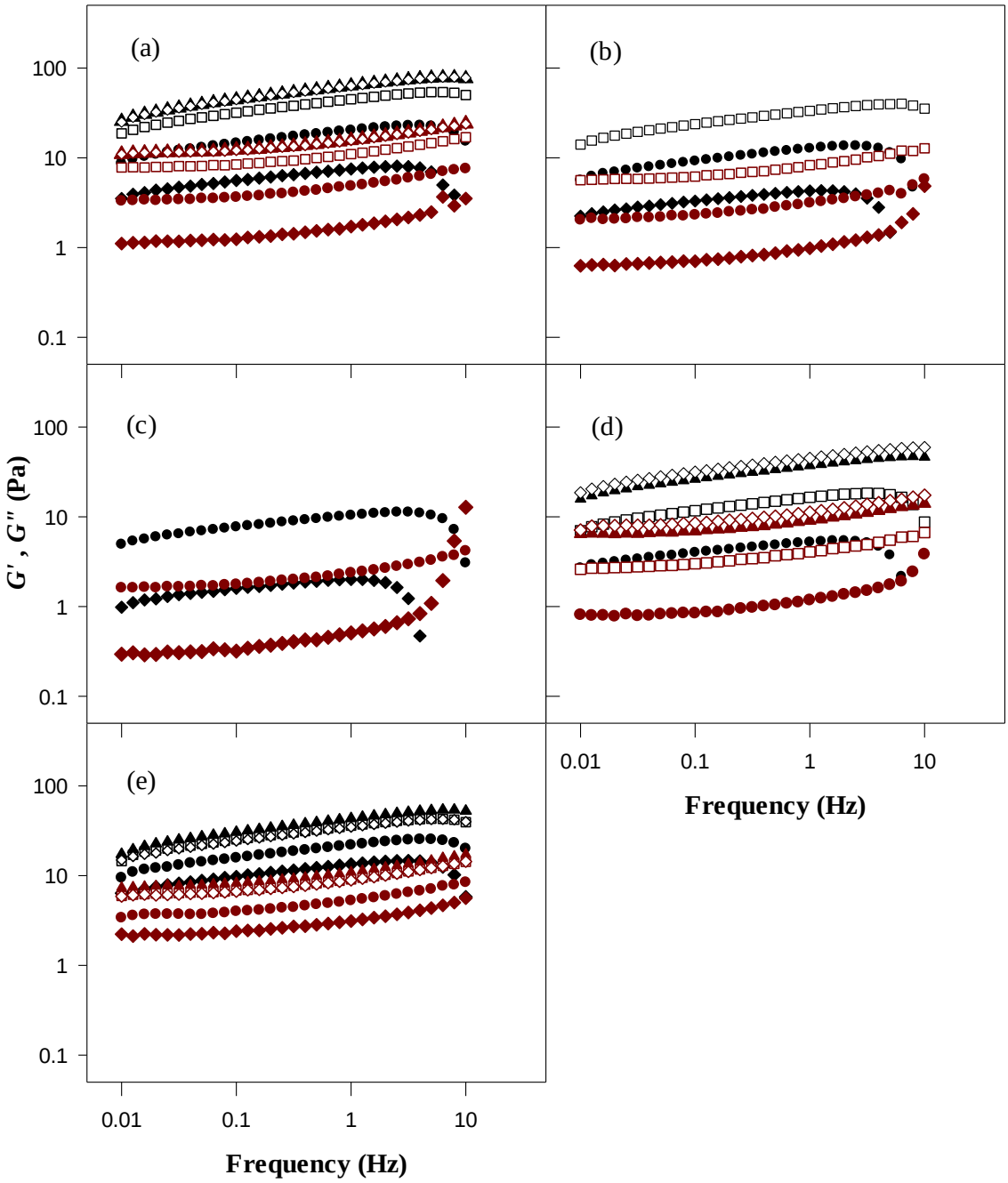


Figure 4-8: Typical plots showing the G' (black symbols) and G'' (red symbols) of the calcium-induced skim milk gels as a function of frequency with added (a) calcium chloride; (b) calcium lactate; (c) calcium gluconate; (d) calcium lactobionate and (e) calcium iodide: concentration of added calcium salt was 12.5 mmol L^{-1} (◆); 15 mmol L^{-1} (●); 20 mmol L^{-1} (□); 30 mmol L^{-1} (▲) and 40 mmol L^{-1} (◇).

Overall, the results suggest that at the same molar concentration of calcium salt added, calcium salts with higher a_{Ca}^{2+} and lower pH resulted in higher final G', e.g. skim milks with added calcium chloride (Figure 4-1, Figure 4-7a). However, although all calcium salts showed a similar sigmoidal shape when the final G' was presented with respect to the a_{Ca}^{2+} (Figure 4-7b), an increase in a_{Ca}^{2+} with calcium lactobionate led to a greater increase in final G' when compared to the same increase in a_{Ca}^{2+} with calcium chloride. This result shows that a_{Ca}^{2+} alone may not explain the differences in the gelation induced by the different calcium salts. Other ionic species in the skim milk may also influence the protein interactions. For example, positively-charged CaL^+ present in the skim milks with added calcium lactobionate, calcium lactate and calcium gluconate may also interact with the negatively-charged sites on the casein micelles (Figure 4-7b). The effect of CaL^+ on the calcium equilibrium in skim milk is also not known. As there are limited information available on CaL^+, it is unclear how the gelation of the milk proteins may be influenced in the presence of CaL^+. However, it was noted that for the organic calcium salts, the order of the final G' of the gels at the same concentration of added calcium salts was in the reverse order to the observed particle size. For example, calcium lactobionate, which had the largest particle size amongst the organic calcium salts, produced gels with the lowest in final G' (Table 4-5, Figure 4-7). It is therefore possible that interactions between CaL^+ and proteins may hinder protein aggregation and gelation due to steric hindrance.

Anions such as Cl^- or I^- may also influence the interactions between proteins by binding with the positively charged amino acid residues, such as lysine, arginine and histidine, or on the non-polar surfaces of the proteins (Bringe & Kinsella, 1991; Lund et al., 2010). Although Cl^- ions are higher in electronegativity and therefore have a stronger

affinity for the positively charged amino acid residues, the larger I^- ions have a higher affinity to non-polar regions on the proteins via hydrophobic interactions (Lund et al., 2010). Therefore, the overall affinity of I^- ions on the protein surface could be higher than Cl^- ions. As the pH of the skim milk in this study was above its isoelectric point, the net charge of the proteins in the skim milk would be negative (Table 4-5). A stronger attraction between the I^- ions with the negatively charged proteins would therefore increase the electrostatic repulsion between the proteins, resulting in reduced protein aggregation as predicted by the Hofmeister series (Kim, Bringe & Kinsella, 1990). This may be the cause for the lower final G' observed in the calcium iodide samples, especially at higher concentrations added (> 20 mmol L^{-1}) where there is a higher concentration of I^- ions available for binding. However, further work is needed to confirm if such interactions occurred and their contribution to the gel strength.

4.3.6 General Discussion

The results presented in this study provided some insights into the intricate relationships between calcium equilibrium, pH of milk, protein stability in milk and possible interactions that may lead to the gelation of skim milk. Although the reduction in zeta-potential of casein micelles as a result of the increased number of cations (Ca^{2+} and H^+) may have contributed to destabilisation of the milk proteins (Table 4-5), it is unlikely to be the sole cause of the observed gelation. Mellema, Leermakers & De Kruif (1999) suggested that calcium-mediated bridging could occur between two neighbouring casein micelles where divalent Ca^{2+} bridges phosphate or carboxylate groups of β- or α_{s1}-casein, or carboxylate groups of κ-casein. The concept of calcium-mediated bridging is in line with the effect of $a_{Ca}{}^{2+}$ and the effect of different calcium salts on the final G' values of milk gels presented in this study. The varying dissociation behaviour of the organic calcium salts resulted in different $a_{Ca}{}^{2+}$, which in turn produced calcium-

induced skim milk gels with different gel strengths. At the same concentration of calcium salt added, calcium chloride produced gels with higher final G' than calcium iodide, which follows the order of the Hofmeister series, where Cl^- ions are more effective than I^- in aggregating proteins. This was presumably due to stronger association between the I^- ions with the proteins on the non-polar regions of the proteins, thus preventing protein-protein interactions through electrostatic repulsion.

4.4 Conclusions

This study showed that addition of five different soluble calcium salts (calcium chloride, calcium lactate, calcium gluconate, calcium lactobionate, calcium iodide) resulted in varying degrees of change in the $a_{Ca^{2+}}$ and pH in milk. At the same concentration of calcium salt added, calcium salts which resulted in higher $a_{Ca^{2+}}$ resulted in gels with higher G'. The effect of the different added calcium salts on the calcium-induced gelation in skim milk was likely caused by the difference in the affinity of the anions (Cl^-, I^-, anionic hydroxycarboxylate groups) with calcium ions, the formation of an intermediate calcium complex, CaL^+, and interactions between these ions and the casein micelles. However, the involvement of these ions deserves further research to gain a better understanding of their contribution to calcium-induced gelation of milk.

Chapter 5 - Effect of temperature, pH and preheat treatment on calcium-induced skim milk gels

5.1 Introduction

The effect of temperature on the formation of the structure in dairy protein gels is well documented. Increasing the temperature of milk results in the reduction in milk pH (Chaplin & Lyster, 1988; Walstra et al., 1999), alterations to the calcium equilibrium (Lewis, 2011; Rose & Tessier, 1959; Sauer & Moraru, 2012) and the denaturation and aggregation of whey proteins (Loveday, 2016; Mulvihill & Kinsella, 1987). These changes lead to a reduction in stability of both casein and whey proteins in milk, which can alter the aggregation, viscosity and gel-forming properties of the milk (O'Connell & Fox, 2003; Singh, 2004). In whey protein and acid- or rennet-induced milk gels, increasing the temperature of the milk during the formation of the gel structure was reported to result in higher gel strength (Harwalkar & Kalab, 1980; McClements & Keogh, 1995). Ramasubramanian (2013) investigated the effect of holding calcium-added milk on its rheological properties up to 70°C, but the effect of holding temperatures > 70°C has yet to be explored.

Preheat treatment (also known as forewarming), typically conducted at 90°C for 2 to 10 min, is commonly applied in the production of yogurts to alter the texture and consistency (Lucey, Teo, Munro & Singh, 1997; O'Connell & Fox, 2003). Compared to non-preheated milk, preheat treatment results in an increased gel strength (G') in acid-milk gels (Donato et al., 2007; Lakemond & van Vliet, 2008) but leads to a decrease in the gel strength in rennet-induced milk gels (Anema, Lee & Klostermeyer, 2011) when compared to non-preheated milk. The influence of preheat treatment on gel strength of the milk gels was reported to be due to the denaturation of whey proteins and their interactions with casein (Anema et al., 2004b; Donato et al., 2007).

The pH of milk at preheating influences the interactions between casein and whey proteins and the rheological properties of milk gels (Anema & Klostermeyer, 1997; Dalgleish & Corredig, 2012; Donato & Dalgleish, 2006). Preheating milk at lower pH (pH < 6.5) was found to result in acid-induced milk gels with lower final *G'* than milk preheated at higher pH (pH > 6.7) (Lakemond & van Vliet, 2008; Vasbinder & De Kruif, 2003). The studies on the effect of preheating pH suggests that the interactions between whey proteins and caseins during preheat treatment determines the conformation of the proteins and thus the formation of gel structure. This chapter explores the effect of temperature and pH during gelation; preheat treatment, and preheating pH on the calcium-induced skim milk gel.

5.2 Materials and methods

The experimental plan and treatments applied to the skim milk in this chapter are summarised in Figure 5-1.

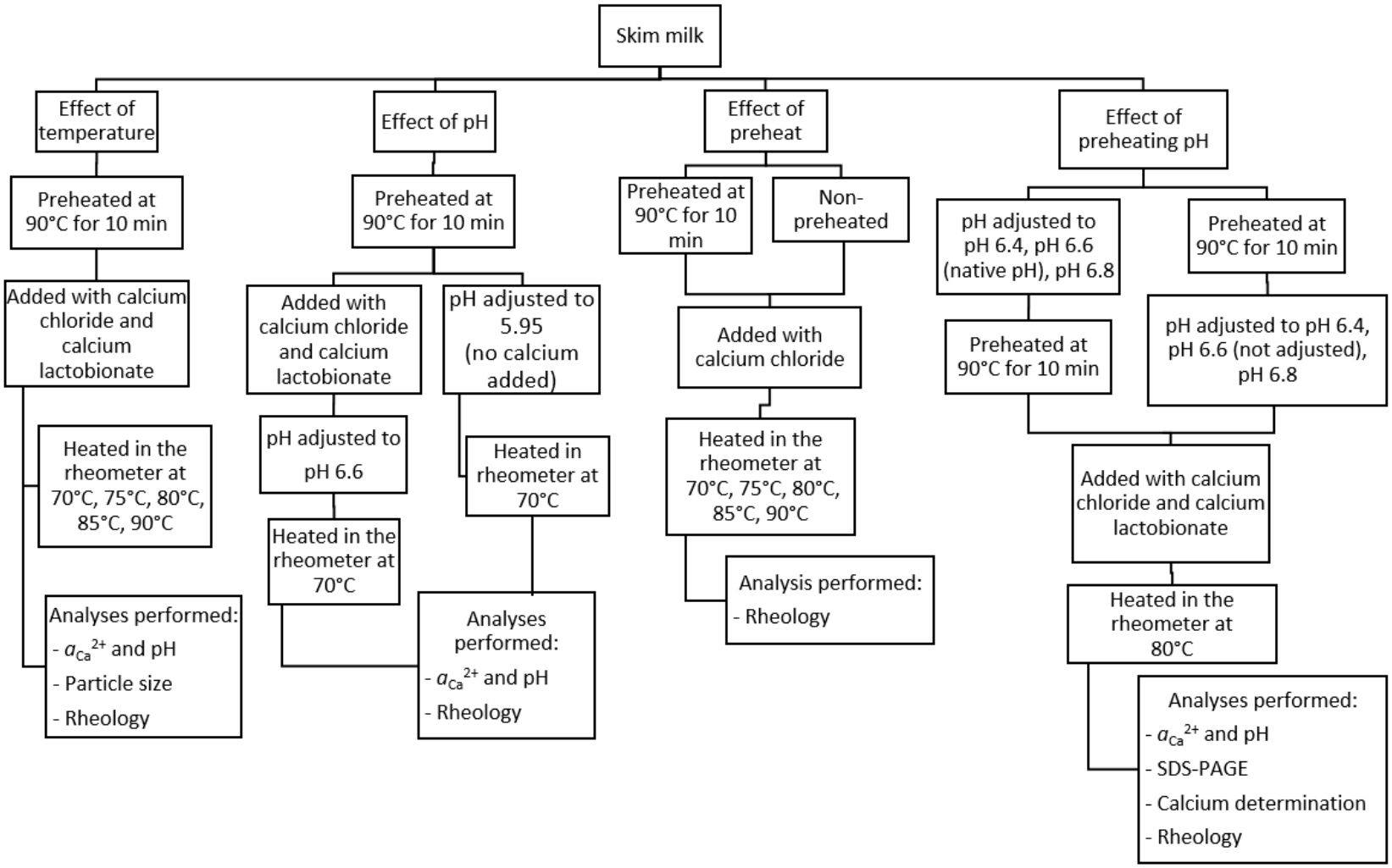


Figure 5-1: Summary of the experimental plan and treatments applied to the skim milk to investigate the effect of temperature, pH and preheated treatment.

5.2.1 Preparation of non-preheated skim milk

Reconstituted skim milk was prepared as described in Section 3.2 and Figure 5-1.

For non-preheated skim milk samples, the calcium chloride stock solutions were added to non-preheated skim milk to achieve final calcium concentrations between 5 and 40 mmol L^{-1}. Distilled water was added to the samples to adjust the final skim milk concentration to 9.6 % total solids (*w/w*) in all samples. A sample with no added calcium salt was also included as a control sample.

5.2.2 Preparation of skim milk with pH adjustment

A baseline experiment to establish the relationship between pH and calcium ion activity of the skim milk was conducted by adjusting the pH of the skim milk from pH 5.60 to pH 7.00 by addition of 1 M HCl or 2 M NaOH.

Skim milk samples with pH adjustments were prepared as described in Figure 5-1 for the investigation of the effect of pH and preheating pH.

To determine the effect of pH on the calcium-induced skim milk gel, pH of the skim milk was adjusted to pH 6.60 using 2 M NaOH after preheating and calcium salt addition to eliminate the effect of the decrease in pH caused by addition of calcium salt. A separate study where the skim milk with no calcium salt added was adjusted to pH 5.95 ± 0.01 (pH of skim milk with 40 mmol L^{-1} of calcium chloride addition) by careful addition of 1 M HCl was also carried out.

The effect of preheating pH on the calcium-induced skim milk gels was investigated by adjusting the skim milk samples to pH 6.40 ± 0.01 or pH 6.80 ± 0.01 by careful addition of 1 M HCl or 2 M NaOH, prior to preheating. The adjusted skim milk samples were left to

equilibrate for at least 12 hours followed by any further readjustments to the required pH if necessary. The pH-adjusted skim milks were then preheated according to the procedures described in Section 3.4. A sample with no added calcium salt was also included as a control sample.

A second set of experiment was carried out where the skim milk samples were preheated at pH 6.60 ± 0.01 (native pH) and subsequently adjusted to pH 6.40 ± 0.01 or 6.80 ± 0.01 by careful addition of 1 M HCl or 2 M NaOH after the preheat treatment.

5.2.3 Collection of skim milk permeate at different temperatures

A 1 L stainless steel (SS) jacketed container used for holding the skim milk was connected to a water bath as shown in Figure 5-2. Temperature-controlled water from the water bath was circulated through the outer jacket of the SS jacketed container to maintain the temperature of the skim milk during filtration. The water was circulated through the SS jacketed container for at least 30 min to allow equilibration of temperature. The temperature of the skim milk in the SS jacketed container was maintained at 20 ± 1°C or 53 ± 2°C during filtration.

Skim milk was prepared according to Section 3.2 and 0 to 20 mmol L^{-1} of calcium chloride was added to the skim milk. The final skim milk concentration was kept constant at 9.6% total solids (*w/w*). The skim milk samples were poured into the SS jacketed container while stirring with an overhead stirrer (RW20, IKA Works GmbH & Co., Staufen, Germany). The skim milk samples were left stirring at 500 rpm for 15 min in the SS jacketed container to allow the skim milk to equilibrate to the filtration temperature. Collection of the permeate of the skim milk was initiated by pumping the skim milk from the SS jacketed

container to the ultrafiltration (UF) cassette which contained a polyethersulfone membrane with a molecular cut-off of 10 kDa (Vivaflow 50, Sartorius Stedim Biotech, Germany) using a peristaltic pump (Masterflex® L/S® Variable-speed economy modular drives, Cole-Parmer Instrument Company, Illinois, USA) (Figure 5-2). The collection of the skim milk permeate ceased when 20 mL of skim milk permeate was collected. Duplicate runs were conducted for skim milk at each temperature and concentration of added calcium chloride. The skim milk permeate were cooled to 20 ± 1°C before calcium ion and pH measurements. The $a_{Ca^{2+}}$ and pH were measured three times for each sample (n =6). Addition of calcium salts at > 20 mmol L^{-1} of calcium chloride was found to result in coagulation of the skim milk at 53 ± 2°C and was therefore not included in this study.

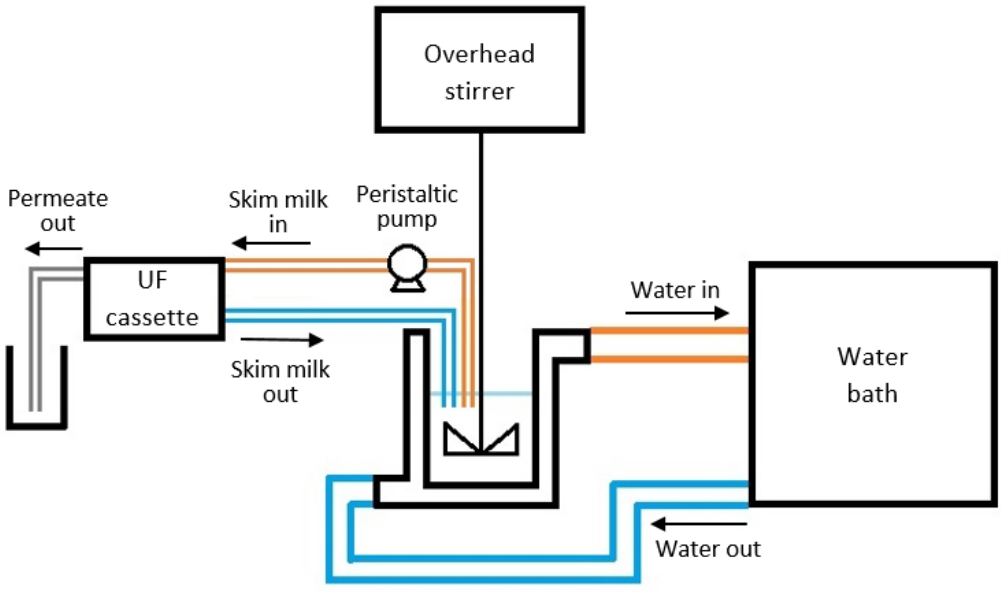


Figure 5-2: Diagram of setup for collection of skim milk permeate at different temperatures.

5.2.4 pH and calcium ion activity measurement in skim milk

The pH and calcium ion activity of the non-preheated and pH-adjusted skim milk samples were measured according the methods described in Section 3.6.

5.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and laser densitometry

SDS-PAGE was carried out on the non-preheated and pH-adjusted skim milk samples according to methods described in Section 3.7 and 3.8.

5.2.6 Determination of calcium concentration in skim milk and serum samples

The calcium concentration in the pH-adjusted skim milk and serum samples were determined using methods described in Section 3.7 and 3.9.

5.2.7 Particle size analysis with temperature ramp

Samples for particle size analysis were prepared as described in Section 3.10. The 'temperature trend' measurement method was selected in the zetasizer (make, model, country) where heat treatment of the sample was performed in situ. The zetasizer was set to heat the sample from 20°C to 80°C. Measurements for particle size were set to be taken at every 5°C increment intervals, with three min equilibration before measurement. The samples were held in a heat-resistant glass cuvette (Zetasizer nano series PCS8501, Malvern, Australia) during the analysis.

5.2.8 Rheological measurements of calcium-induced skim milk gels in the rheometer

Details of the rheological measurements are described in Section 3.11.

The effect of temperature and preheat treatment on the rheological properties of calcium-induced skim milk gels were investigated using the hard-anodised aluminium (HA) cup

with holding temperatures based on the rheometer set temperatures of 70°C, 75°C, 80°C, 85°C, and 90°C. The effect of pH on the rheological properties of the calcium-induced skim milk gels were investigated using the HA cup with holding temperature at 70°C (rheometer set temperature).

A comparison between the rheological results using the HA cup and stainless steel (SS) cup were carried out at 70°C, 80°C and 90°C. The effect of preheating pH on the rheological properties of calcium-induced skim milk gels were investigated using the SS cup with holding temperature at 80°C (sample temperature). As discussed in Section 3.11, the rheometer set temperature was altered when using the SS cup to ensure the sample temperature was held at 80°C for 60 min (Figure 3-4). The G' values were only compared between samples using the same cup for measurement.

5.3 Results and Discussion - Effect of temperature

5.3.1 Effect of temperature on calcium ion activity and pH in skim milk

The effect of heat and calcium addition on the $a_{Ca^{2+}}$ and pH in milk has been studied extensively (Chandrapala, McKinnon, Augustin & Udabage, 2010; Fox & McSweeney, 1998; Kaombe, Du & Lewis, 2012; On-Nom, Grandison & Lewis, 2010; Rose & Tessier, 1959). However, there is little information on how temperature affects the $a_{Ca^{2+}}$ in calcium-added skim milk. Investigating the effect of temperature on the $a_{Ca^{2+}}$ and pH in calcium-added skim milk may provide information on how the changes in calcium equilibrium during heat treatment may affect the gelation of calcium-induced skim milk gels. The two temperatures were investigated to determine the difference in the physico-chemical

properties of skim milk at room temperature (20°C) and heated skim milk (53°C). The skim milk was only heated to 53°C as the maximum operating temperature of the ultrafiltration membrane was 60°C.

Table 5-1: The effects of added calcium chloride and temperature of skim milk on the calcium ion activity ($a_{Ca^{2+}}$) and pH of skim milk permeate (n= 6). At the same concentration of added calcium chloride, different superscript letters (a, b) indicates significant difference in the $a_{Ca^{2+}}$ or pH in the skim milk permeate collected at filtration temperature of 20°C and 55°C. Results presented are mean ± standard deviation (n =3).

	Concentration of added calcium chloride (mmol L^{-1})	**Filtration temperature (°C)**		**Difference between 20 ± 1°C and 53 ± 2°C**	**Percentage difference (%)**
		20 ± 1	**53 ± 2**		
$a_{Ca^{2+}}$	0	0.6 ± 0.1 [a]	0.3 ± 0.1 [b]	0.3	50.0
	10	1.7 ± 0.1 [a]	1.2 ± 0.1 [b]	0.5	29.4
	20	3.7 ± 0.1 [a]	3.1 ± 0.1 [b]	0.6	16.2
pH	0	6.67 ± 0.01 [a]	6.43 ± 0.02 [b]	0.24	
	10	6.42 ± 0.01 [a]	6.05 ± 0.01 [b]	0.37	
	20	6.23 ± 0.01 [a]	5.84 ± 0.01 [b]	0.39	

Increasing the temperature of skim milk resulted in significant reduction in calcium ion activity ($a_{Ca^{2+}}$) and pH whether or not calcium salt was added ($p < 0.05$) (Table 5-1). Reduction in $a_{Ca^{2+}}$ on heating was attributed to the decrease in the solubility of calcium phosphate (Chandrapala, McKinnon, et al., 2010; de la Fuente & Juarez, 2015). The equilibrium between calcium ions and phosphate shifts towards the formation of calcium phosphate precipitate on increase in temperature, thus resulting in the decrease in $a_{Ca^{2+}}$ (Fox & McSweeney, 1998; van Boekel et al., 1989). It has been reported that the precipitated calcium phosphate is likely to transfer from the serum phase to the casein micelles, which decrease casein micelle stability (Philippe et al., 2003; Walstra et al.,

1999). The decrease in a_{Ca}^{2+} suggested that heat treatment may have resulted in interactions between the calcium ions and the proteins, or that the precipitated calcium phosphate associated with the casein micelles. The interactions between the calcium phosphate or calcium ions with casein micelles may have led to protein instability and hence aggregation and gelation on heating.

The percentage difference between the a_{Ca}^{2+} at 20°C and 53°C decreased as the concentration of added calcium salt increased (Table 5-1). As calcium ion activity is an indication of the concentration of calcium ions in the serum phase, the smaller percentage difference in calcium ion activity suggested that on heating, a smaller proportion of the added calcium transferred into the colloidal phase (i.e. a higher proportion of added calcium ions remained in the serum phase) with increasing concentration of added calcium salt. On-Nom et al. (2012) reported a similar trend in the calcium ion concentration of permeate collected from skim milk at 20°C and skim milk heated to 115°C. This observation indicated that the interactions that occurred during heating between the added calcium ions or precipitated calcium phosphate with the casein micelles may be approaching saturation with increasing concentration of added calcium salt.

The reduction in pH appears to be higher with increasing concentration of added calcium salt (Table 5-1). On heating to 53°C, the precipitation of primary and secondary calcium phosphate as tertiary phosphates with the concomitant release of H^+ ions was likely the primary cause of the reduction in pH (van Boekel et al., 1989). The reduction in pH on heating skim milk could also be due to hydrolysis of organic (casein) phosphate and its subsequent precipitation as $Ca_3(PO_4)_2$ with release of H^+ (Fox, 1981).

5.3.2 Particle size of casein micelles with increasing temperature

As mentioned in Section 4.3.4, based on the z-average (240.5 ± 0.5 nm), the particles measured at 20°C were likely the casein micelles. Further, as this was a skim milk system, the particle size range representing fat globules was not considered. The effect of temperature on the aggregation of casein micelles was determined by measuring the changes in the particle size in the skim milk with increasing temperature.

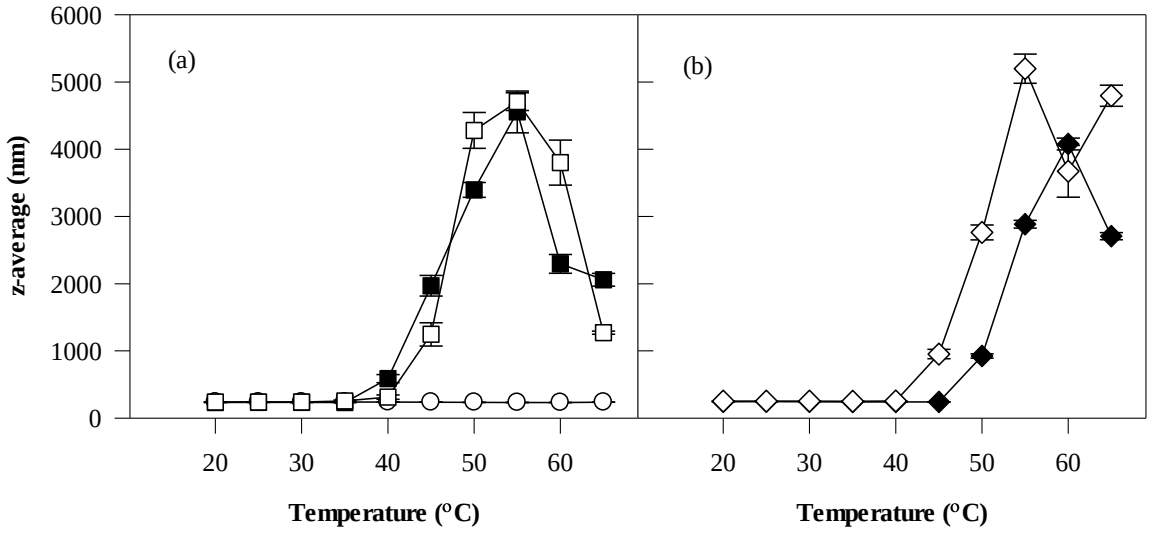


Figure 5-3: Changes in the size of casein particles in skim milk as a function of temperature. Skim milk was preheated at 90°C for 10 min, then (a) calcium chloride and (b) calcium lactobionate were added at 0 mmol L^{-1} (○), 20 mmol L^{-1} (■, ◆) and 40 mmol L^{-1} (□, ◇) of, prior to dilution with the corresponding skim milk permeate (n = 6).

When skim milk was heated without the addition of calcium salts, the size of the casein particles remained unchanged over the temperature range of 20°C to 65°C (Figure 5-3). The results showed that the casein micelles were stable to heat-induced aggregation up to 65°C when no calcium salts were added. When 20 mmol L^{-1} and 40 mmol L^{-1} of calcium chloride were added to the skim milk, an increase in particle size was observed at 40°C,

indicating casein micelle aggregation (Figure 5-3a). For calcium lactobionate, the increase in casein particle size was observed at 50°C for 20 mmol L^{-1}, and 45°C for 40 mmol L^{-1} of its addition (Figure 5-3b). The higher temperature required for calcium lactobionate to initiate aggregation is in agreement with the results presented in Figure 4-7. Increasing temperature results in increasing movement of particles and thus a higher frequency of collision (McClements & Keogh, 1995). Bond formation occurs when the attractive forces of the collided particles are strong enough. If the attractive or cohesive forces are too weak, the particles will not form a permanent attachment even when they collide (Dalgleish, 1983). As calcium lactobionate released fewer calcium ions (Ca^{2+}), there were fewer available calcium ions to destabilise the proteins. Hence, a higher temperature may be required for sufficient collision and bond formation between the particles in the calcium lactobionate-added skim milk for aggregation to occur.

The size of the casein particles peaked at 55°C to 60°C for all the samples with added calcium, then decreased as temperature increased (Figure 5-3). The decrease in particle size was likely to be due to sedimentation of the aggregated particles that are too dense to remain suspended in the solution. The glass cuvette was removed after the analysis and sedimentation of the aggregated particles was observed (Figure 5-4). The difference in the results obtained between the rheological measurements (Section 4.3.5), where skim milk samples with added 20 and 40 mmol L^{-1} of calcium salts gelled and the results obtained for the particle size analysis, where casein micelles in the skim milk samples with added 20 and 40 mmol L^{-1} of calcium salts aggregated but did not gel, was likely due to the concentration of the total solids/ total proteins. As mentioned in the materials and methods section, the skim milk samples were diluted with skim milk permeate at 1:200 (*v/v*) before

particle size analysis. Totosaus et al. (2002) proposed that for gelation to occur, a minimum concentration of protein is required for the crosslinking of the particles to form the continuous three-dimensional network, below which the structure cannot be formed. Thus, it is likely that the protein concentration in calcium-added skim milk was sufficient for the formation of a gel structure, but in the diluted samples for the particle size analysis, the protein concentration was sufficient for aggregation to occur but not the formation of a gel structure.

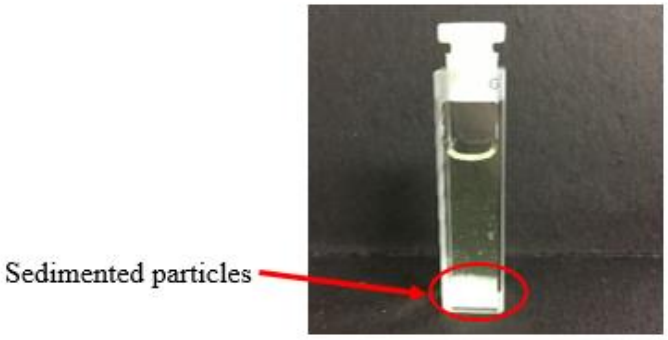


Figure 5-4: Appearance of glass cuvette immediately after particle size analysis with aggregated particles sedimented.

It should be noted that as temperature increase and the casein particles aggregate, the distribution of the particle size changes from monomodal to multimodal. However, as z-average is described as the harmonic intensity averaged particle diameter, a value that describes the mean diameter of the particles in a dispersion or solution derived by intensity-based calculations (Malvern Instruments, 2011), the increase in z-average values still indicated that the averaged particle size in the sample increased at higher temperature. Hence, although the samples had a multimodal distribution, it does not alter the conclusion

that the particle size increased, and this was presumably due to aggregation of the casein micelles.

5.3.3 Effect of holding temperature on rheological properties of calcium-induced skim milk gels

Skim milk samples with various concentrations of added calcium chloride and calcium lactobionate were placed in the sample cup of the rheometer and the G' of the skim milk ranging from 20°C to 90°C was monitored (Figure 5-5). An exponential growth in G' was observed with increase in temperature in samples where gelation was observed (Figure 5-5). This indicated that the rate of reaction was increasing with temperature and suggests an Arrhenius relationship between gelation rate and temperature. Moreover, increasing the salt concentration in solutions reduces the electrostatic repulsion between the proteins. According to the DLVO theory, this leads to a reduction in the activation energy required for reaction to occur between the protein particles on collision (De Young et al., 1993). A lower activation energy required could mean that a larger fraction of protein particles would react on collision. Hence, this may be the reason for the lower gelation temperature at higher concentrations of added calcium salt (Table 5-2).

At the same concentration of added calcium salt, the onset of gelation (as indicated by an increase in G' to > 1 Pa) in calcium chloride added skim milk was observed at a significantly lower temperature ($p < 0.05$). For example, when 20 mmol L^{-1} of calcium chloride was added, gelation was observed 67.40 ± 1.17°C, while for 20 mmol L^{-1} of calcium lactobionate added, gelation was only observed at 75.69 ± 0.59°C (Table 5-2). Similar to the results observed in the change in the particle size of the casein micelles (Section 5.3.2), a lower temperature was required to initiate gelation in skim milk with

added calcium chloride (Figure 5-5). Further, for the same type of added calcium salt, increasing concentration of added calcium salt significantly decreased the gelation temperature ($p < 0.05$) (Table 5-2). The lower gelation temperature observed in calcium chloride-added skim milk and at higher concentrations of added calcium salt could again be attributed to the effect of the calcium salt addition on the $a_{Ca^{2+}}$. As discussed in Chapter 4, a higher $a_{Ca^{2+}}$ indicated a higher concentration of calcium ions available for interactions with the proteins in the calcium chloride-added skim milk, which may then lead to increased interactions between the proteins and gelation.

It should also be noted that while aggregation of the casein micelles was initiated at approximately 40°C to 50°C (Figure 5-3), gelation was initiated only at between 55°C to 60°C (Figure 5-5). According to De Kruif et al. (1995), the formation of a gel network involves three steps: (1) destabilisation of the colloidal dispersion, which could be induced by salt, pH, temperature, pressure etc.; (2) aggregation; (3) aggregated particles forming a gel network. Hence, temperatures between 40°C to 50°C appear to be sufficient to initiate aggregation amongst the casein micelles of the calcium-added skim milk, but a higher temperature of 55°C to 60°C was required for sufficient aggregation to form a gel network as detected by the rheometer.

At all holding temperatures, the G' increased rapidly for the first 20 min during holding, followed by a plateau after 20 min of holding (Figure 5-6). This indicated that formation of the gel network occurred mainly during the first 20 min of holding regardless of the holding temperature. The exponential growth curves also suggest that the reactions occurring during gelation may be cooperative. Cooperative binding occurs when the binding of one molecule induces structural changes that result in altered molecular binding affinities in the

remaining bindings sites (Marangoni, 2003). The calcium ion-protein and protein-protein interactions could be occurring cooperatively, resulting in molecules with higher numbers of binding sites and thus increased chances for reactions on collision. The plateaus towards the end of the holding curves suggest a reduction in the rate at which the proteins interact. Parker & Dalgleish (1977) suggested that as aggregation proceeds in a calcium-induced α_{s1}-casein aggregation, there comes a point when the rate in which the molecules interact is not limited by the number of binding sites on the molecule but by the rate at which collisions occur. In the calcium-induced skim milk gel system, the plateau could indicate that the gel matrix may have been formed and therefore limiting the number of further particle collisions, resulting in the reduction in rate of gel formation.

Overall, the holding temperature appears to be more critical in determining the strength of the gel than the holding time (Figure 5-6). For instance, when 20 mmol L^{-1} of calcium chloride was added, holding the skim milk at the rheometer set temperature of 90°C for 20 min achieved a G' of approximately 20 Pa, but even after holding at 70°C for 60 min, the G' was only approximately 10 Pa (Figure 5-6c). This indicated that the temperature during gelation was crucial for determining the rheological properties of the gel during the initial stages of the formation of the gel network by influencing the frequency of collision and bond formation between the particles.

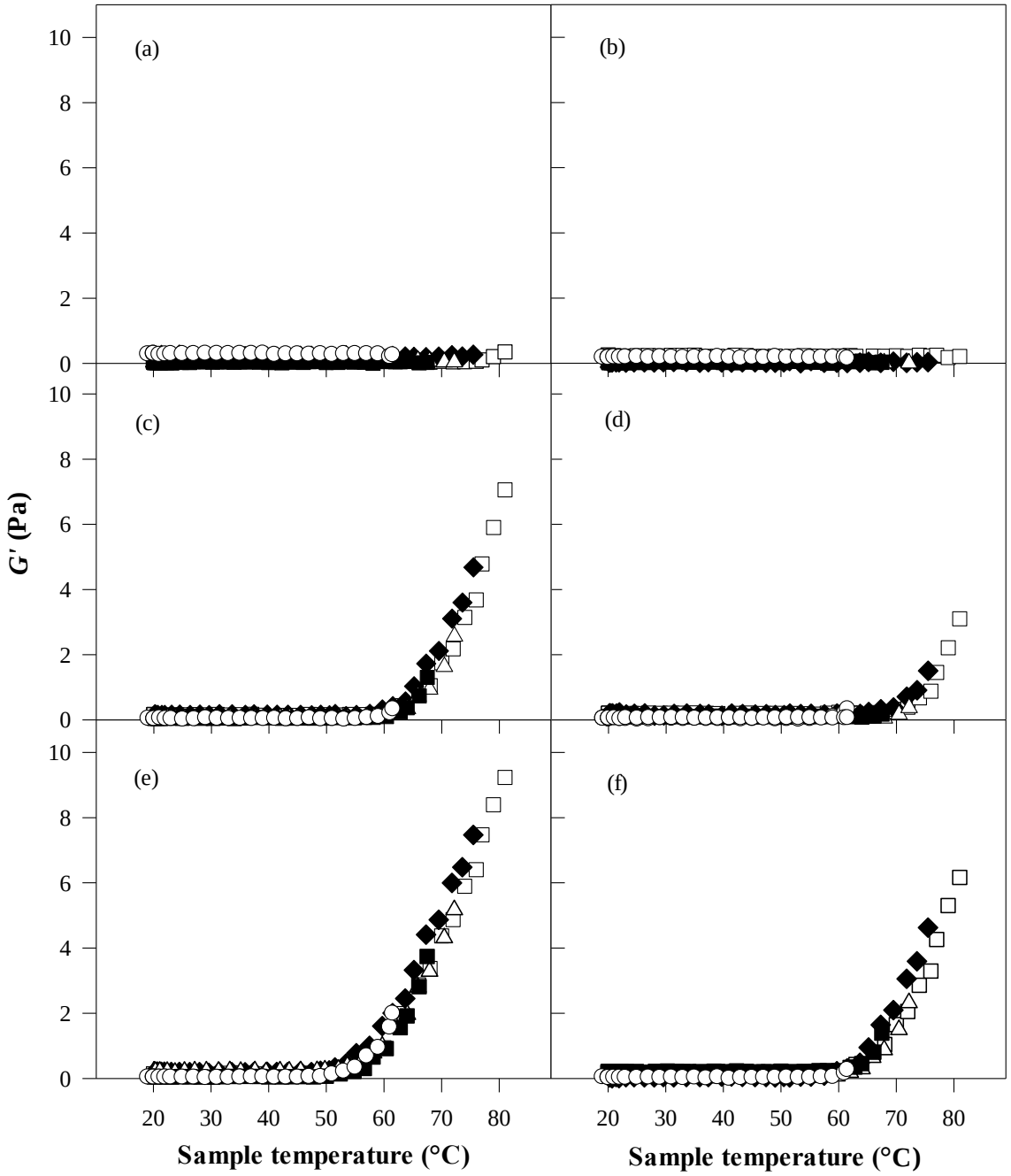


Figure 5-5: Typical plots showing the change in G' during heating from 20°C to the rheometer set temperature of 70°C (○), 75°C (■), 80°C (△), 85°C (◆), and 90°C (□) for skim milk with 10 mmol L^{-1} (a, b), 20 mmol L^{-1} (c, d) and 40 mmol L^{-1} (e, f) of added calcium chloride (a, c, e) and calcium lactobionate (b, d, f).

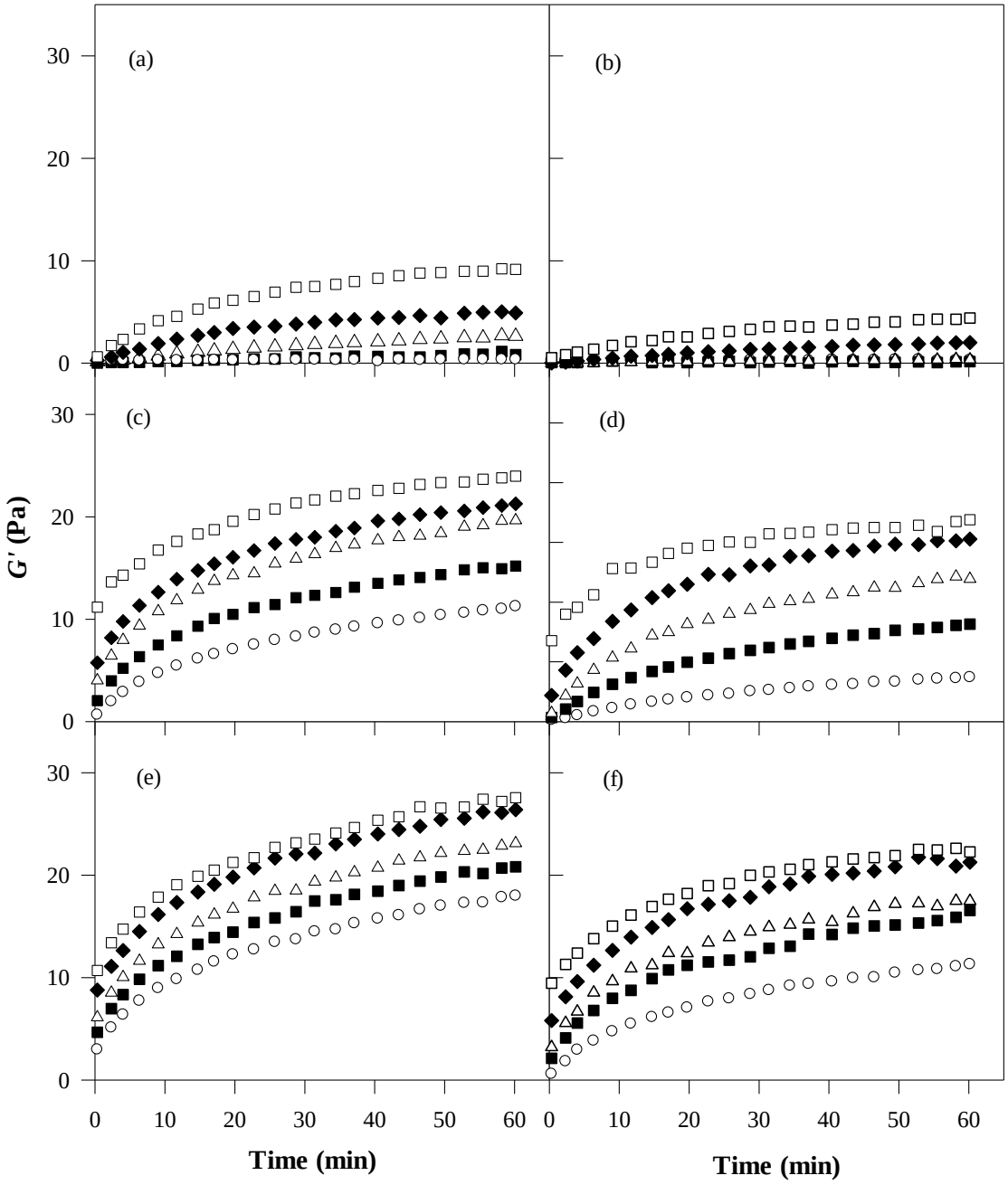


Figure 5-6: Typical plots showing the change in G' during holding at rheometer set temperature of 70°C (○), 75°C (■), 80°C (△), 85°C (◆), and 90°C (□) for skim milk with 10 mmol L^{-1} (a, b), 20 mmol L^{-1} (c, d) and 40 mmol L^{-1} (e, f) of added calcium chloride (a, c, e) and calcium lactobionate (b, d, f).

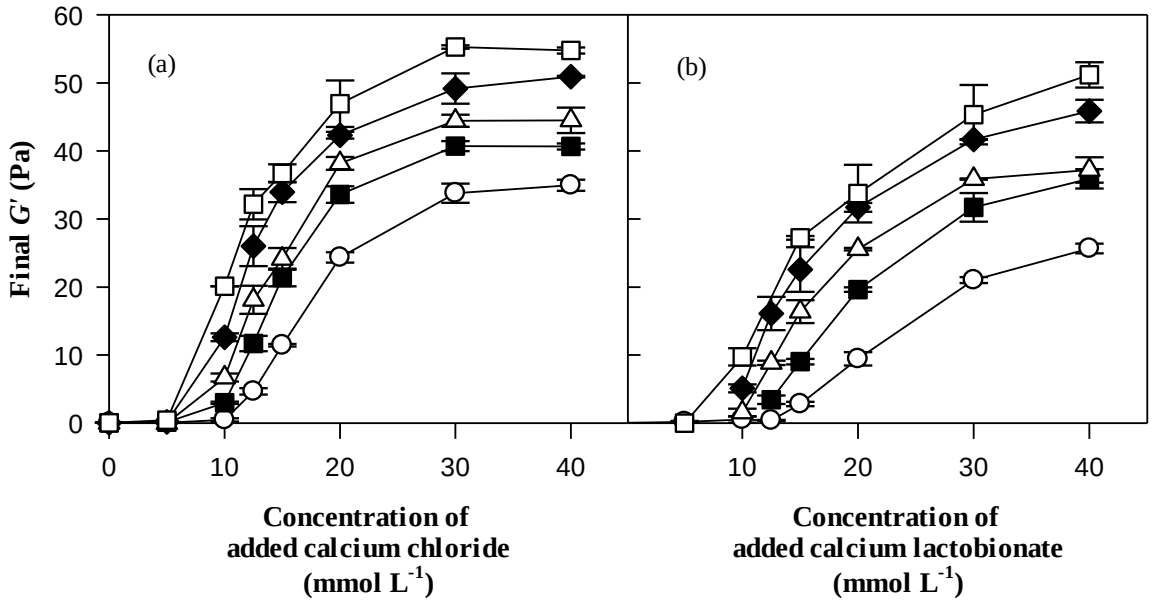


Figure 5-7: Relationship between final G' at 20°C and calcium salt concentration in calcium-induced skim milk gels with added (a) calcium chloride and (b) calcium lactobionate. Calcium-induced skim milk gels were heated in the rheometer at holding temperatures of 70°C (○), 75°C (■), 80°C (△), 85°C (◆), and 90°C (□). Data points are mean values ± standard deviation (n = 3).

Table 5-2: Gelation temperature of calcium-induced skim milk gels at various concentrations of added calcium salt. Results presented are means ± standard deviation (n = 9). For each type of calcium salt, different superscript letters (a, b, c) indicate significant difference across the different concentration of added calcium salt. At each concentration of added calcium salt, different superscript Greek letters (χ, γ) indicate significant difference across the different type of added calcium salt (95% confidence level).

Type of added calcium salt	Concentration of added calcium salt (mmol L^{-1})	Gelation temperature[+] (°C)
Calcium chloride	20	$67.40 \pm 1.17^{a,\chi}$
	30	$61.72 \pm 0.49^{b,\chi}$
	40	$59.79 \pm 0.67^{c,\chi}$
Calcium lactobionate	20	$75.69 \pm 0.59^{a,\gamma}$
	30	$69.76 \pm 0.55^{b,\gamma}$
	40	$67.98 \pm 0.74^{c,\gamma}$

[+] Temperature at which the skim milk achieved G' of > 1 Pa

At the same holding temperature, all plots displayed a sigmoidal shape curve where there is a sharp increase in final G' at between 5 to 20 mmol L^{-1} of added calcium salt, followed by smaller increases in final G' from 20 to 40 mmol L^{-1} of added calcium salt (Figure 5-7). As explained, cooperative binding between calcium ions and proteins may be occurring during gelation, increasing the binding affinities of the protein molecules. Hence, when the concentration of added calcium salt increased from 5 to 20 mmol L^{-1}, the higher number of reacting calcium ions may increase the binding affinities and hence gelation (G') of the proteins exponentially. However, as discussed in Section 5.3.1, it appears that there may be a maximum concentration of added calcium that can interact with casein micelles before saturation occurs. This may explain why the final G' began to plateau at higher concentrations of added calcium salts when heated at the same holding temperature. Despite this, increasing the holding temperature in the rheometer increases the final G' of both calcium chloride- and calcium lactobionate-added skim milk across the concentrations of calcium salt added (Figure 5-7). As described in Section 2.9.3, reactions can be diffusion-limiting or reaction-limiting. The dependency of the final G' on the holding temperature as shown in Figure 5-7 suggests that the interactions may be limited by the frequency in which the particles collide (diffusion-limiting) rather than by the probability of the particles interacting on collision (reaction-limiting).

Besides increasing the frequency of collision among the protein particles with increasing temperatures, the increased final G' at higher temperatures could also be due to increased binding of calcium with the proteins. Pappas and Rothwell (1991) reported an increase in moles of calcium bound to casein when casein solutions were heat treated at 80°C or 95°C for 30 min. Dalgleish & Parker (1980) also found that binding of calcium ions to α_{s1}-casein

increased with increasing temperature. The increased binding between calcium and casein may have facilitated increased hydrophobic interactions due to reduced electrostatic repulsion, and calcium bridging between the proteins (Crowley et al., 2014; Walstra et al., 1999), thus resulting in the higher final G' when skim milk was heated to a higher temperature. The increase in calcium binding at higher temperature may also explain why a lower concentration of calcium salt addition was required to induce gelation when skim milk was heated at higher temperatures. For example, when skim milk was heated at 70°C, addition of 12.5 mmol L^{-1} of calcium chloride was required for gelation but when skim milk was heated at > 75°C, gelation was observed at 10 mmol L^{-1} of calcium chloride addition (Figure 5-7).

5.4 Results and Discussion - Effect of pH

5.4.1 Calcium ion activity in skim milk

The effect of pH on the calcium ion activity has been extensively reviewed (Faka et al., 2009; Lewis, 2011; Lucey et al., 1996; Tsioulpas et al., 2007). An increase in $a_{Ca}{}^{2+}$ with decreasing pH was observed in the skim milk (Figure 5-8). This result could be attributed to the shift in calcium equilibrium in milk, which resulted in the solubilisation of the colloidal calcium phosphate from the casein micelles into calcium ions in the serum (Gaucheron, 2005; Philippe et al., 2003).

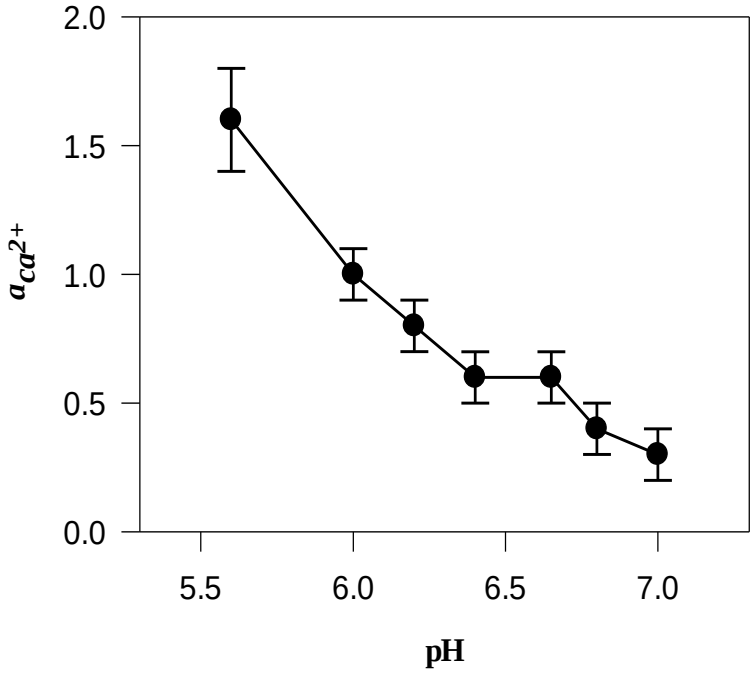


Figure 5-8: The relationship between pH and the calcium ion activity of skim milk, without added calcium (n =6).

5.4.2 Rheological properties of the calcium-induced skim milk gels

To determine the effect of the decrease in pH with calcium salt addition on the gelation of the calcium-induced skim milk gels, the skim milk pH was adjusted back to pH 6.6 (native pH of the skim milk) prior to rheological measurements.

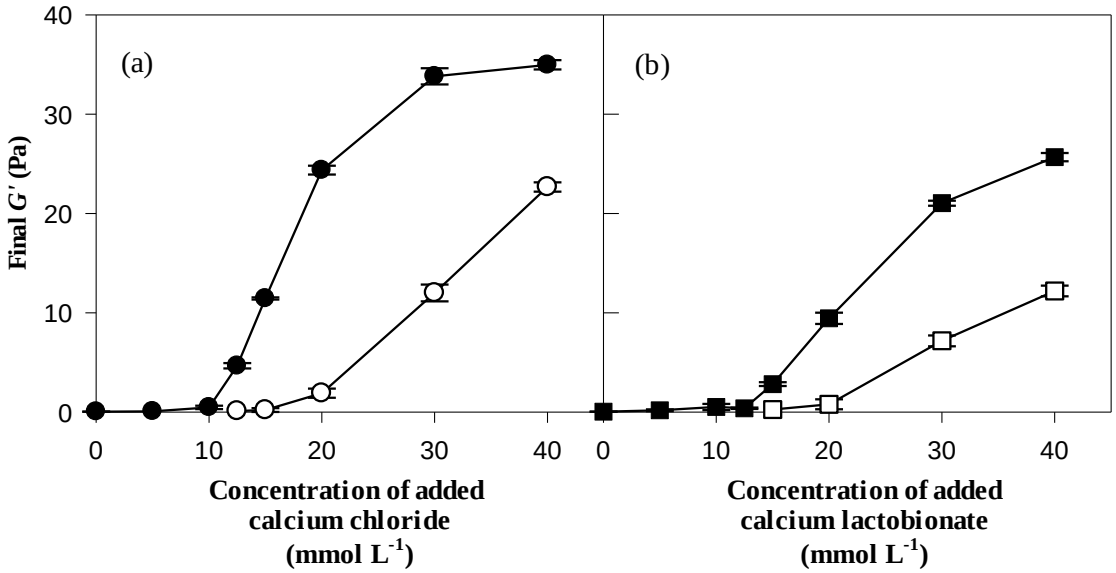


Figure 5-9: Relationship between final *G′* of calcium-induced skim milk gels at rheometer set temperature of 20°C with added (a) calcium chloride and (b) calcium lactobionate. Closed symbols (●, ■) represents samples with no pH adjustment and open symbols (○, □) represents samples with pH adjusted to 6.6. Data points are mean values ± standard deviation (n= 3).

On increasing the pH of the skim milk to pH 6.6 after calcium addition, the final *G′* in both calcium chloride and calcium lactobionate samples were lower than their corresponding pH-unadjusted samples at the same concentration of calcium salt added (Figure 5-9). This result indicated that the decrease in skim milk pH with the calcium addition contributed to the gelation of milk. The higher concentration of H^+ ions in the system would decrease the negative charge on the casein micelle, leading to instability and promoting aggregation of the casein (Mishra et al., 2005; Teo, Munro, Singh & Hudson, 1996). To determine the effect of pH without added calcium salt, skim milk samples were adjusted to pH 5.95 ± 0.01 (the lowest pH of the skim milk after calcium salt addition (40 mmol L^{-1} of added calcium chloride) using 1 M HCl. With the same heating, holding and cooling protocol, no

gelation (final $G' < 1$ Pa) was observed. In unheated acid-induced milk gels, gelation occurs at close to the isoelectric point of casein at a pH of approximately 4.8, while in milk preheated at 90°C for 10 min, gelation occurs at approximately pH 5.5 (Vasbinder et al., 2003). The gelation observed at pH 5.5 in preheated milk by Vasbinder et al. (2003) was likely due to the whey proteins on the surface of casein micelles approaching the isoelectric point of whey proteins (pH 5.2), where the whey proteins acted as bridges for formation of gel network between casein micelles. At pH 5.95, the electrostatic repulsion may still be too high for bonds to form between the whey proteins on the surface of casein micelles, hence no gelation was observed in this study. The results demonstrated that while the pH reduction induced by addition of calcium salt contributed to the strength of the gel, the reduction in pH to 5.95 was insufficient to result in gelation of the skim milk, and that the increase in acidity of the calcium-added skim milk was not the primary cause of gelation.

5.5 Results and Discussion - Effect of preheat treatment

5.5.1 Rheological properties of calcium-induced skim milk gels

To determine the effect of preheat treatment on the calcium-induced gelation of skim milk, the rheological properties of preheated and non-preheated skim milk with added calcium chloride were determined at various holding temperatures.

At the same concentration of added calcium salt, the gelation temperature for preheated skim milk was lower than for non-preheated skim milk (Figure 5-10). Addition of calcium salts to denatured whey protein results in screening of electrostatic repulsion, thus facilitating hydrophobic interactions, and formation of calcium bridges between the whey proteins (Hongsprabhas & Barbut, 1997; Mudgal, Daubert & Foegeding, 2011). Hence,

during heating to 70°C in the rheometer, the denatured whey proteins on the surface of the casein micelles may start to interact and form a gel network as described by Ramasubramanian et al. (2013). On the other hand, in non-preheated skim milk, no gelation occurred at < 65°C across all concentrations of added calcium chloride (Figure 5-10b, d, f). The lower gelation temperature observed in preheated skim milk could be attributed to the interactions between the denatured whey proteins during heating, which contributed to the increase in G'. In the non-preheated skim milk, the whey proteins were likely to be still in their native state and hence may not interact with casein micelles or with each other to contribute to the gel strength.

At the same holding temperature and concentration of calcium chloride added, the final G' of the skim milk gel was lower when no preheat treatment was applied (Figure 5-11). Ramasubramanian et al. (2013) also reported a lower final G' from non-preheated whole milk compared to that from preheated whole milk with up to 20 mmol L^{-1} added calcium chloride. Preheat treatment to temperatures > 70°C results in the denaturation of whey proteins, which can then interact with themselves or with casein (Anema, 2007; Schorsch et al., 2001). These interactions were reported to result in higher gel firmness in acid-induced milk gels due to the participation of the denatured whey proteins in the gel structure (Anema et al., 2004b; Lucey, Teo, et al., 1997; Schorsch et al., 2001). Complexes of denatured β-lactoglobulin with κ-casein on the casein micelles have been found to appear as hairy appendages surrounding the casein micelles under electron microscopy (Mottar & Bassier, 1989; Ramasubramanian et al., 2013). Ramasubramanian et al. (2013) suggested that the poorly developed appendages in non-preheated milk resulted in fusion of casein micelles in calcium-induced milk coagulum, which gave a soft and compact texture. On the

other hand, a calcium-induced milk coagulum produced from milk which had been preheated at 90°C for 10 min showed casein micelles which were crosslinked by the appendages, leading to longer micellar chains with fewer pores and a firmer texture (Ramasubramanian, 2013). The lower final *G′* of the non-preheated calcium-induced skim milk gels could be due to reduced participation of the whey proteins in the gel structure.

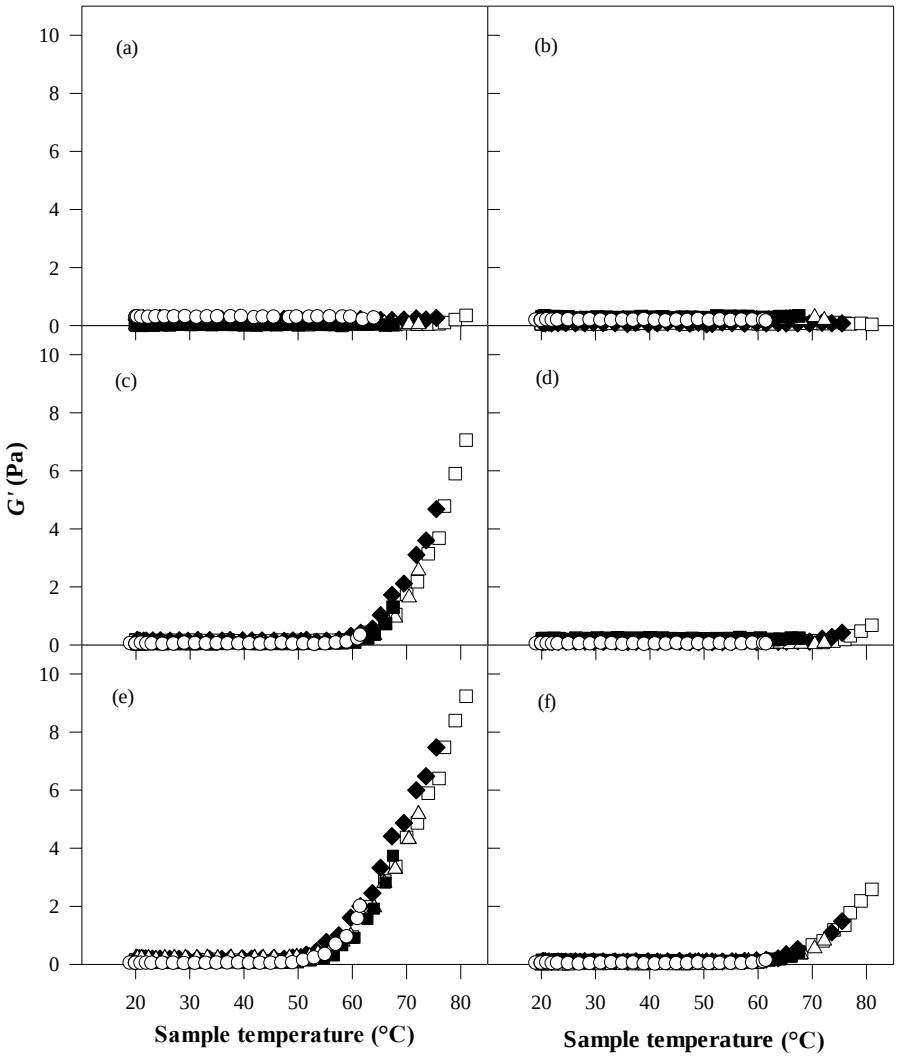


Figure 5-10: Typical plots showing the change in *G′* during heating from 20°C to the rheometer set holding temperature of 70°C (○), 75°C (■), 80°C (△), 85°C (◆), and 90°C (□) for skim milk preheated at 90°C for 10 min (a, c, e) and non-preheated skim milk (b, d, f) with 10 mmol L^{-1} (a, b), 20 mmol L^{-1} (c, d) and 40 mmol L^{-1} (e, f) of added calcium chloride.

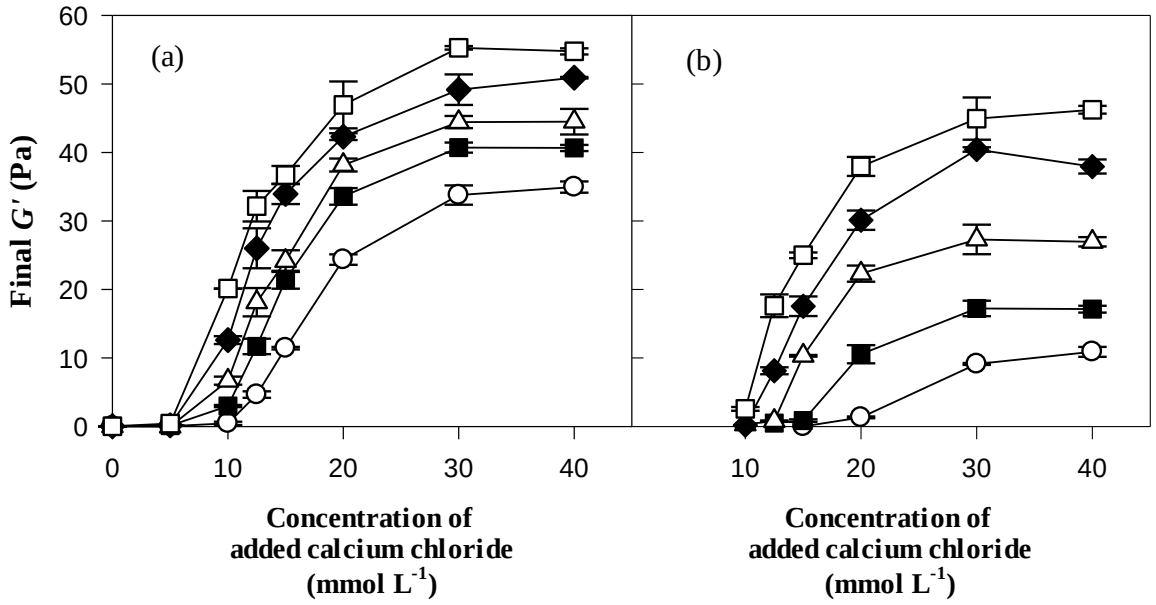


Figure 5-11: Relationship between final G' at rheometer set temperature of 20°C and calcium chloride concentration for skim milk (a) preheated at 90°C for 10 min and (b) non-preheated. Calcium-induced skim milk gels were heated in the rheometer and held at rheometer set temperatures of 70°C (○), 75°C (■), 80°C (△), 85°C (◆), and 90°C (□). Data points are mean values ± standard deviation (n= 2 to 3).

5.6 Results and Discussion - Effect of hard-anodised aluminium (HA) cup versus stainless steel (SS) cup on the rheological properties at different temperatures

To determine the difference between measurements using the HA and SS cup, the rheological properties of the calcium-induced milk gels at various temperatures using the two cups were compared.

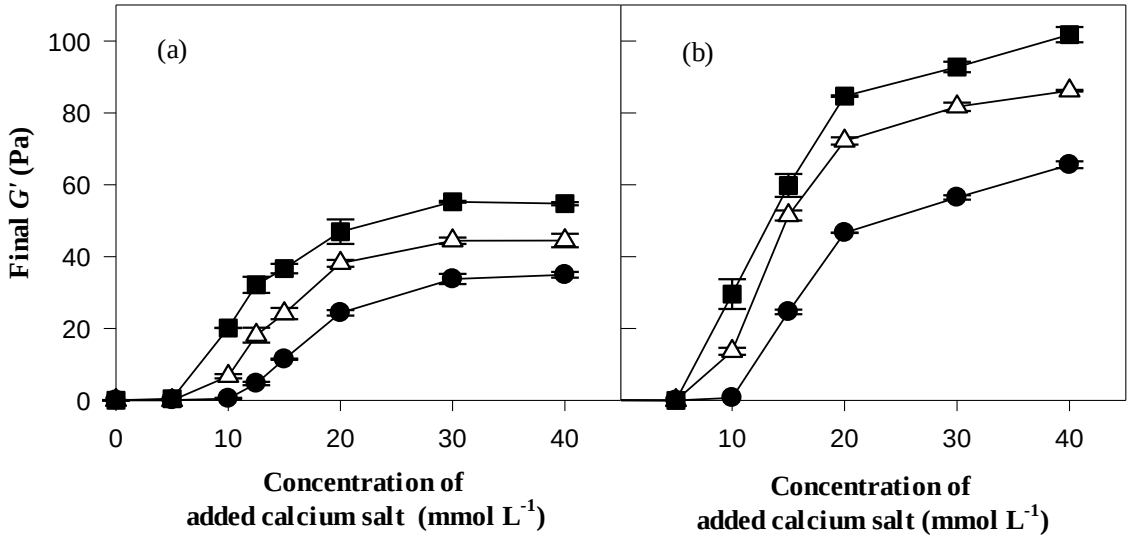


Figure 5-12: Comparison of final G' at 20°C of skim milk with various concentrations of added calcium chloride using the (a) HA cup and (b) SS cup. Skim milk with added calcium were heated in the rheometer at holding temperatures of 70°C (●), 80°C (△), and 90°C (■). Data points are mean values ± standard deviation (n = 2 to 3).

The final G' of the calcium-induced skim milk gels were higher using the SS cup (Figure 5-12). As mentioned in Section 3.11, the protocol was modified by using a higher rheometer set temperature for the SS cup. Once the desired holding temperature was achieved, the holding time was the same for both cups. As the SS cup heated up and cooled down at a slower rate, the sample was exposed to higher temperatures for a longer period than in the HA cup. The difference in the heating protocols may have contributed to the difference in the absolute G' values. Another reason for the higher G' obtained from the SS cup could be due to the difference in the diameter of the two cups (HA cup: 34 mm; SS cup: 30 mm). As G' is a stress component generated from the applied strain, the smaller gap between the geometry and SS could have resulted in a higher G' for the same sample (Rao, 2014). However, both cups gave the same trend in which increasing calcium concentration and increasing holding temperatures increased the final G'. This finding was confirmed

with the manufacturer where the trend using the two cups would remain the same, although the absolute G' values would be different. Therefore, a decision was made to replace the HA cup with the SS cup to reduce the problems with calcium deposits. It should be noted that the G' values were only compared between samples using the same cup for measurement.

5.7 Results and Discussion - Effect of pH at preheating

5.7.1 Calcium ion activity and pH

As the pH was not readjusted after preheating the skim milk at pH 6.40, 6.60, and 6.80, skim milk that was preheated at pH 6.40 had the lowest final pH after addition of both calcium chloride and calcium lactobionate (Figure 5-13c, d) Conversely, skim milk preheated at pH 6.8 had the highest final pH (Figure 5-13c, d). While pH was known to affect the calcium equilibrium and therefore $a_{Ca^{2+}}$ (Croguennec et al., 2016), the $a_{Ca^{2+}}$ values were not significantly different ($p < 0.05$) between preheating pH 6.40 and 6.60 (Figure 5-13a, b). However, at preheat pH 6.8, the $a_{Ca^{2+}}$ values were significantly lower ($p > 0.05$) than at preheat pH 6.40 and 6.60 with 20 to 40 $mmol^{-1}$ of calcium salt added (Figure 5-13a, b). A comparative study where skim milk was preheated at pH 6.60 and subsequently the pH adjusted to pH 6.40 and pH 6.80, or with no pH adjustment (pH 6.60) found a similar trend where the $a_{Ca^{2+}}$ values were generally not different between the three pHs at all added calcium salt concentrations (Figure 5-14a, b).

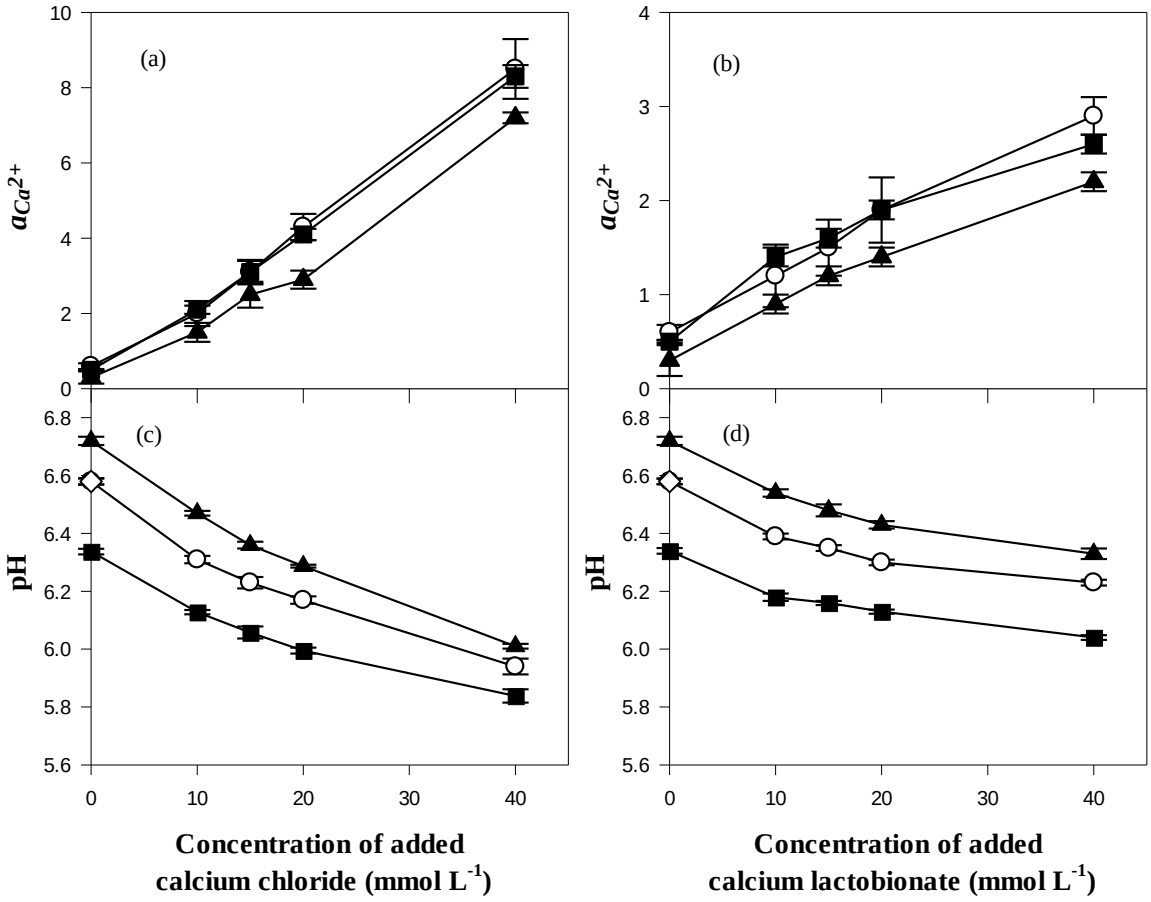


Figure 5-13: The effect of preheating skim milk at pH 6.4 (■), pH 6.6 (○) and pH 6.8 (▲) at 90°C for 10 min and subsequent addition of calcium chloride (a, c) and calcium lactobionate (b, d) on the calcium ion activity, $a_{Ca^{2+}}$ (a, b) and pH (b, d). Data points are mean values ± standard deviation (n= 6).

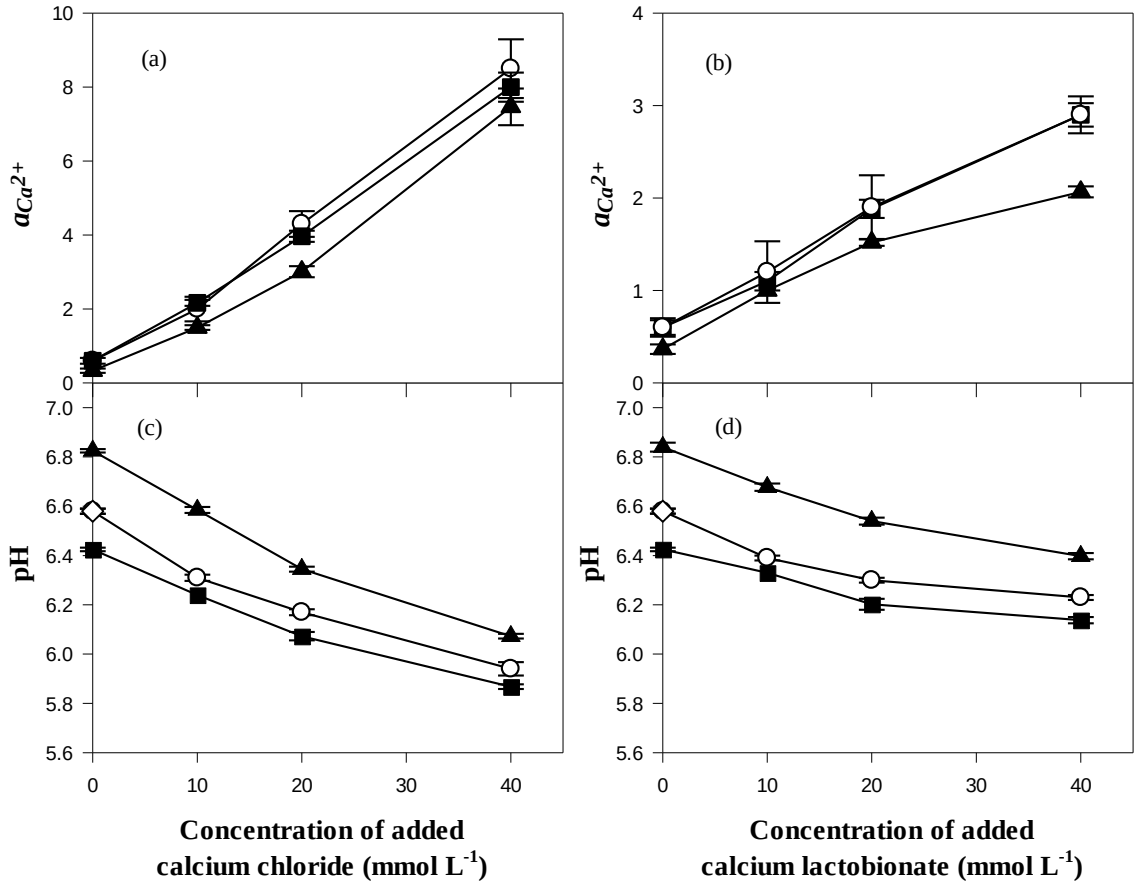


Figure 5-14: The effect of preheating skim milk at pH 6.6 at 90°C for 10 min with subsequent pH adjustment to pH 6.4 (■) and pH 6.8 (▲), with no adjustment (○) and with added calcium chloride (a, c) and calcium lactobionate (b, d) on the calcium ion activity, $a_{Ca^{2+}}$ (a, b) and pH (b, d). Data points are mean values ± standard deviation (n= 6).

5.7.2 Protein concentration in serum phase at different calcium concentrations

Preheating milk at different pH results in different amount of casein, β-lactoglobulin and α-lactalbumin in the serum phase (Anema & Klostermeyer, 1997; Donato & Dalgleish, 2006). In Chapter 4, the addition of different calcium salts was found to significantly decrease the concentration of casein in the serum phase. SDS-PAGE analysis was carried out to determine if the concentration of proteins in the serum phase was influenced by the preheating pH and calcium addition.

When no calcium salt was added, the concentration of casein in the serum phase increased significantly ($p < 0.05$) as preheating pH increased from 6.40 to 6.80 (Table 5-3). Visual assessment of the SDS-PAGE gel also showed that the intensity of the casein bands increased as preheating pH increased (Figure 5-15). The concentration of β-lactoglobulin in the serum also increased significantly ($p < 0.05$) with increasing preheating pH at when no calcium salt was added (Table 5-3). For example, the concentration of serum casein increased from 2.4 ± 0.1 mg g^{-1} to 4.6 ± 0.4 mg g^{-1}, and the concentration of β-lactoglobulin increased significantly ($p < 0.05$) from 0.8 ± 0.3 mg g^{-1} to 4.2 ± 0.1 mg g^{-1} when the preheating pH increased from pH 6.40 to 6.80 in skim milk with no calcium chloride added (Table 5-3). As discussed in Chapter 4, preheat treatment of skim milk at its native pH results in the association of denatured β-lactoglobulin with κ-casein on casein micelles, hence resulting in reduction in serum β-lactoglobulin after preheat treatment.

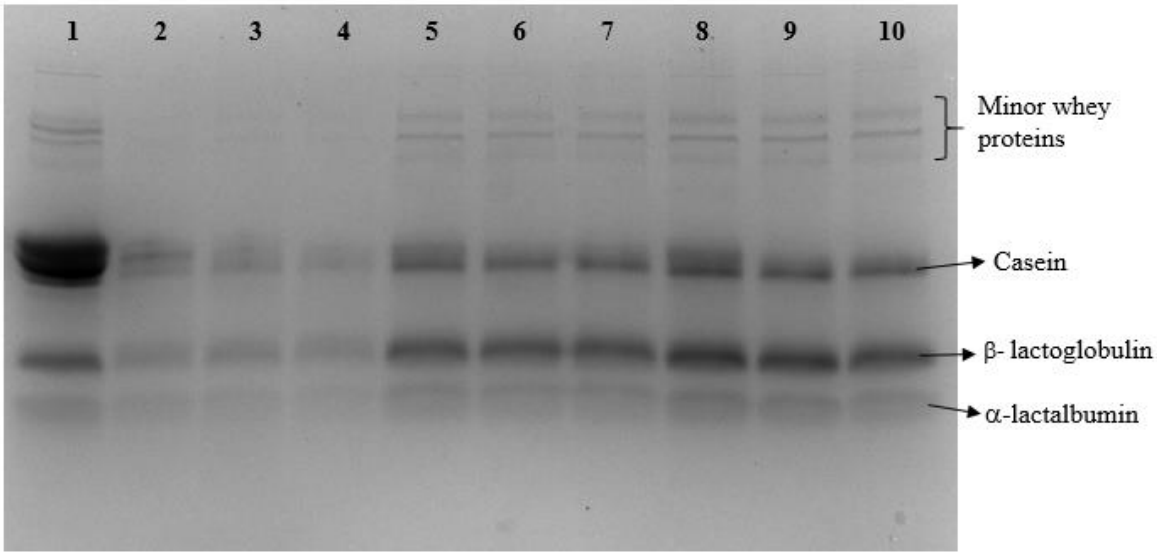


Figure 5-15: SDS-PAGE patterns of skim milk (lane 1) and serum of skim milk preheated at pH 6.4 (Lane 2 to 4), pH 6.6 (Lane 5 to 7) and pH 6.8 (Lane 8 to 10) with 0 mmol L^{-1} (Lanes 2, 5, 8), 20 mmol L^{-1} (Lane 3, 6, 9) and 40 mmol L^{-1} (Lane 4, 7, 10) of calcium chloride added.

Table 5-3: Concentration (mg g^{-1}) of casein, β-lactoglobulin, and α-lactalbumin in skim milk or serum phase of skim milk at various concentrations of calcium salt added determined by SDS-PAGE. Results presented are means ± standard deviation (n = 2 to 4). At each preheating pH, different superscript letters (a, b, c) indicate significant difference across the different concentration of added calcium salt. At each concentration of added calcium salt, different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different preheating pH (95% confidence level).

Calcium salt added	Preheat pH	Concentration of added calcium salt (mmol L^{-1})	Casein (mg g^{-1})	β-lactoglobulin (mg g^{-1})	α-lactalbumin (mg g^{-1})
Calcium chloride	6.4	0	2.4 ± 0.1 $^{a, \chi}$	0.8 ± 0.3 $^{a, \chi}$	0.3 ± 0.1 $^{a, \chi}$
		10	1.1 ± 0.1 $^{b, \chi}$	0.8 ± 0.1 $^{a, \chi}$	0.2 ± 0.1 $^{a, \chi}$
		20	1.2 ± 0.3 $^{b, \chi}$	0.7 ± 0.1 $^{a, \chi}$	0.1 ± 0.1 $^{a, \chi}$
		40	1.0 ± 0.3 $^{b, \chi}$	0.9 ± 0.2 $^{a, \chi}$	0.2 ± 0.1 $^{a, \chi}$
	6.6 (native pH)	0	3.9 ± 0.2 $^{a, \gamma}$	3.3 ± 0.3 $^{a, \gamma}$	0.8 ± 0.3 $^{a, \gamma}$
		10	2.4 ± 0.2 $^{b, \gamma}$	3.2 ± 0.1 $^{a, \gamma}$	0.8 ± 0.1 $^{a, \gamma}$
		20	2.3 ± 0.2 $^{b, \gamma}$	3.2 ± 0.2 $^{a, \gamma}$	0.7 ± 0.1 $^{a, \gamma}$
		40	2.2 ± 0.1 $^{b, \gamma}$	3.0 ± 0.2 $^{a, \gamma}$	0.7 ± 0.1 $^{a, \gamma}$
	6.8	0	4.6 ± 0.4 $^{a, \phi}$	4.2 ± 0.1 $^{a, \phi}$	1.1 ± 0.3 $^{a, \gamma}$
		10	3.1 ± 0.2 $^{b, \phi}$	4.3 ± 0.2 $^{a, \phi}$	0.9 ± 0.3 $^{a, \gamma}$
		20	2.5 ± 0.1 ^{c,}	3.9 ± 0.4 $^{a b,}$	0.8 ± 0.1 $^{a, \gamma}$
		40	2.3 ± 0.1 $^{c, \gamma}$	3.3 ± 0.3 $^{b, \gamma}$	0.9 ± 0.3 $^{a, \gamma}$
Calcium lactobionate	6.4	0	2.4 ± 0.1 $^{a, \chi}$	0.8 ± 0.3 $^{a, \chi}$	0.3 ± 0.1 $^{a, \chi}$
		10	1.2 ± 0.2 $^{b, \chi}$	1.1 ± 0.2 $^{a, \chi}$	0.4 ± 0.1 $^{a, \chi}$
		20	0.9 ± 0.1 $^{b, \chi}$	0.7 ± 0.3 $^{a, \chi}$	0.3 ± 0.2 $^{a, \chi}$
		40	0.9 ± 0.3 $^{b, \chi}$	0.8 ± 0.3 $^{a, \chi}$	0.1 ± 0.1 $^{a, \chi}$
	6.6 (native pH)	0	3.9 ± 0.2 $^{a, \gamma}$	3.6 ± 0.4 $^{a, \gamma}$	0.9 ± 0.3 $^{a, \gamma}$
		10	2.2 ± 0.2 $^{b, \gamma}$	3.2 ± 0.2 $^{a, \gamma}$	0.8 ± 0.2 $^{a, \gamma}$
		20	2.1 ± 0.3 $^{b, \gamma}$	3.3 ± 0.4 $^{a, \gamma}$	0.7 ± 0.1 $^{a, \gamma}$
		40	2.2 ± 0.1 $^{b, \gamma}$	3.3± 0.4 $^{a, \gamma}$	0.6 ± 0.2 $^{a, \gamma}$
	6.8	0	4.6 ± 0.4 $^{a, \phi}$	4.2 ± 0.1 $^{a, \phi}$	1.0 ± 0.3 $^{a, \gamma}$
		10	2.8 ± 0.4 $^{b, \phi}$	3.4 ± 0.3 $^{a, \gamma}$	0.7± 0.2 $^{a, \gamma}$
		20	2.5 ± 0.2 $^{b, \gamma}$	3.6 ± 0.5 $^{a, \gamma}$	0.8 ± 0.3 $^{a, \gamma}$
		40	2.6 ± 0.3 $^{b, \gamma}$	3.5 ± 0.1 $^{a, \gamma}$	0.6 ± 0.2 $^{a, \gamma}$

The trends in decreasing concentration of serum casein and β-lactoglobulin with increasing preheating pH were in agreement with the findings reported in literature (Anema & Klostermeyer, 1997; Donato & Dalgleish, 2006; Lakemond & van Vliet, 2008). Anema & Klostermeyer (1997) reported that on heating milks above 60°C for 15 min at lower preheating pH (6.3 to 6.5), the proportion of casein and β-lactoglobulin decreased in the serum phase when compared to milk preheated at its native pH. As preheating pH increases, higher amounts of β-lactoglobulin and α-lactoglobulin remained in the serum phase, along with increased dissociation of κ-casein from the casein micelle (Anema & Klostermeyer, 1997). Whey proteins denature on heating above 70°C, which results in aggregation amongst themselves and with κ-casein, via hydrophobic and sulphydryl-disulphide interactions (Anema et al., 2004b; Donato et al., 2007; Schorsch et al., 2001). The interaction between β-lactoglobulin and κ-casein was found to be influenced by the pH at which heating occurred (Anema et al., 2004b; Lakemond & van Vliet, 2008). Lakemond & van Vliet (2008) found that the percentage of β-lactoglobulin attached to casein micelles (via κ-casein) decreased with decreasing heating pH, while the percentage of aggregated β-lactoglobulin in the serum phase increased with increasing heating pH. The results observed in the present study could be explained by the difference in the distribution of the whey protein - κ-casein complex at different preheating pH. At lower preheating pH (pH < 6.5), the complex was likely attached the casein micelles, hence sedimenting with the casein micelles on centrifugation. At higher preheating pH (pH > 6.7), the complex was likely to be found in the serum phase along with soluble denatured whey proteins (Anema et al., 2004b).

The concentration of casein found in the serum phase at different preheating pH was also influenced by the concentration of added calcium salt (Table 5-3). At a low concentration of calcium salt addition (10 mmol L^{-1}), the concentration of casein in the serum phase increased significantly ($p < 0.05$) from 1.1 ± 0.1 to 3.1 ± 0.2 mg g^{-1} as preheating pH increased from pH 6.40 to pH 6.80 (Table 5-3). However, when addition of calcium salt increased to 20 mmol L^{-1} and 40 mmol L^{-1}, a significant increase in serum casein concentration was observed when preheating pH increased from pH 6.40 to pH 6.60 (at 20 mmol L^{-1}: increase from 1.2 ± 0.3 to 2.3 ± 0.2 mg g^{-1}), but no significant difference was observed from preheat pH 6.60 to pH 6.80 (at 20 mmol L^{-1}: from 2.3 ± 0.2 to 2.5 ± 0.2 mg g^{-1}). As discussed in Chapter 4, casein aggregation could occur through bindings with calcium ions, which may result in the sedimentation of the aggregated casein on centrifugation (Koutina et al., 2015; Swaisgood, 2003). At 10 mmol L^{-1} of calcium chloride addition, calcium ions available for binding in the system are limited. It is possible there may have been insufficient calcium ions available for binding with the higher concentration of casein in the serum phase at higher preheating pH 6.80, leading to the higher amount of casein remaining in the serum phase when 10 mmol L^{-1} of calcium salt was added. As concentration of calcium salt added increased to 20 mmol L^{-1} and 40 mmol L^{-1}, calcium ions available for binding in the system increased. Hence, there may have been sufficient calcium ions to bind with the serum caseins at higher preheating pH. The order of sensitivity of the casein subunits to calcium binding is α_{s2}-> α_{s1}-> β- > κ- casein (Farrell et al., 2004; Swaisgood, 2003). Therefore, casein remaining in the serum phase on calcium addition of up to 40 mmol L^{-1} could be casein subunits that are less sensitive to calcium bindings (e.g. κ-casein).

When the concentration of added calcium chloride or calcium lactobionate increased from 0 to 10 mmol L^{-1}, a significant decrease ($p < 0.05$) in serum casein was observed at all three preheating pH (Table 5-3). For example, the serum casein decreased from 2.4 ± 0.1 mg g^{-1} (0 mmol L^{-1} calcium chloride) to 1.1 ± 0.1 mg g^{-1} (10 mmol L^{-1} calcium chloride) for preheating pH 6.40, and from 4.6 ± 0.4 mg g^{-1} (0 mmol L^{-1} calcium chloride) to 3.1 ± 0.1 mg g^{-1} (10 mmol L^{-1} calcium chloride) for preheating pH 6.80. On further increase in calcium concentrations from 10 to 40 mmol L^{-1}, there was no significant decrease in serum casein at preheating pH 6.40 and 6.60, but a significant decrease in serum casein was observed when calcium salt added increased from 10 to 20 mmol L^{-1} at pH 6.8 (3.1 ± 0.2 mg g^{-1} to 2.5 ± 0.1 mg g^{-1}). As explained earlier, a higher concentration of casein was found in the serum phase when milk was preheated at pH 6.80 compared to at pH 6.40 and 6.60 (Table 5-3). A higher concentration of serum casein in the preheat pH 6.8 system may be available for interactions with calcium ions. Hence, increasing calcium salt addition resulted in a significant decrease in serum casein when skim milk was preheated at pH 6.80 (Table 5-3). The concentration of β-lactoglobulin and α-lactalbumin did not change significantly as the concentration of calcium salt added increased at all preheating pH for both calcium chloride and calcium lactobionate.

To determine the effect of pH, a separate study where skim milk was preheated at pH 6.6 and subsequently readjusted to pH 6.4, pH 6.8 or not readjusted (pH 6.6) was conducted. The results showed that when skim milk was preheated at the same pH (pH 6.6) with added calcium salts (10 to 40 mmol L^{-1}), the concentration of proteins remaining in the serum phase was not significantly different regardless of the pH to which the skim milk was

Table 5-4: Concentration (mg g^{-1}) of casein, β-lactoglobulin, α-lactalbumin of skim milk or serum phase of skim milk at various concentrations of calcium salt added determined by SDS-PAGE. Results presented are means ± standard deviation (n = 2 to 4). At each preheating pH, different superscript letters (a, b) indicate significant difference across the different concentration of added calcium salt. At each concentration of added calcium salt, different superscript Greek letters (χ, γ) indicate significant difference across the different preheating pH (95% confidence level).

Calcium salt added	pH readjusted to after preheating	Concentration of added calcium salt (mmol L^{-1})	Casein (mg g^{-1})	β-lactoglobulin (mg g^{-1})	α-lactalbumin (mg g^{-1})
Calcium chloride	6.4	0	$3.9 \pm 0.2^{a\,\chi}$	$3.2 \pm 0.2^{a\,\chi}$	$0.9 \pm 0.1^{a\,\chi}$
		10	$2.6 \pm 0.2^{b\,\chi}$	$2.9 \pm 0.2^{a\,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		20	$2.4 \pm 0.2^{b\,\chi}$	$2.8 \pm 0.3^{a\,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		40	$2.1 \pm 0.1^{b\,\chi}$	$2.4 \pm 0.2^{b\,\chi}$	$0.8 \pm 0.1^{a\,\chi}$
	6.6 (not readjusted)	0	$3.9 \pm 0.2^{a\,\chi}$	$3.3 \pm 0.3^{a\,\chi}$	$0.8 \pm 0.3^{a\,\chi}$
		10	$2.4 \pm 0.1^{b\,\chi}$	$3.2 \pm 0.1^{a\,\chi}$	$0.8 \pm 0.1^{a\,\chi}$
		20	$2.3 \pm 0.2^{b\,\chi}$	$3.2 \pm 0.2^{a\,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		40	$2.2 \pm 0.1^{b\,\chi}$	$3.0 \pm 0.2^{a\,\gamma}$	$0.7 \pm 0.1^{a\,\chi}$
	6.8	0	$3.8 \pm 0.3^{a\,\chi}$	$3.2 \pm 0.1^{a\,\chi}$	$0.6 \pm 0.1^{a\,\chi}$
		10	$2.7 \pm 0.2^{b\,\chi}$	$3.2 \pm 0.3^{a\,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		20	$2.7 \pm 0.1^{b\,\chi}$	$3.0 \pm 0.2^{a\,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		40	$1.9 \pm 0.1^{b\,\chi}$	$2.5 \pm 0.4^{b\,\chi}$	$0.6 \pm 0.1^{a\,\chi}$
Calcium lactobionate	6.4	0	$3.9 \pm 0.2^{a\,\chi}$	$3.2 \pm 0.2^{a\,\chi}$	$0.9 \pm 0.1^{a\,\chi}$
		10	$2.4 \pm 0.2^{b\,\chi\gamma}$	$3.2 \pm 0.2^{a\,\chi}$	$0.9 \pm 0.2^{a\,\chi}$
		20	$2.4 \pm 0.3^{b\,\chi}$	$3.4 \pm 0.2^{a\,\chi}$	$0.9 \pm 0.1^{a\,\chi}$
		40	$2.1 \pm 0.4^{b\,\chi}$	$3.0 \pm 0.3^{a\,\chi}$	$0.6 \pm 0.1^{a\,\chi}$
	6.6 (not readjusted)	0	$3.9 \pm 0.2^{a\,\chi}$	$3.6 \pm 0.4^{a,\gamma}$	$0.9 \pm 0.3^{a\,\chi}$
		10	$2.2 \pm 0.2^{b,\chi}$	$3.2 \pm 0.2^{a,\chi}$	$0.8 \pm 0.2^{a\,\chi}$
		20	$2.1 \pm 0.3^{b,\chi}$	$3.3 \pm 0.4^{a,\chi}$	$0.7 \pm 0.1^{a\,\chi}$
		40	$2.2 \pm 0.1^{b,\chi}$	$3.3 \pm 0.4^{a,\chi}$	$0.6 \pm 0.2^{a\,\chi}$
	6.8	0	$3.8 \pm 0.3^{a\,\chi}$	$3.2 \pm 0.1^{a\,\chi}$	$0.6 \pm 0.1^{a\,\chi}$
		10	$2.6 \pm 0.2^{b\,\gamma}$	$3.4 \pm 0.2^{a\,\chi}$	$0.8 \pm 0.3^{a\,\chi}$
		20	$2.5 \pm 0.4^{b\,\chi}$	$3.3 \pm 0.1^{a\,\chi}$	$0.7 \pm 0.3^{a\,\chi}$
		40	$2.3 \pm 0.3^{b\,\chi}$	$3.1 \pm 0.2^{a\,\chi}$	$0.7 \pm 0.3^{a\,\chi}$

readjusted to after the preheat treatment (Table 5-4). This indicated that the difference observed in the concentration of proteins in the serum phase in Table 5-3 was a result of the different preheating pH, not the effect of the pH of the skim milk.

5.7.3 Quantification of calcium in the sediment and serum phases of skim milk

To determine the changes in the calcium equilibrium in skim milk preheated at different pH values, quantification of calcium in the serum phase of the calcium-added skim milk was carried out. Addition of both calcium chloride and calcium lactobionate showed significant increase in both serum and sediment calcium at preheating pH 6.40, 6.60 and pH 6.80 (Table 5-5). Similar to the findings reported in Chapter 4, skim milk with added calcium lactobionate resulted in higher calcium in the serum phase regardless of the preheating pH. This was presumably due to the presence of CaL^+ ions in the serum phase of calcium lactobionate-added skim milk (Vavrusova, Munk & Skibsted, 2013). Unlike the Ca^{2+} ions which can interact with phosphate ions and precipitate as calcium phosphate in the casein micelles (Philippe et al., 2003), the CaL^+ ions may not be able to interact with phosphate ions and therefore remain in the serum phase.

At the same concentration of added calcium salt, increasing preheating pH decreased the calcium in the serum phase (Table 5-5). The pH of milk is known to influence the calcium equilibrium between the colloidal and serum calcium in milk (Gaucheron, 2005). The final pH of the skim milk was the highest in skim milk with the highest preheating pH (Figure 5-13). As discussed in Chapter 4 (Equation 4-1, 4-2), increasing pH leads to the $H_2PO_4^-$ dissociating into H^+ and HPO_4^{2-} ions to restore the equilibrium. With the HPO_4^{2-} increase in the system, the calcium equilibrium thus shifts towards the formation of insoluble $CaHPO_4$ between Ca^{2+} and HPO_4^{2-}. The resulting $CaHPO_4$ could either sediment on centrifugation,

or associate and sediment with the casein micelles on centrifugation (Lewis, 2011; Lucey et al., 1996).

When skim milk was preheated at pH 6.6 with subsequent pH adjustment to pH 6.4 and pH 6.8, the distribution of calcium between the serum and sediment also showed a similar trend where calcium in the serum phase decreased with increasing pH (Table 5-6). This could again be attributed to the shift in calcium equilibrium with change in skim milk pH as discussed earlier. The results in Table 5-6 suggests that the difference in the concentration of calcium in the serum phase at different preheating pH (Table 5-5) was likely due to the effect of the pH of skim milk, rather than an effect of the different pH at preheating of the skim milk.

Table 5-5: Concentration of calcium (mg g^{-1}) in skim milk, serum and sediment of skim milk at various concentrations and preheating pH (n = 3 to 6). At each preheating pH, different superscript letters (a, b, c, d) indicate significant difference across the different concentration of added calcium salt. At each concentration of added calcium salt, different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different preheating pH (95% confidence level).

Calcium salt added	Preheating pH	Concentration of added calcium salt (mmol L^{-1})	Mass of calcium added (mg g^{-1})	Total calcium in skim milk^{+} (mg g^{-1})	Serum calcium^{+} (mg g^{-1})	Sediment calcium* (mg g^{-1})
Calcium chloride	6.4	0	0	1.16	0.30 $^{a\chi}$	0.86 ^{a}
		10	0.4	1.56	0.54 $^{b\chi}$	1.02 ^{b}
		20	0.8	1.96	0.88 $^{c\chi}$	1.08 ^{c}
		40	1.6	2.76	1.52 $^{d\chi}$	1.24 ^{d}
	6.6 (native pH)	0	0	1.18	0.28 $^{a\chi}$	0.90 ^{a}
		10	0.4	1.60	0.52 $^{b\chi}$	1.08 ^{b}
		20	0.8	1.98	0.80 $^{c\gamma}$	1.18 ^{c}
		40	1.6	2.76	1.44 $^{d\gamma}$	1.32 ^{d}
	6.8	0	0	1.16	0.24 $^{a\gamma}$	0.92 ^{a}
		10	0.4	1.56	0.46 $^{b\gamma}$	1.10 ^{b}
		20	0.8	1.96	0.72 $^{c\phi}$	1.24 ^{c}
		40	1.6	2.76	1.28 $^{d\phi}$	1.48 ^{d}
Calcium lactobionate	6.4	0	0	1.16	0.30 $^{a\chi}$	0.86 ^{a}
		10	0.4	1.56	0.58 $^{b\chi}$	0.98 ^{b}
		20	0.8	1.98	0.92 $^{c\chi}$	1.06 ^{c}
		40	1.6	2.76	1.62 $^{d\chi}$	1.14 ^{d}
	6.6 (native pH)	0	0	1.18	0.28 $^{a\chi}$	0.90 ^{a}
		10	0.4	1.58	0.52 $^{b\gamma}$	1.06 ^{b}
		20	0.8	1.98	0.84 $^{c\gamma}$	1.12 ^{c}
		40	1.6	2.72	1.48 $^{d\gamma}$	1.24 ^{d}
	6.8	0	0	1.16	0.24 $^{a\gamma}$	0.92 ^{a}
		10	0.4	1.56	0.48 $^{b\gamma}$	1.08 ^{b}
		20	0.8	1.98	0.78 $^{c\phi}$	1.20 ^{c}
		40	1.6	2.78	1.46 $^{d\gamma}$	1.32 ^{d}

Pooled standard deviation: ± 0.02

$^{+}$ Concentration determined by EDTA titration

* Concentration determined by difference between milk and serum calcium

Table 5-6: Concentration of calcium (mg g^{-1}) in skim milk, serum and sediment of skim milk at various pH adjusted after preheating at pH 6.60 (n = 3 to 6). Different superscript letters (a, b, c, d) indicate significant difference across the calcium concentration added at each adjusted pH. Different superscript Greek letters (χ, γ, ϕ) indicate significant difference across the different adjusted pH at each calcium concentration (95% confidence level).

Calcium salt added	pH adjusted after preheating at pH 6.6	Concentration of added calcium salt (mmol L^{-1})	Mass of calcium added (mg)	Total calcium in skim milk^{+} (mg g^{-1})	Serum calcium^{+} (mg g^{-1})	Sediment calcium* (mg g^{-1})
Calcium chloride	6.4	0	0	1.16	0.30 $^{a\,\chi}$	0.86 ^{a}
		10	0.4	1.56	0.54 $^{b\,\chi}$	1.02 ^{b}
		20	0.8	1.94	0.84 $^{c\,\chi}$	1.10 ^{c}
		40	1.6	2.74	1.46 $^{d\,\chi}$	1.28 ^{d}
	6.6	0	0	1.16	0.28 $^{a\,\chi}$	0.88 ^{a}
		10	0.4	1.58	0.52 $^{b\,\chi}$	1.06 ^{b}
		20	0.8	1.96	0.80 $^{c\,\chi}$	1.16 ^{c}
		40	1.6	2.76	1.44 $^{d\,\chi}$	1.32 ^{d}
	6.8	0	0	1.18	0.26 $^{a\,\gamma}$	0.92 ^{a}
		10	0.4	1.54	0.42 $^{b\,\gamma}$	1.12 ^{b}
		20	0.8	1.94	0.68 $^{c\,\gamma}$	1.26 ^{c}
		40	1.6	2.74	1.22 $^{d\,\gamma}$	1.52 ^{d}
Calcium lactobionate	6.4	0	0	1.16	0.30 $^{a\,\chi}$	0.86 ^{a}
		10	0.4	1.56	0.64 $^{b\,\chi}$	0.92 ^{b}
		20	0.8	1.96	0.96 $^{c\,\chi}$	1.00 ^{c}
		40	1.6	2.74	1.62 $^{d\,\chi}$	1.12 ^{d}
	6.6	0	0	1.16	0.28 $^{a\,\chi\gamma}$	0.88 ^{a}
		10	0.4	1.56	0.52 $^{b\,\gamma}$	1.04 ^{b}
		20	0.8	1.98	0.84 $^{c\,\gamma}$	1.12 ^{c}
		40	1.6	2.76	1.48 $^{d\,\gamma}$	1.26 ^{d}
	6.8	0	0	1.18	0.26 $^{a\,\gamma}$	0.92 ^{a}
		10	0.4	1.54	0.48 $^{b\,\gamma}$	1.06 ^{b}
		20	0.8	1.96	0.78 $^{c\,\phi}$	1.18 ^{c}
		40	1.6	2.74	1.46 $^{d\,\gamma}$	1.28 ^{d}

Pooled standard deviation: ± 0.02

$^{+}$ Concentration determined by EDTA titration

* Concentration determined by difference between milk and serum calcium

5.7.4 Rheological properties of calcium-induced gelation in skim milk

Micelle-bound and serum denatured whey protein aggregates were found to modify the texture and rheological properties of acid gels differently (Anema et al., 2004b). As discussed in Section 5.7.2, the distribution of casein and whey proteins in the colloidal and serum phases varied depending on the preheating pH. Therefore, the effect of the distribution of proteins between the colloidal and serum phases on the rheological properties of the calcium-added skim milk was investigated by varying the preheating pH of the skim milk in the range of pH 6.40 to pH 6.80.

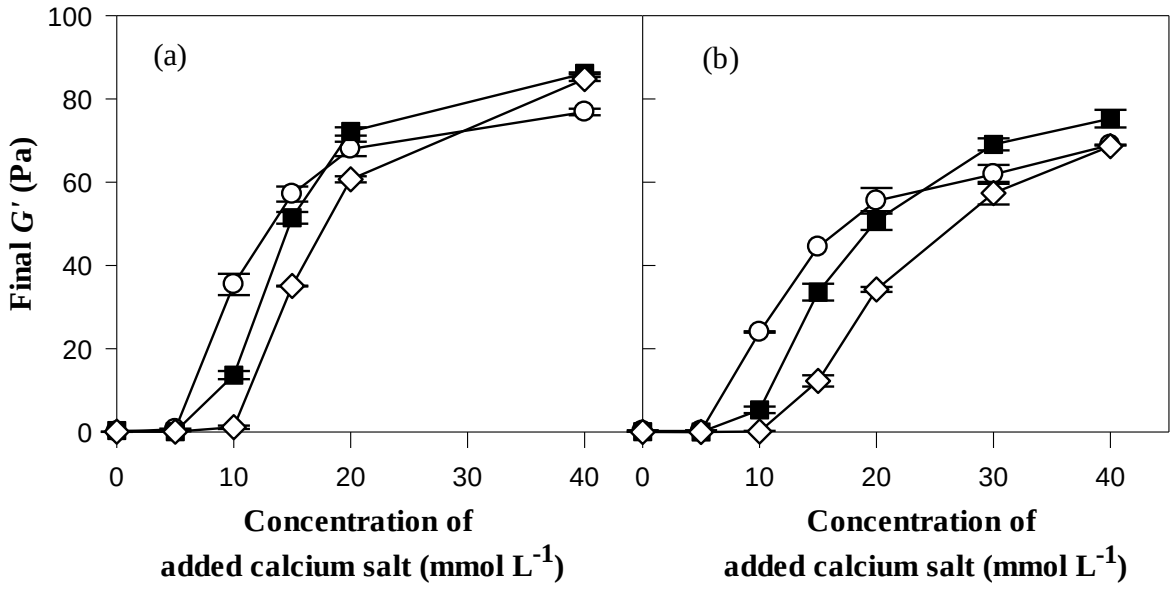


Figure 5-16: Relationship between final G' of calcium-induced skim milk gels preheated at pH 6.4 (○); 6.6 (native milk pH) (■); 6.8 (◇) and concentration of added (a) calcium chloride and (b) calcium lactobionate. Data points are mean values ± standard deviation (n = 2 to 4).

The pH at which skim milk was preheated resulted in different final G' of the skim milk gels (Figure 5-16). At lower concentrations of calcium salt added (10 to 15 mmol L^{-1}),

statistical analysis showed that the final G' of the skim milk decreased significantly when preheating pH increased from pH 6.40 to 6.80 ($p < 0.05$). Skim milk that was preheated at preheating pH 6.4 had the highest final $a_{Ca^{2+}}$ and lowest pH (Figure 5-13), both of which would favour association between the proteins and gelation in skim milk (Crowley et al., 2014; Tsioulpas et al., 2007). However, when the concentration of calcium salt was added at between 20 and 40 mmol L^{-1}, the final G' of the skim milk preheated at pH 6.40 began to plateau, while the final G' of skim milk preheated at pH 6.60 and pH 6.80 continued to increase (Figure 5-16). The samples at 40 mmol L^{-1} calcium salt addition were repeated four times and the result was found to be reproducible. At 40 mmol L^{-1} of added calcium chloride, skim milk that was preheated at pH 6.40 resulted in significantly lower ($p < 0.05$) final G' than skim milk preheated at pH 6.60 and pH 6.80 (Figure 5-16a). At 40 mmol L^{-1} of added calcium lactobionate, skim milk preheated at pH 6.4 also resulted in significantly lower final G' than at preheat pH 6.60 ($p < 0.05$), but no significant difference ($p < 0.05$) was observed in final G' values between preheat pH 6.40 and preheat pH 6.80 (Figure 5-16b).

To separate the effect of skim milk pH from the effect of preheating pH on the rheological properties of the calcium-induced skim milk gels, rheological measurements of skim milk preheated at pH 6.6 and subsequently readjusted to pH 6.40 and pH 6.80 prior to calcium salt addition was carried out. As shown in Figure 5-17 when skim milk was preheated at the original pH (pH 6.60), the final G' decreased significantly ($p <0.05$) as the pH of milk adjustment increased from pH 6.40 to 6.80 at between 10 to 20 mmol L^{-1} of added calcium chloride and calcium lactobionate. At 40 mmol L^{-1} of added calcium chloride and calcium lactobionate, the final G' of skim milk adjusted to pH 6.40 and 6.60 were not significantly

different, but the final G' of skim milk adjusted to pH 6.80 was significantly lower than than both pH 6.40 and 6.60 ($p < 0.05$). This result was in the same order as the final pH of the skim milk from the lowest to the highest (Figure 5-14). As discussed in Section 5.4, decreasing the pH of skim milk destabilises casein micelles and facilitates aggregation and gelation due to the decrease in net negative charge. Thus, the result observed in Figure 5-17 was likely due to a lower pH of the skim milk after calcium salt addition, leading to a higher final G'.

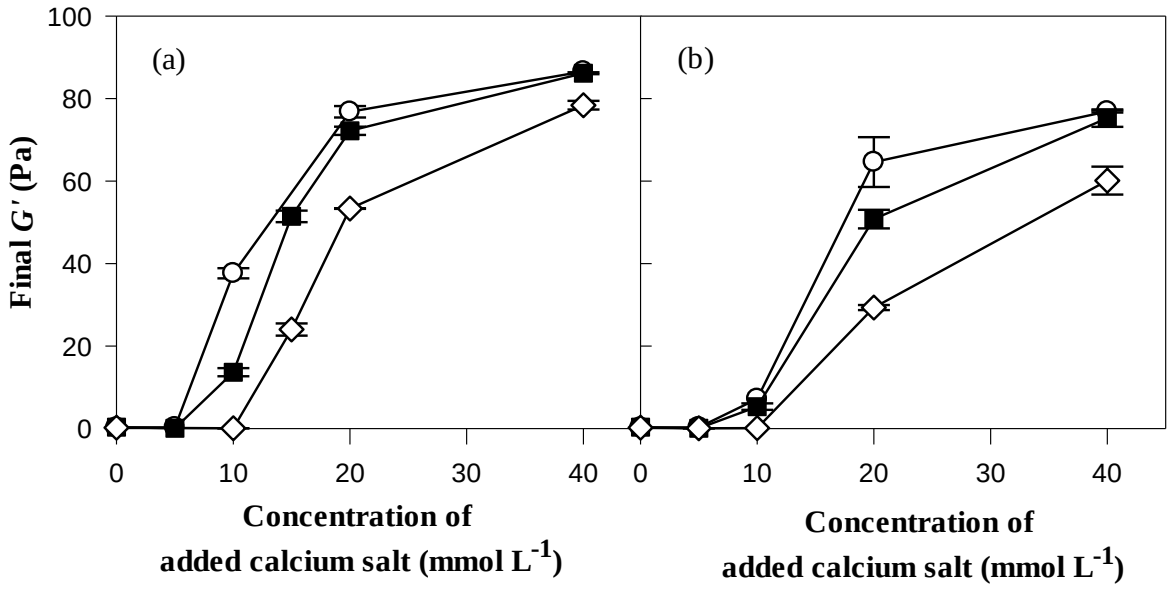


Figure 5-17: Relationship between final G' of calcium-induced skim milk gels preheated at pH 6.6 and subsequent pH adjustment to 6.4 (○); 6.8 (◇) and with no pH adjustment (■) with concentration of added (a) calcium chloride and (b) calcium lactobionate. Data points are mean values ± standard deviation (n = 2 to 3).

A comparison between the skim milk samples that were preheated at different pH (Figure 5-16), and skim milk samples that were preheated at pH 6.60 and subsequently pH adjusted (Figure 5-17) suggests that the effect of preheating pH on the final G' of the calcium-

induced skim milk gel may be dependent on the calcium concentration. When the skim milks were preheated at the original pH (i.e. pH 6.60), the final G' of the skim milk gels followed the predicted trend where a lower final pH resulted in a higher final G' from 10 to 40 mmol L^{-1} of added calcium salt (Figure 5-17). The same trend in skim milk that was preheated at different pH was only observed at lower concentrations of added calcium salt (10 to 15 mmol L^{-1}). At higher concentrations of calcium salt addition (20 to 40 mmol L^{-1}), skim milk that was preheated at pH 6.40, which had the lowest final pH (Figure 5-13c, d) did not result in the highest final G'. The results indicated that the distribution of the protein between the colloidal and serum phase, a consequence of preheating milk at different pH, may influence the gelation behaviour in the calcium-induced skim milk gels.

The influence of the distribution of the proteins between the colloidal and serum phases on the gelation of the calcium-induced skim milk gels appears to be dependent on the concentration of calcium salt added (Figure 5-16). As discussed in Section 5.7.2, a higher proportion of whey proteins were found in the colloidal phase at lower preheating pH (pH 6.40), possibly due to the complexation between whey proteins and κ-casein on the casein micelles. At higher preheating pH (pH 6.80), a higher proportion of the whey protein was found in the serum phase, likely as soluble denatured whey protein aggregates and/ or whey protein-κ-casein complexes that were dissociated from the casein micelles. The G' of a gel is dependent on the number and strength of contact points between the aggregated particles within a defined area of the structure (Anema, Lee, Lowe & Klostermeyer, 2004a). In the skim milks preheated at pH 6.40, there may be fewer available aggregating particles as the aggregation will likely occur only between the whey-protein associated casein micelles during heating in the rheometer. On the other hand, in the skim milks preheated at pH 6.80,

the presence of soluble whey protein aggregates and dissociated whey protein - κ-casein complexes in the serum phase could provide more sites for aggregation and the potential for a more complex gel network (Anema et al., 2004a). The results observed in Figure 5-16 may be due to the balance between the number of aggregating particles (soluble particles and casein micelles) and calcium ions available for interactions in the system. When a lower concentration of calcium salt was added to the skim milk (10 to 15 mmol L^{-1}), the number of calcium ions available for interaction in the system may be the limiting factor. Hence, in the skim milk with lower added calcium salts and preheated at pH 6.40, the added effect of H^+ ions (due to a lower final pH) on charge neutralisation would aid in contributing to a higher final G' than skim milk preheated at pH 6.60 and pH 6.80 (Figure 5-16). As calcium concentration increased to above 15 mmol L^{-1}, the number of available calcium ions that were able to interact with the proteins increased. There may then be sufficient calcium ions to interact with the higher number of aggregating particles in skim milk preheated at a higher pH, thus resulting in a better connected gel network and higher final G'.

The hypothesis that the effect of preheating pH was dependent on the concentration of calcium salt added was supported by the results of the change in G' during heating and holding in the rheometer (Figure 5-18, Figure 5-19). The difference in gelation temperature and G' after heating to 80°C when the added calcium salt increased from 20 to 40 mmol L^{-1} was larger in skim milk preheated at pH 6.80 than those preheated at pH 6.40 (Figure 5-18, Table 5-7). For example, for skim milk preheated at pH 6.40, the gelation temperature reduced by 3.8°C from 77.5 ± 0.7°C to 73.8 ± 0.4°C when the added calcium chloride increased from 20 to 40 mmol L^{-1} (Table 5-7). Skim milk preheated at pH 6.80 resulted in a

6.5°C decrease in gelation temperature (from 81.0 ± 0.1°C to 74.5 ± 0.1°C) when the added calcium chloride increased from 20 to 40 mmol L^{-1} (Table 5-7). The difference in the G' after heating to 80°C between 20 mmol L^{-1} (4.85 ± 0.26 Pa) and 40 mmol L^{-1} (7.21 ± 0.12 Pa) added calcium chloride in skim milk preheated at pH 6.40 was 2.36 Pa. In contrast, the difference in the G' after heating to 80°C between 20 mmol L^{-1} (1.78 ± 0.01 Pa) and 40 mmol L^{-1} (6.77 ± 0.33 Pa) added calcium chloride in skim milk preheated at pH 6.80 was 4.99 Pa. During holding, it was observed that the rate of development of the gel network (indicated by the increase in G') was not significantly different ($p > 0.05$) between 20 and 40 mmol L^{-1} of added calcium chloride for skim milk preheated at 6.40 (Figure 5-19, Table 5-7). On the other hand, increasing the preheating pH to 6.80 resulted in significantly higher ($p < 0.05$) rate of development of gel network between between 20 and 40 mmol L^{-1} of added calcium chloride (Figure 5-19, Table 5-7). A similar trend was observed for calcium lactobionate-added samples (Figure 5-18, Figure 5-19, Table 5-7). The results suggest that at higher concentrations of added calcium salt (> 15 mmol L^{-1}), the development of the gel network was more favourable with increasing preheating pH due to the increased number of aggregating particles participating in the gel network. In contrast, when the skim milk was preheated at the original pH (skim milk preheated at the pH 6.6 and subsequently pH-adjusted), it was presumed that there was a similar number of aggregating particles in the system, thus the strength gel network followed the trend for pH as shown in Figure 5-14.

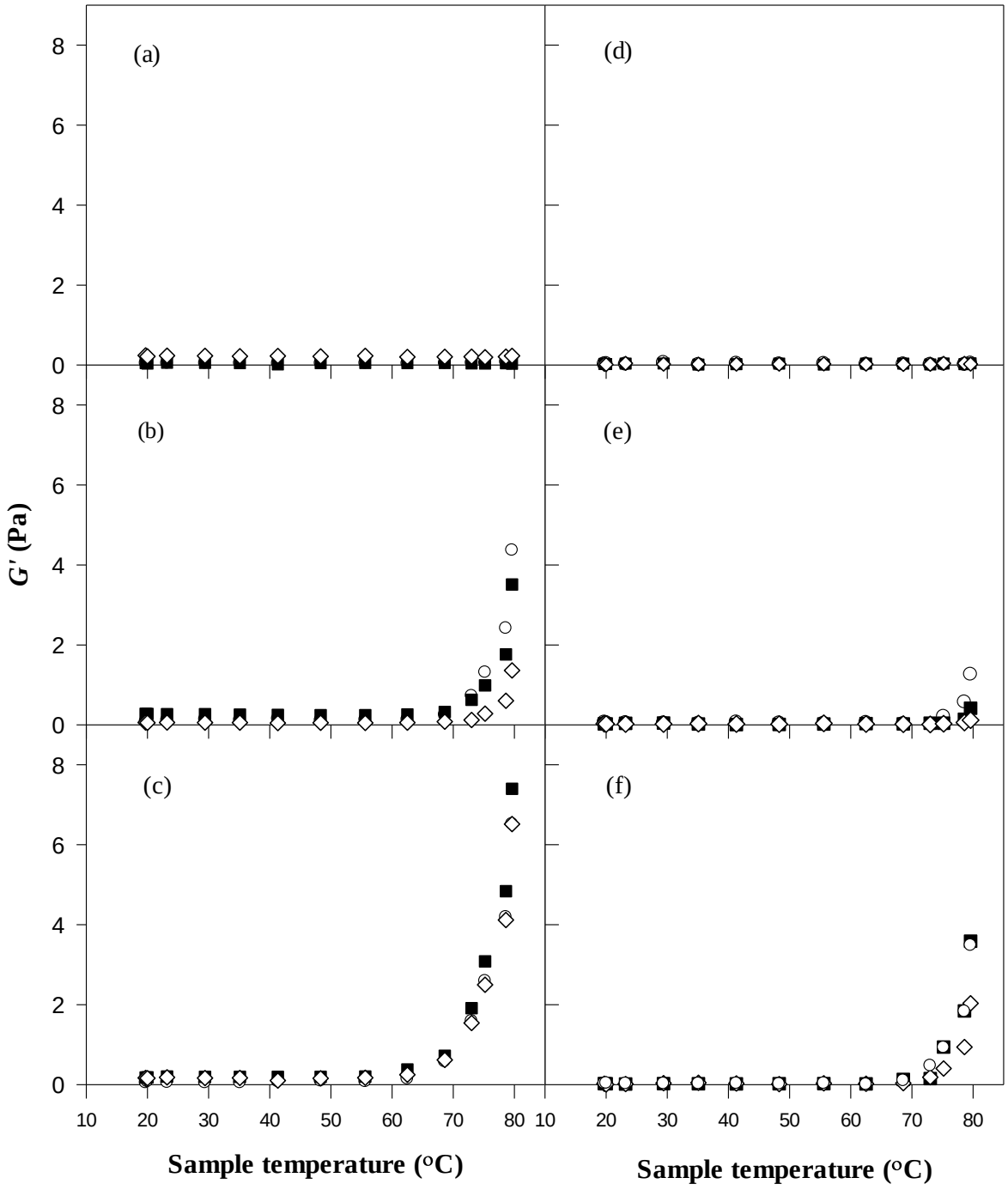


Figure 5-18: Typical plots showing the change in *G′* during heating from 20°C to 80°C for skim milk with 10 mmol L^{-1} (a, d), 20 mmol L^{-1} (b, e) and 40 mmol L^{-1} (c, f) of added calcium chloride (a, b, c) and calcium lactobionate (d, e, f), preheated at pH 6.4 (○); 6.6 (native milk pH) (■); 6.8 (◇).

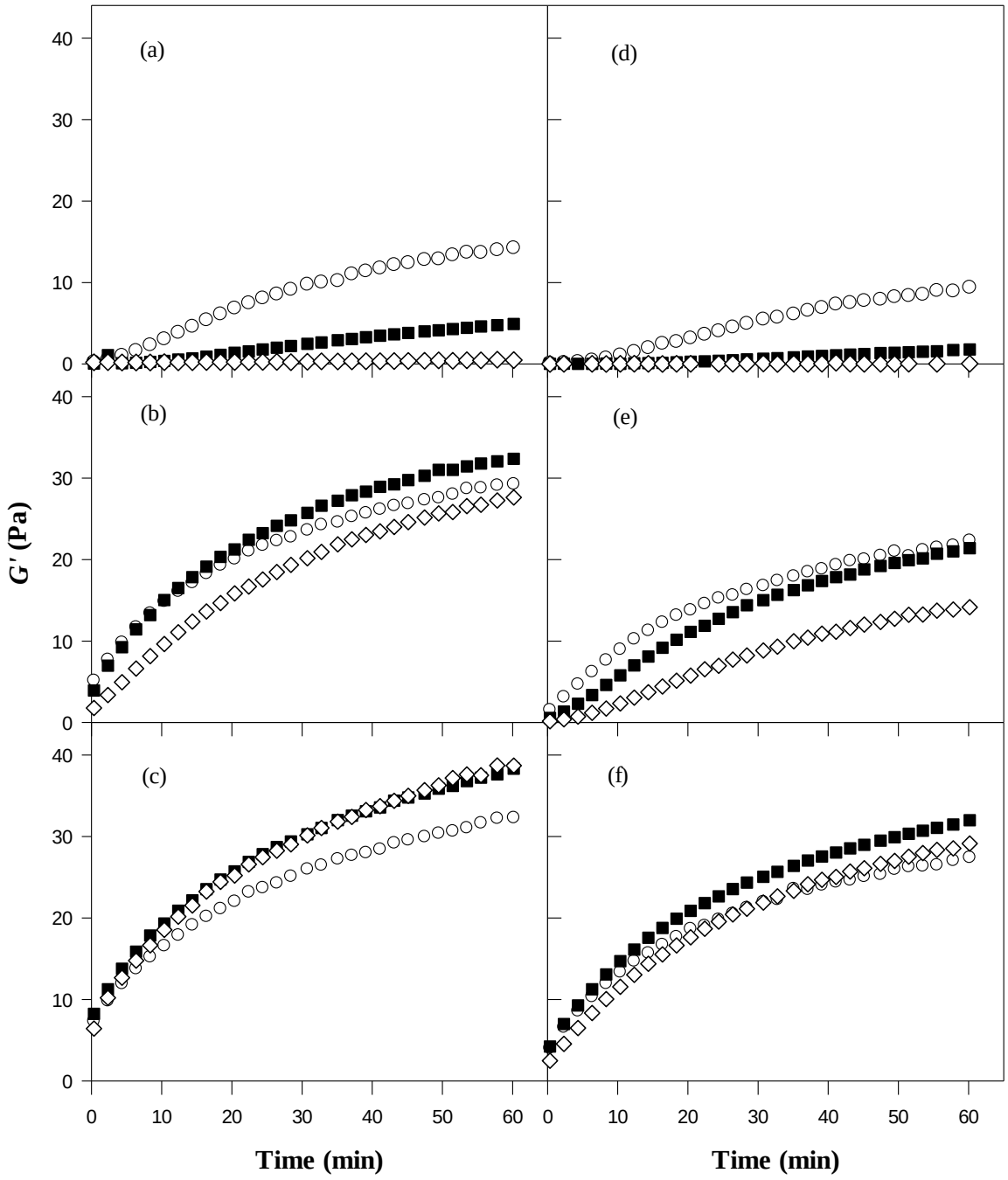


Figure 5-19: Typical plots showing the change in G' during holding at 80°C for 60 minutes for skim milk with 10 mmol L^{-1} (a, d), 20 mmol L^{-1} (b, e) and 40 mmol L^{-1} (c, f) of added calcium chloride (a, b, c) and calcium lactobionate (d, e, f), preheated at pH 6.4 (○); 6.6 (native milk pH) (■); 6.8 (◇).

Table 5-7: Effect of concentration of calcium salt added and preheating pH on the gelation temperature and G' after heating to 80°C in skim milk with added calcium chloride and calcium lactobionate. Results presented are mean ± standard deviation (n = 3). Different letters (a, b) indicate significant difference across the preheating pH at each concentration of added calcium salt. Different superscript Greek letters (χ, γ) indicate significant difference across the different concentration of added calcium salt at the same preheating pH(95% confidence level).

Calcium salt added	Concentration of calcium salt added (mmol L^{-1})	Preheating pH	Gelation temperature[+] (°C)	G' after heating to 80°C (Pa)	Rate of G' development in first 20 min of holding[#] (Pa / min)
Calcium chloride	10	6.4	80.0 (holding)$^{\phi}$	< 1	$0.33 \pm 0.01^{a\chi}$
		6.6	80.0 (holding)$^{\phi}$	< 1	$0.06 \pm 0.01^{b\chi}$
		6.8	DNG[*]	< 1	DNG
	20	6.4	$77.5 \pm 0.7^{a\chi}$	$4.85 \pm 0.26^{a\chi}$	$0.75 \pm 0.01^{a\gamma}$
		6.6	$78.3 \pm 0.1^{b\chi}$	$4.22 \pm 0.43^{a\chi}$	$0.85 \pm 0.03^{b\gamma}$
		6.8	$81.0 \pm 0.1^{b\chi}$	$1.78 \pm 0.01^{b\chi}$	$0.70 \pm 0.01^{a\chi}$
	40	6.4	$73.8 \pm 0.4^{a\gamma}$	$7.21 \pm 0.12^{a\gamma}$	$0.74 \pm 0.02^{a\gamma}$
		6.6	$73.4 \pm 0.7^{a\gamma}$	$8.15 \pm 0.15^{b\gamma}$	$0.87 \pm 0.01^{b\gamma}$
		6.8	$74.5 \pm 0.1^{a\gamma}$	$6.77 \pm 0.33^{a\gamma}$	$0.94 \pm 0.03^{b\gamma}$
Calcium lactobionate	10	6.4	80.0 (holding)$^{\phi}$	< 1	$0.20 \pm 0.01^{\chi}$
		6.6	> 80.0	< 1	DNG
		6.8	> 80.0	< 1	DNG
	20	6.4	$81.1 \pm 0.2^{\chi}$	$1.73 \pm 0.27^{\chi}$	$0.74 \pm 0.01^{a\gamma}$
		6.6	80.0 (holding)$^{\phi}$	< 1	$0.55 \pm 0.05^{b\chi}$
		6.8	DNG	< 1	$0.29 \pm 0.02^{c\chi}$
	40	6.4	$78.7 \pm 0.3^{a\gamma}$	$4.11 \pm 0.14^{a\gamma}$	$0.74 \pm 0.02^{a\gamma}$
		6.6	78.7 ± 0.3^{a}	4.07 ± 0.20^{a}	$0.87 \pm 0.01^{b\gamma}$
		6.8	79.9 ± 0.3^{b}	2.72 ± 0.36^{b}	$0.90 \pm 0.03^{b\gamma}$

[+]Gelation temperature refers to temperature required to achieve G' of 1 Pa

[#]Calculations can be referred to in Appendix 4

$^{\phi}$Gelation occurred during holding at 80°C

[*]DNG: did not gel

5.8 Conclusions

The results in this chapter demonstrated that the temperature and pH of skim milk during gelation are both important parameters in determining the rheological properties of the calcium-induced skim milk gels. Increasing the holding temperature increases the final G' of the gels due to increased frequency of collision and increased calcium ion binding properties of the proteins. The results of skim milk with and without preheat treatment suggests that the denaturation of whey proteins prior to calcium addition and gelation could influence the rheological properties of the gel. In addition, it appears that the pH of the skim milk at which preheating is carried out may influence the rheological properties of the calcium-induced skim milk gels by altering the amount of aggregating particles available for participation in the gel network. At lower concentration (10 mmol L^{-1}) of added calcium salts, a lower preheating pH will result in stronger gels. At higher concentration (10 mmol L^{-1}) of added calcium salts, gelation was favoured when skim milk was preheated at higher pH. The results in this chapter indicated that the influence of casein and whey proteins on the gel network may be dependent on H^+ and Ca^{2+} ions available in the system to facilitate bond formation. Further work on the contribution of casein and whey proteins to the gel network is investigated in Chapter 6.

Chapter 6 - Effect of casein and whey protein on the formation of calcium-induced skim milk gels

6.1 Introduction

The interactions between calcium and casein and whey proteins in milk can influence the stability and gelation properties of the proteins (Croguennec et al., 2004; De Kruif, 1999; Philippe, Graët & Gaucheron, 2005; Pitkowski, Nicolai & Durand, 2009; Zittle, DellaMonica, Rudd & Custer, 1957). Interactions between calcium and caseinates or casein micelles were reported to induce aggregation and improve renneting properties of casein micelles (Dalgleish, 1983; Dalgleish & Parker, 1980; Gaucheron, Graet, Boyaval & Piot, 1997; Sandra et al., 2012; Smialowska, Matia-Merino, Ingham & Carr, 2017). In heat-set and cold-set whey protein gels, the addition of calcium has been shown to promote aggregation and gelation (Pappas & Rothwell, 1991; Riou et al., 2011; Zittle et al., 1957). More recent published data suggest that there may be competition between casein and whey proteins in the binding of calcium ions (Nguyen et al., 2016). In milk systems, varying the levels of casein and whey protein was reported to alter the microstructure and rheological properties of acidified and heated milk (Singh, Chandrapala, Udabage, McKinnon & Augustin, 2015; Zhao, Wang, Tian & Mao, 2016). The results in Chapter 5 suggested that both casein and whey proteins participated in the structural formation of the calcium-induced skim milk gel. The aim of this chapter was to determine the contribution of casein and whey proteins towards the gelation of the calcium-added skim milk.

6.2 Materials and Methods

Solutions comprising pure whey proteins were prepared to eliminate the effect of casein. To study the effect of casein, skim milk was microfiltered to deplete the whey proteins in the skim milk. Skim milk blends with different concentrations of casein and whey proteins were prepared and various concentrations of calcium salt were added to determine if there was preferential binding of the calcium ions to casein or whey proteins. The effects of whey protein denaturation and the interaction of the denatured whey proteins with casein on gelation were investigated by comparing preheated and non-preheated skim milk blends with different concentrations of casein and whey proteins.

6.2.1 Preparation of whey protein solutions

Skim milk permeate from ultrafiltration was prepared as described in Section 3.12 using a membrane with a nominal MWCO of 10 kDa. The pH and calcium ion activity of the permeate were 6.64 ± 0.02 and 0.6 ± 0.1, respectively. The permeate obtained was analysed using SDS-PAGE as described in Section 3.7 and found to contain no casein or whey proteins.

Whey protein isolate (WPI) was obtained from Fonterra New Zealand (93.9% protein, 0.3% fat, < 1.5% carbohydrate, 4.8% moisture, 1.7% ash (*w/w*)). SDS-PAGE analysis found that the residual casein in the WPI was 1.5 ± 0.3% of total protein (*w/w*) (i.e. 92.5% whey proteins in WPI (*w/w*)). WPI was reconstituted in the skim milk permeate at 0.72%, 1.44% and 2.4% of whey protein (*w/w*). The WPI-added permeate solutions (hereinafter called WPI solutions) were stirred for 30 min under magnetic stirring and stored at 20°C for at least 10 hours to ensure full hydration of the WPI before use.

Preparation of the calcium-added WPI solutions was carried out using the same procedure as for calcium added to skim milk preparations (Section 3.4 and 3.5). The WPI solutions were preheated from 20 to 90°C within 5 min in a 94°C water bath (GD100, Grant Instruments Ltd, Cambridge, England), then held at 90 ± 2°C for 10 min. After 10 min, the WPI solutions were cooled to 20 ± 2 °C within 5 min in a 4 ± 2°C water bath. The calcium chloride and calcium lactobionate stock solutions were added after the preheated WPI solutions had been cooled to 20°C, with final added calcium concentrations between 0 and 40 mmol L^{-1}. Distilled water was added to the WPI solutions to achieve final whey protein concentrations of 0.6%, 1.2% and 2.0% (*w/w*). The WPI concentrations were determined by calculations from the percentage of whey proteins in the WPI as determined by SDS-PAGE. The total whey proteins in the reconstituted skim milk (9.6% total solids) was 0.6% (*w/w*). Hence, the WPI solutions was investigated at 0.6% whey protein to determine the contribution of whey proteins to gelation in the absence of casein micelles. The percentage of whey protein was also increased to 1.2% and 2.0% to determine the effect of increasing whey protein concentration. A summary of the preparation of WPI solutions is shown in Table 6-1.

Table 6-1: Percentage of added WPI and percentage of total whey proteins in WPI solutions before and after calcium salt addition.

Percentage of WPI in WPI solution before calcium salt addition (% *w/w*)	Percentage whey protein in WPI solution before calcium salt addition (% *w/w*)	Final percentage whey protein in WPI solution after calcium salt addition (% *w/w*)
0.78	0.72	0.60
1.56	1.44	1.20
2.60	2.40	2.00

6.2.2 Preparation of skim milk blends with different concentrations of casein and whey proteins

Skim milk and whey protein-depleted skim milk (WDSM) were blended to prepare skim milk blends with different concentrations of casein and whey proteins. Skim milk and WDSM were prepared according to the methods described in Sections 3.2, 3.13 and 3.14, respectively.

Table 6-2: Comparison of the protein compositions of skim milk and whey protein-depleted skim milk (WDSM) as determined by SDS-PAGE. Results presented are mean ± standard deviation (n = 5 for skim milk, n = 3 for WDSM).

	Protein (mg/ g)					
	Casein	**β-lactoglobulin**	**α-lactalbumin**	**Other whey proteins**	**Casein (%)**	**Whey proteins (%)**
Skim milk	26.9 ± 0.2	5.1 ± 0.2	1.3 ± 0.2	0.2 ± 0.1	80.3	19.7
WDSM	27.6 ± 0.6	0.8 ± 0.3	0.0 ± 0.0	0.6 ± 0.1	95.2	4.8

The pH and calcium ion activity of the WDSM were 6.62 ± 0.01 and 0.7 ± 0.1, respectively. A comparison of the protein compositions of skim milk before microfiltration and the WDSM is shown in Table 6-2.

The skim milk and WDSM obtained was analysed using SDS-PAGE as described in Section 3.8. The average percentage of casein and whey protein relative to the total protein in skim milk was 80.3 ± 0.2% and 19.7% ± 0.2 (*w/w*), respectively. The WDSM comprised of an average of 95.2 ± 0.6% casein, 4.8 ± 0.6% whey protein relative to the total protein in the solution (Table 6-2). Skim milk was prepared at 3.5% and 3.0% total protein (*w/w*) and blended with the WDSM and distilled water to achieve final skim milk with different

percentages of casein and whey proteins as shown in Table 6-3. The total protein of the WDSM prepared according to Section 3.14 was 2.90%. The lower total protein in the WDSM was expected due to the removal of whey proteins and minor casein fractions in the serum phase/ small casein micelles during diafiltration. Hence, the total protein in all the skim milk blends were standardised at 2.90% (*w/w*).

Table 6-3: Quantity of skim milk and whey protein-depleted skim milk (WDSM) added to achieve skim milk blends with different concentrations of casein and whey proteins. Total protein in all skim milk blends were 2.90% (*w/w*).

Ratio of casein/ whey protein in total protein	**WDSM (g)**	**Skim milk (3.0% protein) (g)**	**Skim milk (3.5% protein) (g)**	**Water (g)**	**Casein (g/ 100 g skim milk blend)**	**Whey protein (g/ 100 g skim milk blend)**
95%/ 5%	100	0	0	0	2.76	0.14
93%/ 7%	87.1	0	12.2	0.7	2.70	0.20
90%/ 10%	63.5	36.5	0	0	2.61	0.29
85%/ 15%	24.6	73.8	0	1.7	2.47	0.44
80%/ 20%	0	96.7	0	3.3	2.37	0.53

The skim milk blends were preheated from 20 to 90°C in 5 min in a waterbath, then held at 90 ± 2°C for 10 min. After 10 min, the skim milk was cooled to 20 ± 2 °C within 5 min. Calcium chloride and distilled water were added to the WDSM. Addition of 40 mmol L^{-1} of calcium chloride to WDSM with total protein of 2.90% (*w/w*) using calcium stock solutions prepared as described in Section 3.3 will result in a final total protein concentration of 2.40% (*w/w*). Therefore, the total protein of the final calcium-added skim milk was kept constant at 2.40% (*w/w*) for all samples.

6.2.3 Flow curves of whey protein solutions

It was observed after preheating WPI solutions that gelation occurred prior to heating to 80°C for rheological assessment. Flow curves of the WPI solutions were thus determined to investigate the properties of the solutions before heating in the rheometer. Flow curves were obtained using a rheometer (Discovery HR-3, TA Instrument, New Castle, USA) fitted with starch pasting cell geometry (cup diameter: 37.02 mm; bob diameter: 32.40 mm, bob length: 12.00 mm). The samples were poured into the cup and set to equilibrate for 1 min, followed by a pre-shear step for 1 min at 0.1 s^{-1}. For flow curve measurements, the shear rate was increased from 0.5 to 50 s^{-1} and the shear stress results were recorded. Flow curve measurements were conducted at 20 ± 2°C and initiated at 8 min after calcium salts were added.

6.2.4 Analysis of skim milk

This chapter determines the effect of casein and whey proteins on the calcium-induced gelation of skim milk. The assays and techniques used in this chapter can be found in Section 3.6 to 3.11. All samples were preheated at 90°C for 10 min before all the analysis, unless otherwise stated. For the rheological measurement in Section 3.10, the SS cup was used. The holding temperature in the rheometer was 80°C (sample temperature) for all samples. Rheological measurements of WPI solutions commenced at 8 min after calcium salt was added.

6.3 Results and Discussion

The results and discussion on the contribution of casein micelles and whey protein to the gelation of calcium-added skim milk is separated into three parts. In the first part, the results from the pure whey protein system (WPI solutions) are presented to determine the

gelation properties of whey proteins and added calcium in the absence of casein micelles. The second part of the results examines the effect of varying ratios of casein to whey proteins in calcium-added skim milk blends on the rheological properties. Finally, an overall discussion on the proposed mechanism of the interactions between the two proteins and calcium ions is presented.

6.3.1 Calcium ion activity and pH of WPI solutions

Prior to any addition of calcium salt, preheat treatment of the WPI solutions at 90°C for 10 min with various concentrations of whey protein resulted in significant reductions in calcium ion activity (a_{Ca}^{2+}) and pH ($p < 0.05$) (Table 6-4). Heat treatment is known to result in the precipitation of calcium ions as calcium phosphate, resulting from a reduction in calcium phosphate solubility with increasing temperature (Jeurnink & De Kruif, 1995; van Boekel et al., 1989). This may explain why the calcium ion activity was reduced after heat treatment. Binding of the calcium ions to whey proteins may also take place during heating (Jeyarajah & Allen, 1994; Riou et al., 2011), hence reducing the calcium ion concentration and therefore a_{Ca}^{2+}. Reduction in pH could be attributed to the thermal degradation of lactose to organic acids. This occurs when lactose isomerises to lactulose, and subsequently degrades into several compounds including formic acid (Berg & van Boekel, 1994). The reduction in pH on heating could also be attributed to the precipitation of primary ($H_2PO_4^-$) and secondary (HPO_4^{2-}) calcium phosphate as tertiary calcium phosphate (PO_4^{3-}) with the concomitant release of H^+ ions (Fox, 1981; Fox & McSweeney, 1998; Walstra et al., 1999). The reduction in pH was the greatest with no whey protein present (i.e. skim milk permeate) and the least with the highest concentration of whey protein present (2.0% *w/w*) (Table 6-4). The difference in pH in the various protein systems

could be attributed to the pH buffering capacity of proteins. Proteins may buffer the pH of the system by protonation and de-protonation of the amino acids with basic and acidic side chains (Salaün, Mietton & Gaucheron, 2005; Upreti, Bühlmann & Metzger, 2006). Thus, systems with higher percentages of proteins may have higher pH buffering capacity, which leads to a smaller reduction in the pH of the skim milk after preheat treatment.

Table 6-4: The calcium ion activity and pH of WPI solutions before and after preheating at 90°C for 10 min (n = 6). At each WPI solution condition (before or after preheat treatment), different superscript letters (a, b, c, d) indicate significant difference in across the various whey protein concentrations. At each whey protein concentration, different superscript Greek letters (χ, γ) indicate significant difference before and after preheat treatment (95% confidence level).

Whey protein concentration (%)	a_{Ca}^{2+}		pH	
	Before preheat	After preheat	Before preheat	After preheat
0	$0.6 \pm 0.1^{a, \chi}$	$0.3 \pm 0.1^{a, \gamma}$	$6.68 \pm 0.01^{a, \chi}$	$6.27 \pm 0.01^{a, \gamma}$
0.6	$0.5 \pm 0.1^{b, \chi}$	$0.3 \pm 0.1^{a, \gamma}$	$6.68 \pm 0.01^{ab, \chi}$	$6.34 \pm 0.01^{b, \gamma}$
1.2	$0.5 \pm 0.1^{b, \chi}$	$0.3 \pm 0.1^{a, \gamma}$	$6.66 \pm 0.01^{ab, \chi}$	$6.43 \pm 0.01^{c, \gamma}$
2.0	$0.5 \pm 0.1^{b, \chi}$	$0.3 \pm 0.1^{a, \gamma}$	$6.65 \pm 0.01^{b, \chi}$	$6.50 \pm 0.01^{d, \gamma}$

When calcium chloride and calcium lactobionate were added at the same concentration to the skim milk and WPI solutions, the a_{Ca}^{2+} of skim milk and WPI solutions with different concentrations of whey protein were not significantly different ($p > 0.05$), indicating that the percentage of protein or whether casein micelles were present did not influence the a_{Ca}^{2+} (Figure 6-1a, b). On the other hand, the pH of the WPI solutions decreased significantly with decreasing concentration of whey proteins at the same concentration of calcium salt added (Figure 6-1c, d). As explained, the difference in the pH at different concentrations of whey protein could be due to the buffering capacity of the whey proteins. With increasing concentration of calcium chloride and calcium lactobionate added, both

WPI solutions and skim milk systems decreased in pH (Figure 6-1c, d). This observation could be explained by the calcium equilibrium where the added calcium ions interacts with the free phosphate and citrate ions in the serum phase, thus releasing H^+ ions (Croguennec et al., 2016; Gaucheron, 2005).

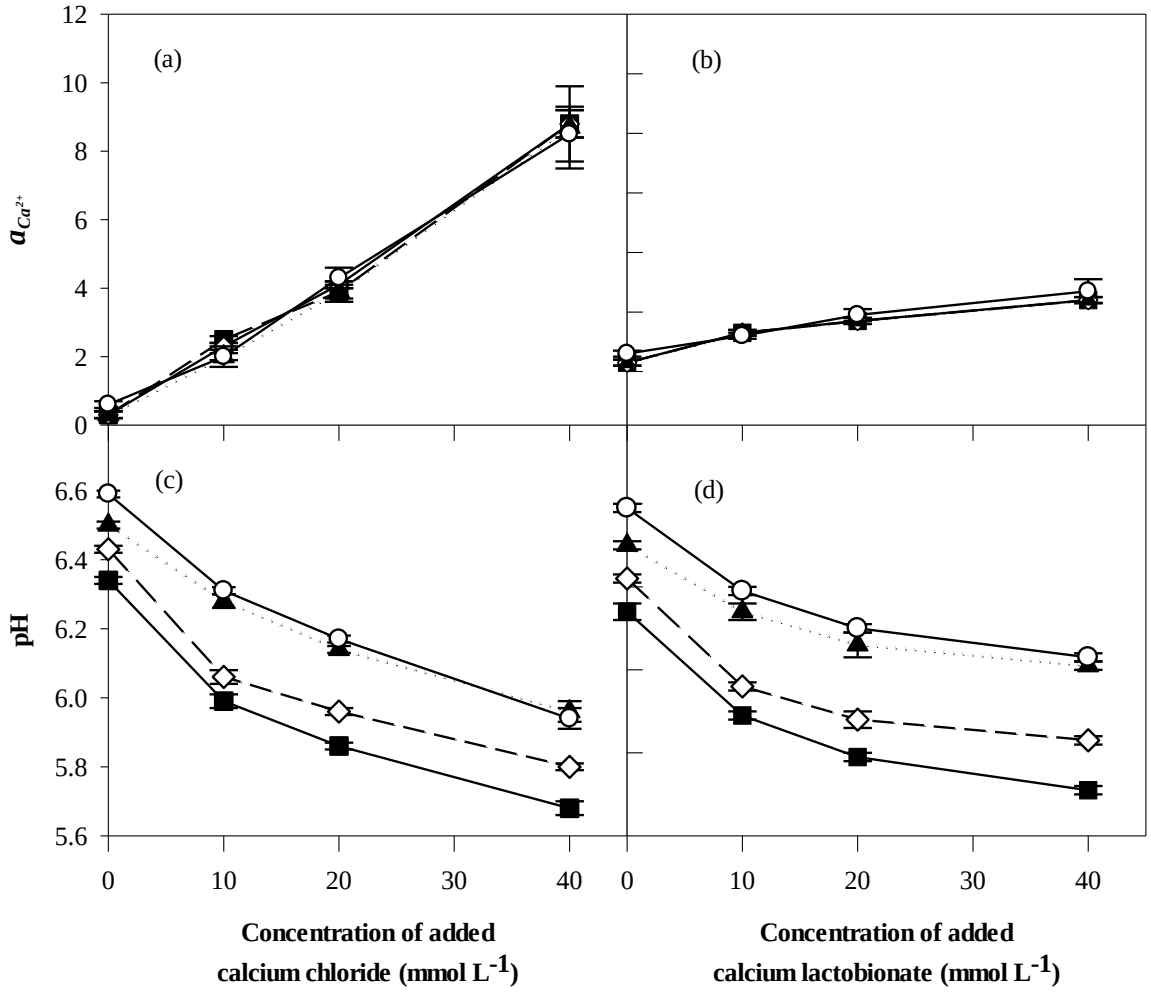


Figure 6-1: The (a, b) calcium ion activity and (c, d) pH of 0.6% (—■—), 1.2% (---◇---), 2.0% (·····▲·····) WPI solution and skim milk (—○—) after preheat treatment at 90°C for 10 min and with added (a, c) calcium chloride and (b, d) calcium lactobionate at various concentrations of calcium salt added. Data points are mean values ± standard deviation (n= 6).

6.3.2 Quantification of calcium in serum and sediment phases in WPI solutions

The concentration of calcium in the WPI solutions and in their serum phases separated from these solutions by centrifugation at 21,000 *g* for 90 min was investigated to determine if calcium salts were binding to whey proteins as any interactions may influence the rheological properties of the calcium-induced gel.

The concentration of calcium in the serum phase decreased significantly ($p < 0.05$) with increasing concentration of whey proteins in the WPI solutions (Table 6-5). Conversely, the concentration of calcium in the sediment increased with increasing percentage of whey proteins. The results indicated that binding of calcium to the whey proteins may have occurred, which resulted in the calcium sedimenting with the whey proteins on centrifugation. The two principal whey proteins in milk, β-lactoglobulin and α-lactalbumin, bind to calcium ions via the carboxyl groups (Pappas & Rothwell, 1991). Native α-lactalbumin is known to bind strongly to calcium ions (one calcium ion per mole α-lactalbumin) (Brew, 2003; Edwards et al., 2009). However, little information is available on the binding capacity of α-lactalbumin to added calcium ions, although added calcium chloride had been suggested to limit the extent of the unfolding of α-lactalbumin during heat treatment (Riou et al., 2011). Studies have shown that denatured whey proteins bind to calcium ions via electrostatic interactions, resulting in a charge screening effect or calcium bridging which leads to the gelation of whey proteins (Jeyarajah & Allen, 1994; Phan-Xuan et al., 2014; Zittle et al., 1957).

Table 6-5: Concentration of calcium (mg g^{-1}) in WPI solution, serum and sediment of WPI solution preheated at 90°C for 10 min, with different casein and whey protein percentages at various concentrations of added calcium salts (n = 3). At the same concentration of added calcium chloride or added calcium lactobionate, different superscript letters (a, b, c, d) indicate significant difference across the WPI solutions with different percentage of whey protein (95% confidence level).

Concentration of calcium salt added (mmol L^{-1})	**Percentage of whey protein (%)**	**Mass of calcium added (mg)**	**Total calcium in WPI solution^{+} (mg g^{-1})**	**Serum calcium^{+} (mg g^{-1})**	**Sediment calcium* (mg g^{-1})**
0	0	0	0.30 ± 0.02	Nil [#]	Nil [#]
Calcium chloride					
0	0.6	0	0.30 ± 0.02	0.18 ± 0.02 [a]	0.12 ± 0.02 [a]
0	1.2	0	0.30 ± 0.02	0.18 ± 0.02 [a]	0.12 ± 0.02 [a]
0	2.0	0	0.30 ± 0.02	0.18 ± 0.02 [a]	0.12 ± 0.02 [a]
20	0.6	0.8	1.08 ± 0.04	0.86 ± 0.04 [a]	0.22 ± 0.04 [a]
20	1.2	0.8	1.06 ± 0.04	0.76 ± 0.06 [b]	0.30 ± 0.06 [ab]
20	2.0	0.8	1.08 ± 0.06	0.74 ± 0.06 [b]	0.34 ± 0.06 [b]
40	0.6	1.6	1.86 ± 0.04	1.56 ± 0.08 [a]	0.30 ± 0.08 [a]
40	1.2	1.6	1.86 ± 0.04	1.50 ± 0.04	0.36 ± 0.04 [a]
40	2.0	1.6	1.84 ± 0.08	1.46 ± 0.06 [b]	0.38 ± 0.06 [b]
Calcium lactobionate					
20	0.6	0.8	1.06 ± 0.02	0.88 ± 0.02 [a]	0.18 ± 0.02 [a]
20	1.2	0.8	1.06 ± 0.02	0.86 ± 0.02	0.20 ± 0.02 [a]
20	2.0	0.8	1.06 ± 0.03	0.80 ± 0.02 [b]	0.26 ± 0.02 [b]
40	0.6	1.6	1.86 ± 0.03	1.48 ± 0.04 [a]	0.38 ± 0.02 [a]
40	1.2	1.6	1.88 ± 0.02	1.44 ± 0.10 [a]	0.44 ± 0.02 [a]
40	2.0	1.6	1.86 ± 0.06	1.36 ± 0.04 [b]	0.50 ± 0.02 [b]

Pooled standard deviation: ± 0.02

[+] Concentration determined by EDTA titration

[*] Concentration determined by difference between milk and serum calcium

[#] No sedimentation after centrifugation at 21,000 *g* for 90 min

6.3.3 Flow curves of WPI solutions

Gelation was observed in the test tubes after calcium salts were added to the preheated and cooled WPI solutions. Gelation of whey proteins after the addition of calcium salts to preheated whey proteins, called cold-set whey protein gelation, has been reported (Ako et al., 2010; Kuhn, Cavallieri & da Cunha, 2010). Flow curves for the WPI solutions were

obtained at 20°C to determine the effect of preheat treatment and calcium salt addition on the flow behaviour of the WPI solutions.

For non-preheated WPI solutions with no added calcium salts, the flow curves showed that the increase in shear stress with increasing shear rate followed a similar shape, regardless of the concentration of WPI (Figure 6-2). After preheating WPI solutions with no added calcium salt, the shear stress at a shear rate of 50 s^{-1} increased significantly for all whey protein concentrations (Table 6-6). For example, in the 2.0% WPI solution, the shear stress increased from 1.76 to 5.82 Pa before and after preheating at a shear rate of 50 s^{-1}. Whey proteins denature and unfold during heat treatment at > 70°C, thus resulting in aggregation via intermolecular disulphide bonds and hydrophobic interactions (Loveday, 2016; Walstra et al., 2006). The increase in the shear stress of the WPI solutions after preheating could be attributed to the aggregation of the denatured whey proteins. The increase in shear stress after preheating was higher in WPI solutions with higher concentrations of whey proteins (Figure 6-5, Table 6-2), likely due to smaller inter-particle distances and therefore increased susceptibility to protein aggregation (Mudgal et al., 2011).

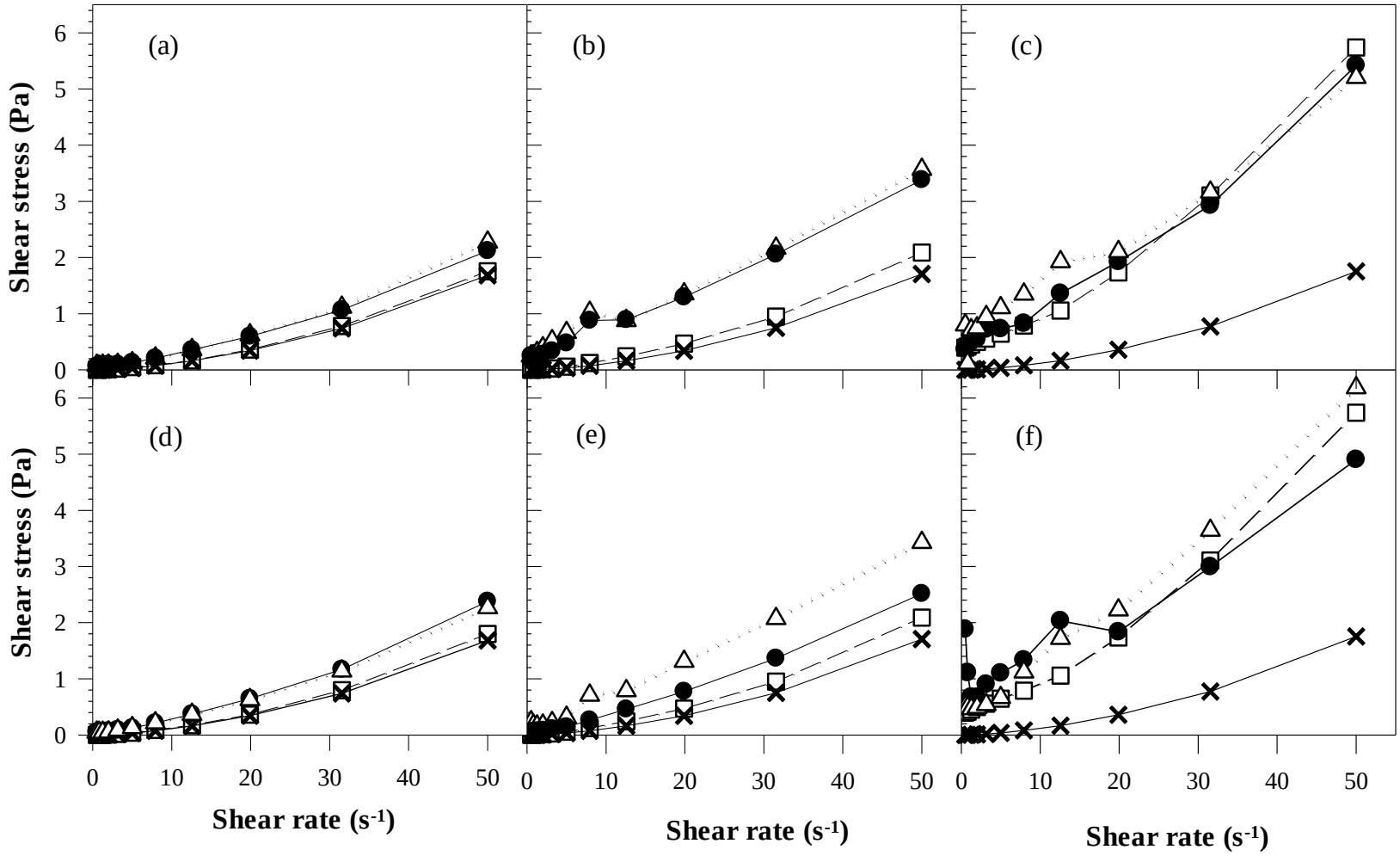


Figure 6-2: Typical flow curves for (a, d) 0.6%, (b, e) 1.2% and (c, f) 2.0% WPI solutions with no preheat (——×——); preheated at 90°C for 10 min (---□---); preheated + 20 mmol L^{-1} (——●——) and preheated + 40 mmol L^{-1} (·····△·····) of calcium chloride (a, b, c) and calcium lactobionate (d, e, f) added (n= 2).

Table 6-6: Shear stress of WPI solutions with 0.6% to 2.0% whey protein at a shear rate of 50 s^{-1}. WPI solutions were either non-preheated, or preheated at 90°C for 10 min, and with added calcium chloride or calcium lactobionate at various concentrations (n =2). At the same whey protein concentration, different superscript letters (a, b) represent significant difference in the shear stress with different concentration of added calcium salt added. At the same whey protein concentration and calcium salt added, different superscript Greek letters (χ, γ) represent significant difference in the shear stress in the non-preheated and preheated samples. Data presented are mean ± standard deviation (95% confidence level).

Whey protein concentration (%)	Concentration of calcium salt added (mmol L^{-1})	Shear stress at 50 s^{-1} (Pa)		
			Preheated	
		Non-preheated	Calcium chloride	Calcium lactobionate
0.6	0	1.69 ± 0.02 $^{a, \chi}$	1.77 ± 0.02 $^{a, \gamma}$	1.77 ± 0.02 $^{a, \gamma}$
	20		2.24 ± 0.05 ^{a}	2.29 ± 0.04 ^{b}
	40		2.28 ± 0.23 ^{a}	2.32 ± 0.08 ^{b}
1.2	0	1.70 ± 0.01 $^{a, \chi}$	2.05 ± 0.06 $^{a, \gamma}$	2.05 ± 0.06 $^{a, \gamma}$
	20		3.58 ± 0.01 ^{b}	2.76 ± 0.35 ^{ab}
	40		3.37 ± 0.02 ^{b}	3.40 ± 0.04 ^{b}
2.0	0	1.76 ± 0.01 $^{b, \chi}$	5.82 ± 0.11 $^{a, \gamma}$	5.82 ± 0.11 $^{a, \gamma}$
	20		5.31 ± 0.15 ^{a}	5.13 ± 0.32 ^{a}
	40		5.30 ± 0.13 ^{a}	6.03 ± 0.21 ^{a}

A significant increase ($p < 0.05$) in shear stress at 50 s^{-1} was observed when the WPI solutions were preheated, regardless of the whey protein concentration (Table 6-6). The results indicated that preheat treatment resulted in aggregation of the whey proteins, which led to solutions with higher shear stress than non-preheated WPI solutions. Addition of calcium chloride to preheated WPI solutions exhibited significantly higher shear stress ($p < 0.05$) at a shear rate of 50 s^{-1} for 1.2% whey proteins, but not for solutions containing 0.6% and 2.0% whey proteins (Table 6-6). There was no significant increase ($p < 0.05$) in shear stress at a shear rate of 50 s^{-1} on further increase in added calcium chloride concentration from 20 to 40 mmol L^{-1}, regardless of the concentration of whey protein (Table 6-6).

Addition of calcium lactobionate resulted in a significant increase ($p < 0.05$) in shear stress at a shear rate of 50 s^{-1} for WPI solutions with 0.6% and 1.2% whey proteins, but not for solutions containing 2.0% whey proteins (Table 6-6). Addition of calcium salts had a significant effect on the shear stress of WPI solutions before heat treatment in the rheometer at lower whey protein concentrations, but at higher whey protein concentrations (2.0%), no significant difference in shear stress was observed on calcium salt addition.

6.3.4 Rheological properties of WPI solutions

A comparison of the rheological properties of the WPI solutions with skim milk was carried out to elucidate the contribution of whey proteins to the gelation occurring in calcium-added skim milks. During heating from 20°C to 80°C, 0.6% WPI solution showed no change in G' for all calcium concentrations added (Figure 6-3a). On the other hand, gelation occurred in skim milk ($G' > 1$ Pa) from approximately 70°C with 20 mmol L^{-1} and 40 mmol L^{-1} of calcium chloride addition, but no gelation was observed at 10 mmol L^{-1} calcium chloride addition (Figure 6-3d). Absence of gelation in 0.6% WPI solution suggested that in skim milk, whey protein is not the primary component responsible for gelation. This could be because at 0.6% whey protein, the protein level may not be sufficient for gelation to occur, or that the presence of casein may be required for gelation to occur. When the concentration of whey proteins was increased to 1.2%, gelation did not occur with no addition of calcium chloride (Figure 6-3b). However, gelation was observed during heating to 80°C in the 1.2% WPI solutions with 10 to 40 mmol L^{-1} added calcium chloride. In 1.2% WPI solutions with 20 and 40 mmol L^{-1} added calcium chloride, the G' was noted to be > 1 Pa at 20°C. As the WPI solutions were preheated prior to the heating step in the rheometer, this result indicated that the denatured whey proteins in the 1.2%

WPI solutions gelled with calcium salt addition, prior to heating in the rheometer to 80°C (Figure 6-3b). Gelation was observed in the 2.0% WPI solutions at 20°C without addition of calcium chloride and with calcium chloride up to 40 mmol L^{-1} (Figure 6-3c).

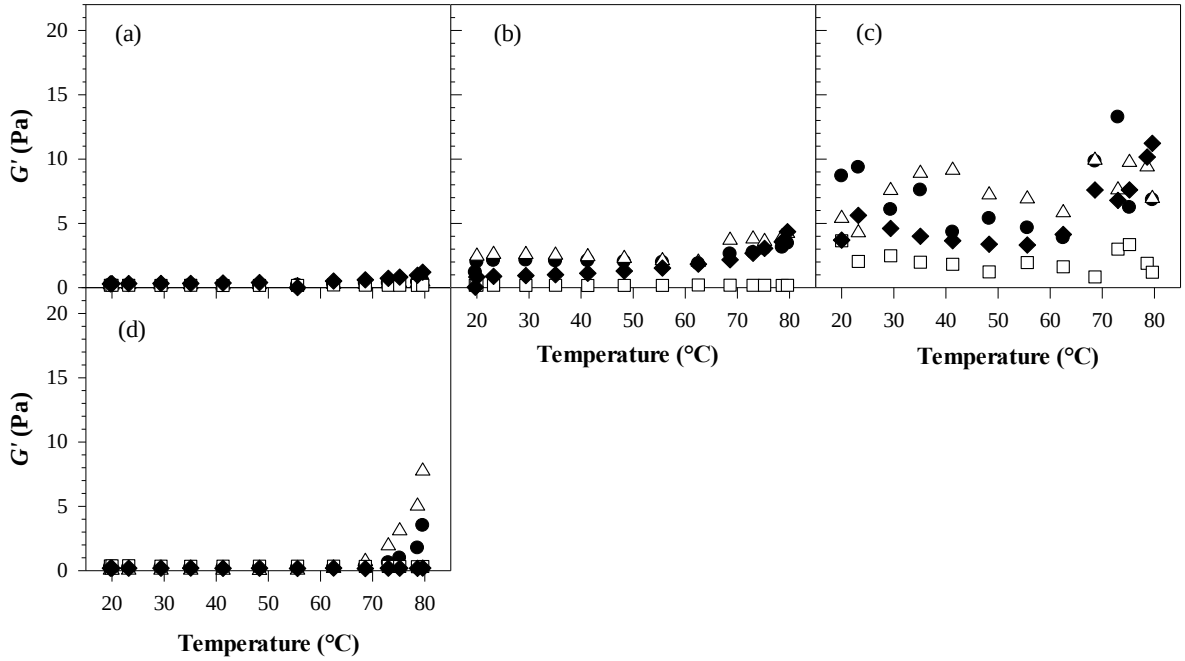


Figure 6-3: Typical plots for *G′* during heating from 20°C to 80°C for preheated (a) 0.6% (b) 1.2%, (c) 2.0% WPI solutions and (d) skim milk with 0 mmol L^{-1} (□);10 mmol L^{-1} (◆), 20 mmol L^{-1} (●) and 40 mmol L^{-1} (△) of added calcium chloride.

The proposed mechanism for whey protein gelation in the presence of calcium ions is linked to the reduction in net negative charge by the charge neutralisation effect between the positively charged calcium ions and negatively charged carboxyl groups on the whey proteins, thereby allowing increased hydrophobic interactions, as well the formation of calcium bridges (Bryant & McClements, 1998; Chung et al., 2013). Heating of β-lactoglobulin to 80°C, and addition of calcium salts, have also been reported to induce conformational changes, leading to the increase in exposed hydrophobic groups on β-

lactoglobulin (Jeyarajah & Allen, 1994; Riou et al., 2011). The change in *G′* during heating of the various concentrations of WPI solutions (Figure 6-3) demonstrated that gelation could occur at < 80°C and was dependent on both whey protein concentration and concentration of calcium salt added, where at lower whey protein concentration (1.2%), calcium chloride was required for gelation but at higher whey protein concentration (2.0%), gelation occurred without the addition of calcium chloride. Gelation of 2.0% whey proteins without the presence of calcium was likely because there was a sufficiently high concentration of whey proteins present that allowed formation of disulphide bonds and hydrophobic interactions, thereby resulting in aggregation and gelation.

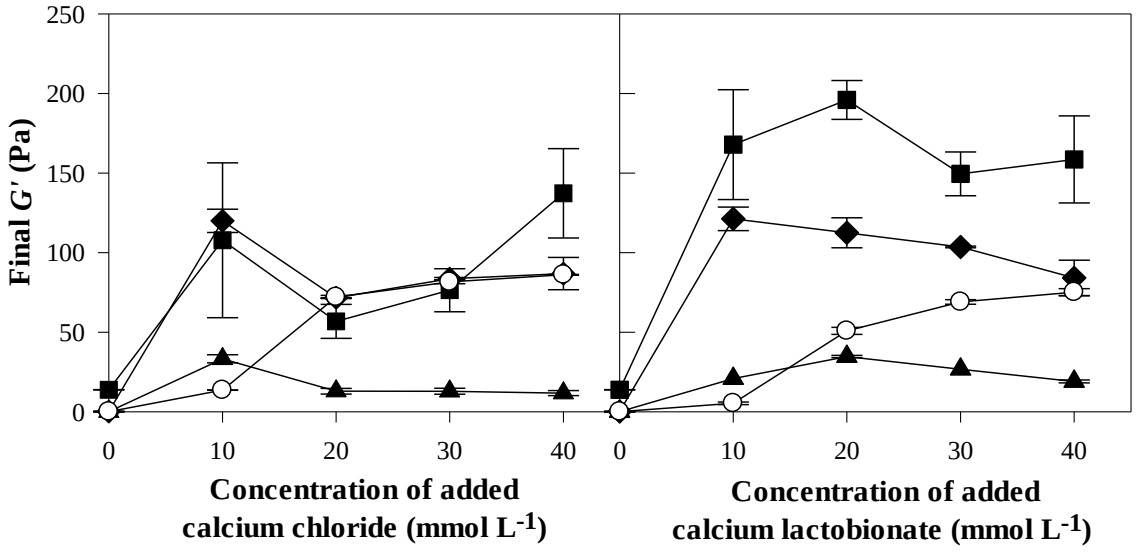


Figure 6-4: Relationship between the final *G′* of WPI solutions with 0.6% (▲), 1.2% (◆), 2.0% (■) whey protein, skim milk (○) at 20°C, and the concentration of added (a) calcium chloride and (b) calcium lactobionate. The WPI solutions and skim milk were preheated at 90°C for 10 min prior to calcium salt addition, and heated in the rheometer at 80°C for 60 min. Data points are mean values ± standard deviation (n = 2 to 4).

The final *G′* values of the WPI solutions and skim milk with various concentrations of added calcium chloride and calcium lactobionate are shown in Figure 6-4. At 10 mmol L^{-1}

addition of either calcium chloride or calcium lactobionate, the final G' of the WPI solutions increased with increasing whey protein concentration. However, skim milk, which had the highest percentage of total protein (3.27%), ands 0.6% whey proteins, had the lowest final G' (Figure 6-4). Nguyen et al. (2016) reported that heat-induced gelation of whey proteins with calcium salt could be inhibited by addition of sodium caseinate, although the inhibition was dependent on both the concentration of calcium salt and sodium caseinate added. Nguyen et al. (2016) attributed the inhibition to the competition for calcium ions between the sodium caseinate and whey proteins. A similar situation could be occurring in the skim milk system where the presence of casein micelles may be competing with whey proteins for the calcium ions, thus resulting in the skim milk achieving a lower final G' at 10 mmol L^{-1} compared to the WPI solutions. Moreover, the presence of casein micelles in skim milk is likely to result in the formation of gels with different structures than in systems with only whey protein present and the resulting gels may have different rheological properties.

On increasing calcium salt addition to between 20 and 40 mmol L^{-1}, the final G' for all WPI solutions decreased, while the final G' for skim milk increased (Figure 6-4). An increase in final G' followed by a decrease in final G' with increasing calcium salt addition in whey protein gels has been reported previously (Riou et al., 2011; Tang et al., 1995). A balance between the attractive and repulsive forces between the protein molecules is required for ordered aggregation of the proteins to form a gel network (Jost, 1993; Lucey, 2009; Oakenfull et al., 1997). Tang et al. (1995) proposed that in the whey protein systems with higher concentrations of added calcium salt, excessive attractive forces (hydrophobic interactions and calcium bridging) may lead to random aggregation rather than the

formation of an ordered gel structure, thus resulting in the lower final *G′*. The proposition that the random interactions between the protein molecules may lead to the formation of insoluble aggregates (precipitate) rather than an ordered gel network may also explain the large standard deviation in the WPI solutions, especially at higher whey protein concentrations (Damodaran & Paraf, 1997). Furthermore, the 1.2% and 2.0% WPI solutions with added calcium lactobionate achieved a higher *G′* than calcium chloride samples (Figure 6-4). As discussed in Chapter 4, calcium lactobionate releases fewer Ca^{2+} ions than calcium chloride on dissolution. The fewer Ca^{2+} ions in calcium lactobionate-added samples may have favoured the formation of a more ordered structure due to the moderate attractive forces compared to calcium chloride-added samples. The rheological measurements of the 2.0% WPI solutions with 40 mmol L^{-1} of added calcium chloride had been repeated four times and the final *G′* was found to be consistently higher than the WPI solutions with 20 and 30 mmol L^{-1} of added calcium chloride. It is unclear why the final *G′* value of the 2.0% WPI solutions with 40 mmol L^{-1} of added calcium chloride increased, although salting out of the protein (precipitation) could be occurring at the concentration of whey protein and calcium chloride added, which may result in gels with different rheological properties.

The difference in the final *G′* of the 0.6% WPI solutions compared to skim milk at 20 to 40 mmol L^{-1} of added calcium chloride and calcium lactobionate suggested that casein micelles were the proteins primarily responsible for the formation of gel network, and whey proteins played a supporting role in enhancing the strength of the gel (Figure 6-4). Nguyen, Chassenieux, Nicolai & Schmitt (2017) studied the microstructures using confocal laser scanning microscopy of gels made from heating mixtures of micellar casein and WPI at

between pH 5.8 to 6.6. Nguyen et al. (2017) reported that during gelation, the network is formed primarily by micellar casein and subsequently reinforced by WPI by binding to the micellar casein which strengthens the junctions of the gel network. A similar structure can be envisaged in the calcium-induced skim milk gel where the gel network is primarily structured by casein micelles, with whey proteins providing a supporting role to the strength of the gel.

6.3.5 Calcium ion activity and pH of skim milk blends with different casein to whey protein ratios

In the skim milk blends, the casein to whey protein ratio was varied while maintaining the total protein at 2.90% (*w/w*), before calcium chloride addition. These blends contained 95%, 93%, 90%, 85% and 80% casein, with whey proteins making up the remainder of the protein to give 100%. It should be noted that the final total protein present in all skim milk blends was 2.40% (*w/w*) after calcium chloride addition.

The $a_{Ca^{2+}}$ of the skim milk blends decreased significantly ($p < 0.05$) as the ratio of casein in the skim milk blends decreased (Table 6-7). Conversely, the pH of the skim milk increased significantly as the ratio of casein in the skim milk blend decreased (Table 6-7). These small but significant differences in the $a_{Ca^{2+}}$ and pH of the skim milk blends could be due to the slight variations in the composition of the SMUF used to prepare the WDSM compared to the skim milk permeate. Although the $a_{Ca^{2+}}$ and pH of the SMUF had been adjusted to mimic the $a_{Ca^{2+}}$ and pH of the skim milk permeate (6.64 ± 0.02), the exact compositions of the skim milk permeate and SMUF were expected to be different as the concentration of the salts in the SMUF were based on estimation. The salts in the skim milk

permeates and SMUF, such as phosphate, citrate, lactate, and carbonate, may influence the calcium equilibrium and pH buffering capacity of the system after preheat treatment and calcium salt addition differently (Salaün et al., 2005). Therefore, skim milk blends with a higher proportion of WDSM may result in the observed difference in a_{Ca}^{2+} and pH compared to skim milk blends with more of the reconstituted skim milk (Table 6-7).

Table 6-7: The $a_{Ca^{2+}}$ and pH of skim milk blends with different casein to whey protein ratios. The skim milk blends were added with 0 mmol L^{-1}, 10 mmol L^{-1}, 20 mmol L^{-1} and 40 mmol L^{-1} of calcium chloride. At the same concentration of added calcium chloride, different superscript letters (a, b, c, d) indicates significant difference in the $a_{Ca^{2+}}$ or pH across the skim milk blends with different casein to whey protein ratio (95% confidence level). Results presented are mean ± standard deviation (n =6 to 9).

Ratio of casein/ whey protein in total protein	**Concentration of added calcium chloride (mmol L^{-1})**							
	$a_{Ca^{2+}}$				**pH**			
	0	**10**	**20**	**40**	**0**	**10**	**20**	**40**
95%/ 5%	0.7 ± 0.1 ^{a}	2.8 ± 0.1 ^{a}	5.0 ± 0.3 ^{a}	11.1 ± 0.4 ^{a}	6.63 ± 0.01 ^{a}	6.29 ± 0.01 ^{a}	6.08 ± 0.01 ^{a}	5.88 ± 0.01 ^{a}
93%/ 7%	0.7 ± 0.1 ^{a}	2.8 ± 0.1 ^{ab}	5.1 ± 0.1 ^{a}	10.9 ± 0.2 ^{a}	6.64 ± 0.01 ^{ab}	6.31 ± 0.01 ^{a}	6.13 ± 0.01 ^{bc}	5.93 ± 0.01 ^{bc}
90%/ 10%	0.5 ± 0.1 ^{b}	2.7 ± 0.1 ^{b}	5.3 ± 0.2 ^{a}	10.6 ± 0.3 ^{ab}	6.64 ± 0.01 ^{ab}	6.39 ± 0.01 ^{b}	6.12 ± 0.01 ^{b}	5.91 ± 0.01 ^{b}
85%/ 15%	0.5 ± 0.1 ^{b}	2.4 ± 0.1 ^{c}	5.1 ± 0.3 ^{a}	10.3 ± 0.4 ^{b}	6.65 ± 0.01 ^{ab}	6.34 ± 0.01 ^{ab}	6.14 ± 0.01 ^{c}	5.93 ± 0.01 ^{bc}
80%/ 20%	0.5 ± 0.1 ^{b}	2.3 ± 0.1 ^{c}	5.4 ± 0.4 ^{a}	10.2 ± 0.2 ^{b}	6.66 ± 0.01 ^{b}	6.33 ± 0.03 ^{b}	6.16 ± 0.01 ^{d}	5.95 ± 0.02 ^{c}

6.3.6 Protein composition in the serum and sediment phases of skim milk blends with different casein to whey protein ratios

Using SDS-PAGE, the concentrations of casein and whey proteins in the skim milk blend, and the distribution of the proteins between serum and sediment phases with calcium salt addition were determined. The results from the SDS-PAGE analysis confirmed that as the skim milk blends went from 95% to 85% casein, the casein concentration decreased significantly, and there was a corresponding increase in β-lactoglobulin and α-lactalbumin (Table 6-8). When no calcium chloride was added (0 mmol L^{-1}), a significantly higher ($p < 0.05$) amount of serum casein was observed in the 95% casein skim milk blend (3.8 ± 0.3 mg g^{-1}) compared to the 80% casein skim milk blend (2.3 ± 0.3 mg g^{-1}) (Table 6-8). However, on addition of calcium chloride between 10 mmol L^{-1} and 40 mmol L^{-1}, the amount of serum casein were not significantly different ($p > 0.05$) in the skim milk blends with different casein and whey protein ratio. For example, serum casein in the 95% casein skim milk blend was 2.2 ± 0.2 mg g^{-1}, which was not significantly different ($p > 0.05$) from the serum casein in the 80% casein skim milk (1.8 ± 0.1 mg g^{-1}). The results indicated that the added calcium chloride interacted with the serum casein which may have sedimented on centrifugation in all skim milk blends.

In the 95% casein skim milk blend, the serum phase contained approximately four times more casein than whey proteins when no calcium chloride was added (3.8 ± 0.3 mg g^{-1} casein, 0.9 ± 0.1 mg g^{-1} whey protein) (Table 6-8). On addition of 40 mmol L^{-1} of calcium chloride to the 95% casein skim milk blend, casein was only approximately twice the amount of whey proteins in the serum phase (1.5 ± 0.3 mg g^{-1} casein, 0.7 ± 0.1 mg g^{-1} whey protein).

Table 6-8: Concentration (mg g^{-1}) of casein, β-lactoglobulin, α-lactalbumin in the skim milk blends (total protein: 2.40%) or serum phase of skim milk blends at various concentrations of calcium salt added determined by SDS-PAGE. Results presented are means ± standard deviation (n = 2 to 4). At the same concentration of added calcium chloride, different superscript letters (a, b, c, d) indicate significant difference across the skim milk blends with different casein to whey protein ratios for each type of protein (95% confidence level).

Ratio of casein / whey protein in total protein	Sample	Concentration of added calcium chloride (mmol L^{-1})	Casein (mg g^{-1})	β-lactoglobulin (mg g^{-1})	α-lactalbumin (mg g^{-1})
95%/ 5%	Skim Milk	0	23.2 ± 0.1 ^{a}	1.3 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}
	Serum	0	3.8 ± 0.3 ^{a}	0.8 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
	Serum	10	2.2 ± 0.2 ^{a}	0.6 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
	Serum	20	1.6 ± 0.2 ^{a}	0.6 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
	Serum	40	1.5 ± 0.3 ^{a}	0.6 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
93% / 7%	Skim Milk	0	23.5 ± 0.2 ^{a}	1.8 ± 0.1 ^{b}	0.2 ± 0.1 ^{a}
	Serum	0	3.6 ± 0.1 ^{a}	0.7 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
	Serum	10	2.1 ± 0.1 ^{a}	0.7 ± 0.1 ^{ab}	0.1 ± 0.1 ^{a}
	Serum	20	1.4 ± 0.1 ^{a}	0.7 ± 0.1 ^{ab}	0.1 ± 0.1 ^{ab}
	Serum	40	1.2 ± 0.1 ^{a}	0.7 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}
90%/ 10%	Skim Milk	0	22.6 ± 0.1 ^{a}	2.1 ± 0.1 ^{c}	0.4 ± 0.1 ^{b}
	Serum	0	3.3 ± 0.2 ^{ab}	1.3 ± 0.1 ^{b}	0.3 ± 0.1 ^{b}
	Serum	10	1.8 ± 0.1 ^{a}	1.2 ± 0.1 ^{b}	0.2 ± 0.1 ^{ab}
	Serum	20	1.7 ± 0.3 ^{a}	1.1 ± 0.1 ^{b}	0.2 ± 0.1 ^{bc}
	Serum	40	1.3 ± 0.1 ^{a}	1.2 ± 0.1 ^{c}	0.2 ± 0.1 ^{b}
85%/ 15%	Skim Milk	0	21.0 ± 0.6 ^{b}	2.7 ± 0.1 ^{cd}	0.5 ± 0.1 ^{c}
	Serum	0	2.7 ± 0.2 ^{bc}	1.9 ± 0.1 ^{bc}	0.4 ± 0.1 ^{c}
	Serum	10	1.9 ± 0.3 ^{a}	2.0 ± 0.3 ^{c}	0.4 ± 0.1 ^{c}
	Serum	20	1.6 ± 0.2 ^{a}	2.0 ± 0.3 ^{c}	0.4 ± 0.1 ^{cd}
	Serum	40	1.8 ± 0.1 ^{a}	1.7 ± 0.1 ^{d}	0.4 ± 0.1 ^{bc}
80%/ 20%	Skim Milk	0	20.0 ± 0.2 ^{b}	3.2 ± 0.1 ^{d}	0.7 ± 0.1 ^{d}
	Serum	0	2.3 ± 0.1 ^{c}	2.2 ± 0.1 ^{c}	0.7 ± 0.1 ^{d}
	Serum	10	1.8 ± 0.1 ^{a}	2.3 ± 0.2 ^{c}	0.8 ± 0.1 ^{d}
	Serum	20	1.5 ± 0.1 ^{a}	2.2 ± 0.1 ^{c}	0.7 ± 0.1 ^{d}
	Serum	40	1.3 ± 0.1 ^{a}	2.1 ± 0.1 ^{e}	0.7 ± 0.1 ^{c}

On the other hand, for the 80% casein skim milk blends, the ratio of serum casein to whey proteins was approximately 1:1 when no calcium salt was added (2.3 ± 0.2 mg g^{-1} casein, 2.9 ± 0.1 mg g^{-1} whey protein), but serum casein decreased to approximately half of serum whey proteins on addition of 40 mmol L^{-1} (1.3 ± 0.1 mg g^{-1} casein, 2.8 ± 0.1 mg g^{-1} whey protein) The results suggest that the distribution of the proteins between the serum and sediment phase is dependent on both the total casein and whey proteins in the skim milk, as well as the concentration of added calcium chloride.

6.3.7 Quantification of calcium in the serum and sediment phases of skim milk blends with different casein to whey protein ratios

The colloidal phase in milk (casein micelles) contains approximately two-thirds of the total calcium in milk (Deeth & Lewis, 2015; Fox & McSweeney, 1998). Different ratios of casein to whey proteins may influence the distribution of the calcium between the colloidal and serum phase. Quantification of the calcium between the serum and sediment was conducted to elucidate how the calcium equilibrium may be influenced by the ratio of casein to whey proteins.

The total concentration of calcium in the skim milk blends decreased with decreasing proportion of casein in the skim milk blends (Table 6-9). At the same concentration of added calcium chloride, the amount of serum calcium was not significantly different across the skim milk blends with different casein percentages ($p > 0.05$). However, the sediment calcium increased with increasing percentage of casein ($p < 0.05$) (Table 6-9). The distribution of calcium between the colloidal and serum phases in native skim milk is approximately 20 mmol L^{-1} and 10 mmol L^{-1}, respectively (Deeth & Lewis, 2015; Fox & McSweeney, 1998). Skim milk blends with a higher proportion of casein may contain a

Table 6-9: Concentration of calcium (mg g^{-1}) in skim milk, serum and sediment of skim milk blends preheated at 90°C for 10 min, with different casein to whey protein ratios at various concentrations of added calcium salts (n = 3). At the same concentration of added calcium chloride, different superscript letters (a, b, c, d) indicate significant difference across the skim milk blends with different casein to whey protein ratio for each type of sample (skim milk, serum or sediment) (95% confidence level).

Ratio of casein / whey protein in total protein	Concentration of added calcium chloride (mmol L^{-1})	Mass of calcium added (mg)	Total calcium in skim milk^{+} (mg g^{-1})	Serum calcium^{+} (mg g^{-1})	Sediment calcium* (mg g^{-1})
95% / 5%	0	0	1.08 ^{a}	0.22 ^{a}	0.86 ^{a}
	10	0.4	1.46 ^{a}	0.44 ^{ab}	1.02 ^{a}
	20	0.8	1.94 ^{a}	0.76 ^{a}	1.18 ^{a}
	40	1.6	2.70 ^{a}	1.48 ^{a}	1.22 ^{a}
93% / 7%	0	0	1.06 ^{a}	0.22 ^{a}	0.84 ^{a}
	10	0.4	1.44 ^{a}	0.42 ^{b}	1.02 ^{a}
	20	0.8	1.84 ^{b}	0.74 ^{a}	1.10 ^{b}
	40	1.6	2.70 ^{a}	1.48 ^{a}	1.22 ^{a}
90% / 10%	0	0	0.96 ^{b}	0.20 ^{a}	0.78 ^{b}
	10	0.4	1.44 ^{a}	0.46 ^{a}	0.98 ^{b}
	20	0.8	1.80 ^{bc}	0.74 ^{a}	1.06 ^{b}
	40	1.6	2.60 ^{ab}	1.46 ^{a}	1.14 ^{b}
85% / 15%	0	0	0.92 ^{c}	0.22 ^{a}	0.70 ^{c}
	10	0.4	1.30 ^{b}	0.44 ^{ab}	0.86 ^{c}
	20	0.8	1.72 ^{cd}	0.78 ^{a}	0.94 ^{c}
	40	1.6	2.52 ^{b}	1.46 ^{a}	1.06 ^{bc}
80% / 20%	0	0	0.86 ^{d}	0.20 ^{a}	0.66 ^{d}
	10	0.4	1.28 ^{b}	0.46 ^{a}	0.82 ^{d}
	20	0.8	1.68 ^{d}	0.82 ^{a}	0.86 ^{c}
	40	1.6	2.50 ^{b}	1.48 ^{a}	1.02 ^{c}

Pooled standard deviation: ± 0.02

$^{+}$ Concentration determined by EDTA titration

* Concentration determined by difference between milk and serum calcium

higher amount of total calcium as a result of more calcium being associated in the casein micelles as colloidal calcium phosphate (CCP). This hypothesis is supported by the higher concentration of sediment calcium found in skim milk blends with higher percentage of casein.

6.3.8 Rheological properties of skim milk blends with different casein to whey protein ratios

The contribution of casein and whey proteins towards the gelation of skim milk blends with added calcium chloride was investigated by rheological measurements of the skim milk blends with different ratios of casein to whey proteins. When the skim milk blends were heated from 20°C to 80°C, preheated skim milk blends with higher proportion of whey proteins resulted in higher G' at 80°C for all concentrations of added calcium salt (Figure 6-5, Table 6-10). Although casein may be the primary protein responsible for the overall gel network (as discussed in Section 6.3.4), it appeared that during heating from 20°C to 80°C, the initial association and gelation of whey proteins in the preheated skim milk blends was favoured over the gelation of casein. As discussed in Section 6.3.4, interactions between calcium ions and whey proteins could result in aggregation and gelation. As the added calcium chloride dissociates into calcium and chloride ions in the serum phase, interactions between the serum whey proteins and the calcium ions in the serum phase can occur and result in aggregation and gelation. Furthermore, as discussed in Chapter 5, preheat treatment results in the interaction of whey proteins with the κ-casein on the surface of casein micelles. The whey proteins on the surface of the casein micelles may also interact with other whey proteins (in the serum phase or attached to other casein micelles) which could promote the first stage of the development of the gel structure (during heating

from 20°C to 80°C). The G' after heating from 20°C to 80°C increased with increasing concentration of added calcium chloride (Figure 6-5, Table 6-10), indicating that binding between the whey proteins and the calcium ions contributed to the gelation of the whey proteins.

Subsequently, during holding at 80°C for 60 min, the development of the gel network appeared to be dependent on both the ratio of casein to whey proteins, and calcium ions present (Figure 6-6, Table 6-10). At 10 mmol L^{-1} of added calcium chloride, increasing whey proteins from 5% to 10% (or decreasing casein from 95% to 90%) resulted in no significant difference in the change in G' during holding ($p > 0.05$), but further increase in whey proteins at between 15% and 20% appeared to favour the formation of a stronger gel (Figure 6-6, Table 6-10). For instance, at 10 mmol L^{-1} calcium chloride addition, the change in G' during holding at 80°C for 60 min was 1.09 ± 0.06 Pa and 6.08 ± 0.31 Pa for 95% and 80% casein skim milk blends, respectively (Table 6-10). In contrast, increasing the amount of added calcium chloride (20 mmol L^{-1} and 40 mmol L^{-1}) appeared to facilitate the gelation of skim milk blends with higher proportion of casein during holding at 80°C (Figure 6-6, Table 6-10). For example, at 40 mmol L^{-1} of added calcium chloride, the 95% casein skim milk blend resulted in a 16.29 ± 0.97 Pa change in G', while 80% casein skim milk blend resulted in a 13.20 ± 0.48 Pa change in G' during holding at 80°C (Table 6-10). In addition, the 93% casein skim milk blend had lower G' when compared to the 85% casein skim milk blend throughout the holding period at 10 mmol L^{-1} and 20 mmol L^{-1} of calcium chloride addition (Figure 6-6). On increasing the calcium chloride addition to 40 mmol L^{-1}, the 93% casein skim milk blend achieved a higher G' than the 85% casein skim milk by the end of holding period (Figure 6-6).

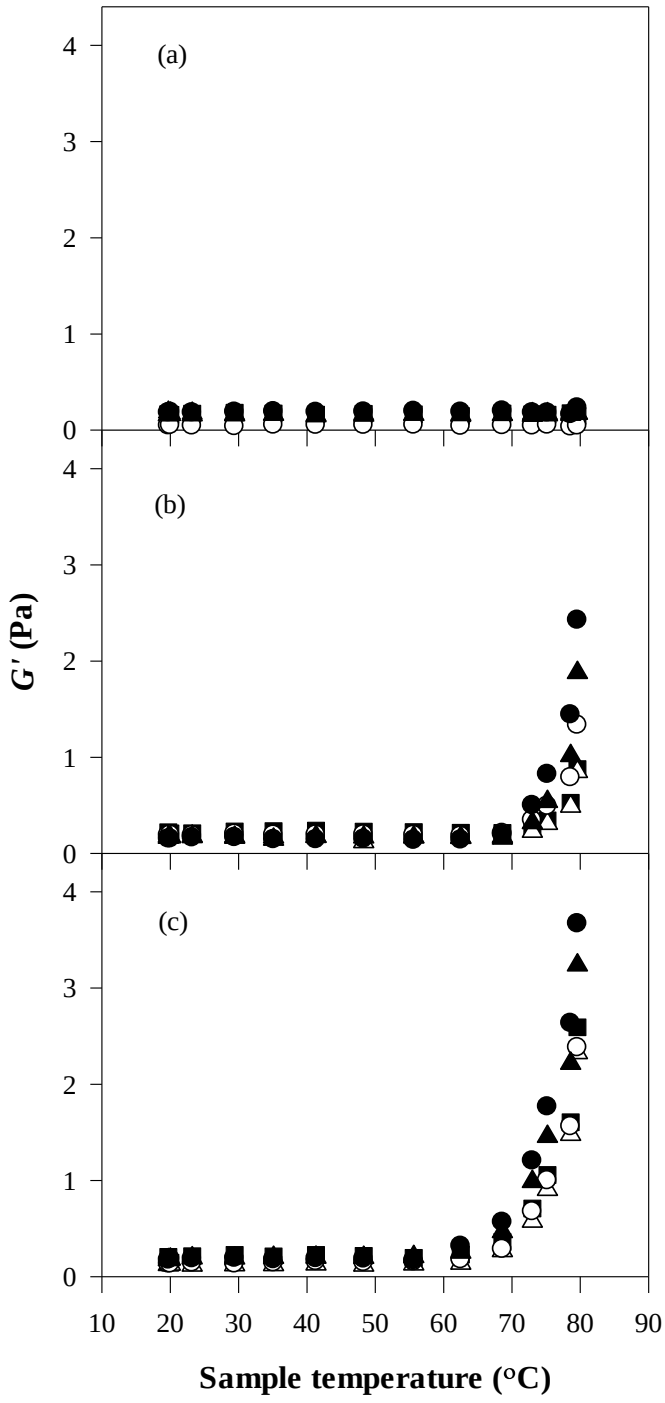


Figure 6-5: Typical plots showing the change in *G′* during heating from 20°C to 80°C for skim milk blends with (a) 10 mmol L^{-1}, (b) 20 mmol L^{-1} and (c) 40 mmol L^{-1} of added calcium chloride. The protein composition of the skim milk blends was 95% casein, 5% whey protein (■); 93% casein, 7% whey protein (△); 90% casein, 10% whey protein (○); 85% casein, 15% whey protein (▲); and 80% casein, 20% whey protein (●).

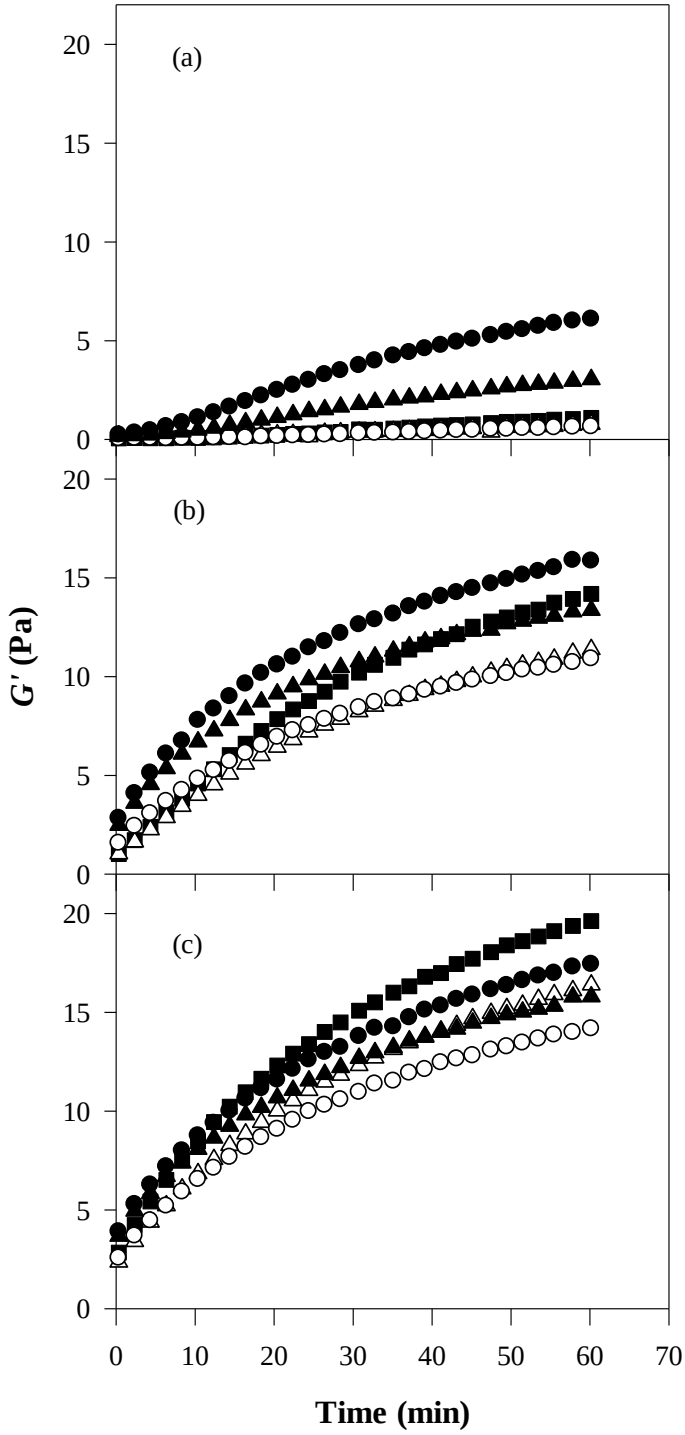


Figure 6-6: Typical plots showing the change in G' during holding at 80°C for 60 minutes for skim milk blends with (a) 10 mmol L^{-1}, (b) 20 mmol L^{-1} and (c) 40 mmol L^{-1} of added calcium chloride. The protein composition of the skim milk blends was 95% casein, 5% whey protein (■); 93% casein, 7% whey protein (△); 90% casein, 10% whey protein (○); 85% casein, 15% whey protein (▲); and 80% casein, 20% whey protein (●).

Table 6-10: The G' of skim milk blends with various casein to whey protein ratio after heating from 20°C to 80°C, and the change in G' during holding at 80°C for 60 min in the rheometer for skim milk blends with added calcium chloride: 10 mmol L^{-1} to 40 mmol L^{-1}. At the same concentration of added calcium chloride, different superscript letters (a, b, c) indicate significant difference in the G' values of the skim milk with different casein to whey protein ratio (95% confidence level). Results presented are mean ± standard deviation (n =2 to 3).

Ratio of casein / whey protein in total protein	***G'* after heating from 20°C to 80°C (Pa)**			**Change in *G'* from t= 0 min to t= 60 min during holding at 80°C (Pa)**		
	10 mmol L^{-1}	**20 mmol L^{-1}**	**40 mmol L^{-1}**	**10 mmol L^{-1}**	**20 mmol L^{-1}**	**40 mmol L^{-1}**
95% / 5%	0.10 ± 0.05 [a]	0.97 ± 0.13 [ab]	2.25 ± 0.48 [a]	1.09 ± 0.06 [a]	13.49 ± 0.44 [a]	16.29 ± 0.96 [a]
93% / 7%	0.15 ± 0.03 [a]	0.80 ± 0.07 [a]	2.21 ± 0.17 [a]	0.89 ± 0.40 [a]	10.34 ± 0.01 [bc]	14.38 ± 0.49 [b]
90% / 10%	0.10 ± 0.05 [a]	1.35 ± 0.02 [b]	2.36 ± 0.03 [ab]	0.62 ± 0.03 [a]	9.48 ± 0.20 [c]	11.88 ± 0.40 [c]
85% / 15%	0.11 ± 0.08 [a]	2.01 ± 0.19 [c]	3.34 ± 0.14 [ab]	3.86 ± 1.38 [b]	11.14 ± 0.36 [b]	12.10 ± 0.02 [c]
80% / 20%	0.19 ± 0.02 [a]	2.43 ± 0.01 [d]	3.42 ± 0.35 [b]	6.08 ± 0.31 [b]	12.87 ± 0.22 [a]	13.20 ± 0.48 [bc]

After the heating, holding and cooling protocol in the rheometer, no gelation was observed in preheated skim milk blends with no calcium chloride addition, regardless of the ratio of casein to whey proteins (Figure 6-7). On addition of 10 to 40 mmol L^{-1} of calcium chloride, gelation (G' > 1 Pa) was observed in all preheated skim milk blends with the various ratios of casein to whey proteins (Figure 6-7). The order of the final G' from the highest to the lowest for the preheated skim milk blends with different ratios of casein and whey proteins was dependent on the concentration of calcium chloride added (Figure 6-7). For instance, at 10 mmol L^{-1} of added calcium chloride, the order of the final G' from the highest to the lowest was 80% > 85% > 95% > 93% > 90% casein skim milk blends, but at 40 mmol L^{-1} of added calcium chloride, the order was 95% > 80% > 93% > 85% > 90% casein skim milk blends.

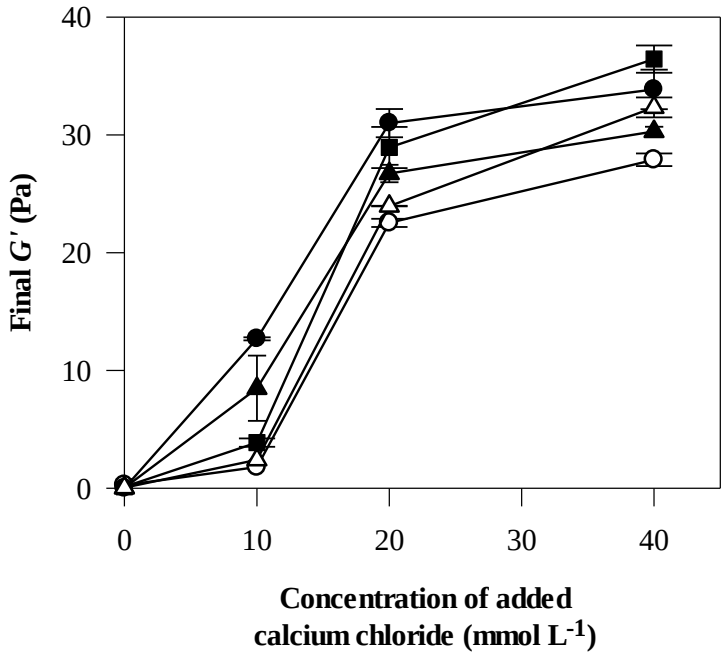


Figure 6-7: Relationship between final G' of calcium-induced skim milk gels at 20°C, after cooling, and concentration of calcium chloride added to the skim milk. The protein composition of the skim milk blends was 95% casein, 5% whey protein (■); 93% casein, 7% whey protein (△); 90% casein, 10% whey protein (○); 85% casein, 15% whey protein (▲); and 80% casein, 20% whey protein (●). Skim milk was preheated at 90°C for 10 min prior to calcium chloride addition, and heated in the rheometer at 80°C for 60 min. Data points are mean values ± standard deviation (n= 2 to 3).

In Chapter 5, preheat treatment was shown to increase the final G' of the calcium-added skim milk. To investigate the effect of native and denatured whey proteins in skim milk blends with different casein to whey protein ratios, 20 mmol L^{-1} of calcium chloride was added to non-preheated skim milk blends and their rheological properties were compared to those of the preheated skim milk blends.

Table 6-11: The G' of skim milk blends with various casein to whey protein ratio after heating from 20°C to 80°C. The skim milk blends were either non-preheated or preheated at 90°C for 10 min and with 20 mmol L^{-1} of added calcium chloride. Results presented are mean ± standard deviation (n = 2 to 3). Within each type of sample (non-preheated or preheated), different superscript letters (a, b, c) indicate significant difference in the G' values in the skim milk blends with different casein to whey protein ratio. At the same casein to whey protein ratio, different superscript Greek letters (χ, γ) represent significant difference in G' values between non-preheated and preheated samples (95% confidence level).

Ratio of casein / whey protein in total protein	***G'* after heating from 20°C to 80°C (Pa)**	
	Non-preheated	**Preheated**
95% / 5%	$1.13 \pm 0.12^{a, \chi}$	$0.97 \pm 0.13^{ab, \chi}$
93% / 7%	$0.77 \pm 0.07^{b, \chi}$	$0.80 \pm 0.07^{a, \chi}$
90% / 10%	$0.69 \pm 0.03^{b, \chi}$	$1.35 \pm 0.02^{b, \gamma}$
85% / 15%	$0.35 \pm 0.02^{c, \chi}$	$2.01 \pm 0.19^{c, \gamma}$
80% / 20%	$0.19 \pm 0.12^{c, \chi}$	$2.43 \pm 0.01^{d, \gamma}$

In preheated skim milk blends, the G' after heating from 20°C to 80°C increased significantly on increasing proportion of whey proteins in preheated skim milks ($p < 0.05$) (Table 6-11). Jeyarajah & Allen (1994) reported that preheat treatment (80°C for 15 min) increased the binding of β-lactoglobulin to calcium ions. The whey proteins in the preheated skim milk may bind more strongly to the added calcium ions after preheating, thus increasing their ability for aggregation and gelation. Furthermore, the binding of calcium ions to preheated β-lactoglobulin may also induce structural changes, such as increasing the number of exposed hydrophobic sites, which may promote hydrophobic interactions between the whey proteins (Jeyarajah & Allen,

1994). Conversely, for the non-preheated skim milk blends with added calcium chloride, the G' after heating from 20°C to 80°C decreased significantly on decreasing casein to whey protein ratio ($p < 0.05$) (Table 6-11). This result was in the reverse order as to what was observed for skim milk blends which had undergone preheat treatment. The decrease in G' with decreasing casein to whey protein ratio in non-preheated skim milk blends suggested that native whey proteins may play a smaller role compared to denatured whey proteins in the gelation in calcium-added skim milk during the heating from 20°C to 80°C in the rheometer. At the same casein to whey protein ratio, a significant difference in G' between non-preheated and preheated skim milk blends was only observed in skim milk blends with 10 to 20% of whey proteins in total protein (Table 6-11). For example, at 95% casein/ 5% whey protein skim milk blends, preheat treatment did not result in a significant difference in the G' values after heating from 20°C to 80°C ($p > 0.05$), but the G' value for 80% casein/ 20% whey protein skim milk blends increased significantly from 0.19 ± 0.12 Pa to 2.43 ± 0.01 Pa with preheat treatment ($p < 0.05$). It appears that presence of denatured whey proteins promoted gelation during heating from 20°C to 80°C, but the promotion of gelation by the whey proteins was not observed if the whey proteins were not preheated prior to calcium addition and heating from 20°C to 80°C.

Table 6-12: The G' of skim milk after cooling, at 20°C, with various casein to whey protein ratios after heating at 80°C in the rheometer for 60 min. The skim milk blends were either non-preheated or preheated at 90°C for 10 min and with 20 mmol L^{-1} of added calcium chloride. Results presented are mean ± standard deviation (n = 2 to 3). Within each type of sample (non-preheated or preheated), different superscript letters (a, b, c) indicate significant difference in the G' values in the skim milk blends with different casein to whey protein ratio. At the same casein to whey protein ratio, different superscript Greek letters (χ, γ) represent significant difference in G' values between non-preheated and preheated samples (95% confidence level).

Ratio casein / whey protein in total protein	**Final G' at 20°C (Pa)**		
	Non-preheated	**Preheated**	**Difference between non-preheated and preheated skim milk**
95% / 5%	$35.75 \pm 1.76^{a, \chi}$	$29.96 \pm 1.75^{ab, \gamma}$	- 6.81
93% / 7%	$28.63 \pm 1.18^{b, \chi}$	$24.31 \pm 0.58^{c, \gamma}$	- 4.66
90% / 10%	$25.00 \pm 1.18^{bc, \chi}$	$23.21 \pm 0.85^{c, \chi}$	- 1.46
85% / 15%	$23.33 \pm 0.18^{c, \chi}$	$26.68 \pm 0.53^{bc, \chi}$	+ 3.4
80% / 20%	$21.71 \pm 0.56^{c, \chi}$	$31.01 \pm 1.20^{a, \gamma}$	+ 9.3

In the non-preheated skim milk blends, the final G' decreased significantly ($p < 0.05$) when the whey protein percentage increased from 5% to 20%, decreasing from 35.75 ± 1.76 Pa to 21.71 ± 0.56 Pa (Table 6-12). In the preheated skim milk blends, the final G' decreased significantly ($p < 0.05$) from 29.96 ± 1.75 Pa to 23.21 ± 0.85 Pa with increasing whey protein from 5 to 10%, then from 10% to 20% whey protein, the final G' increased significantly ($p < 0.05$) to 31.01 ± 1.20 Pa (Table 6-12). Preheat treatment resulted in a significant reduction ($p < 0.05$) in the final G' for 5% whey protein (- 6.81 Pa) and 7% whey protein (- 4.66 Pa) skim milk blends, no significant difference ($p > 0.05$) in the final G' for 10% whey protein skim milk blends, and significant increase in the final G' for 15% whey protein (+ 3.4 Pa) to 20% whey protein (+ 9.3 Pa) skim milk blends (Table 6-12). The results suggested that the contribution of whey proteins

towards the gelation of a calcium-induced skim milk gel is dependent on the ratio of casein to whey protein in the system, as well as whether preheat treatment was applied.

6.3.9 Overall discussion on the effect of casein and whey proteins

Due to the complexity of the interactions among the casein, whey proteins and calcium ions in a milk system, it was technically difficult to distinguish the true contribution of each protein type towards gelation. While recognising the difficulty in separating the effect of casein and whey proteins, the experiments designed in this chapter aimed to elucidate how individual casein and whey proteins may interact to form gels. For instance, systems with only whey proteins provided insights on the gelation of whey proteins with calcium ions, while the contribution of casein was inferred by comparison of the rheological properties of the skim milk with higher ratios of casein to whey proteins to those with lower ratio of casein to whey proteins.

Based on the results presented in this chapter, the following effects of casein and whey protein in a calcium-added skim milk are proposed. The $a_{Ca^{2+}}$ values decreased significantly, while the pH increased significantly as the proportion of casein in the skim milk blends decreased (Table 6-7). As shown in Chapter 4, the $a_{Ca^{2+}}$ and pH can influence the final G' of the gels if the casein to whey protein ratios are kept constant. However, the rheological results of the skim milk blends with different casein to whey protein ratios did not follow the trend of higher $a_{Ca^{2+}}$, lower pH achieving in a higher final G'. This indicated that the differences in the final G' values for the skim milk blends with different casein to whey protein ratios were not due to the differences in the $a_{Ca^{2+}}$ or pH. The total calcium increased with increasing proportion of casein in the skim milk blends (Table 6-9). While increasing total calcium in the skim milk may favour the formation of a stronger gel, the final G' of the preheated skim milk blends did

not increase with increasing proportion of casein at the same concentration of added calcium chloride (Figure 6-7). This indicated that the ratio of casein to whey protein had a stronger influence on the final G' of the skim milk blends than the total calcium concentration.

Binding of calcium ions to casein (Balakrishnan et al., 2018; Dalgleish & Parker, 1980; Smialowska et al., 2017) and to whey proteins (Jeyarajah & Allen, 1994; Lönnerdale & Glazier, 1985; Riou et al., 2011), which induces aggregation and gelation have both been reported. The results in Section 6.3.4 showed that at the concentration of whey proteins in skim milk (0.6%), addition of calcium salts was required for gelation. Casein micelles are known to be heat stable when heated at < 100°C at the native pH of milk (Nguyen et al., 2017). Skim milk blends containing 95% casein did not gel when no calcium chloride was added (Figure 6-7). The results for the 0.6% WPI solutions and the casein-rich skim milk blends indicated that at the native conditions of skim milk, both casein and whey proteins required the addition of calcium salts for gelation to occur. However, the contribution of the two proteins towards the formation of a calcium-induced skim milk gel may differ. Comparisons of the final G' between 0.6% WPI solutions and skim milk indicated that the presence of casein micelles in skim milk had a stronger influence on the gel strength. For example, with 20 mmol L^{-1} of added calcium chloride, the final G' of 0.6% WPI solutions was approximately 10 Pa, but with the presence of casein micelles in skim milk, the final G' was approximately 75 Pa (Figure 6-4a). The difference in the final G' of the 0.6% WPI solutions and skim milk is indicative of the gel strength contributed by casein micelles.

As discussed in Section 6.3.8, gelation was favoured in preheated skim milk blends with higher proportions of whey proteins during heating from 20°C to 80°C, regardless of the concentration of added calcium chloride (Figure 6-5). This suggested that calcium ions

may first interact with the denatured whey proteins compared to the casein micelles during the initial stages of gelation. However, at the same concentration of added calcium salt (between 10 and 40 mmol L^{-1}), the final G' of the preheated skim milk blends showed that an increase in whey protein from 5% to 10% (casein reduced from 95% to 90%) resulted in a significant decrease ($p < 0.05$) in final G' (Figure 6-7). Nguyen et al. (2016) proposed that in a sodium caseinate and whey protein system, gelation was dependent on the concentration of the two proteins and the concentration of calcium salt added, as both proteins could compete for the binding with calcium ions. Hence, competition for calcium ions may also be occurring between the casein micelles and whey proteins in the skim milk. The reduction in the final G' when the whey protein proportion increased from 5% to 10% could be due to the whey proteins competing with casein micelles for binding of calcium ions. With further increase in the proportion of whey protein from 10% to 20%, the concentration of whey proteins present appeared to be substantial in contributing to the strength of the gel network as the final G' of the skim milk blends increased significantly with an increase in whey protein from 10% to 20%. It appears that in preheated skim milk blends, there may be two competing effects involving the denatured whey proteins in order to form skim milk gels in the presence of added calcium. On one hand, whey proteins may compete with casein micelles for binding of calcium ions, thus reducing interactions between casein and calcium. On the other hand, whey proteins are also able to enhance the gel strength by association with each other through hydrophobic interactions and calcium-bridging, and that the gel strength increases as the proportion of whey proteins increased as shown in Section 6.3.4.

The order of the final G' of the preheated skim milk blends with different casein to whey protein ratios (from the highest to the lowest) varied depending on the

concentration of calcium chloride added (Figure 6-7). For example, the final G' of skim milk blends with 95% casein was significantly lower than skim milk blends with 85% casein at 10 mmol L^{-1} of calcium chloride added, but with 40 mmol L^{-1} of added calcium chloride, the final G' of skim milk blends with 95% casein was significantly higher than skim milk blends with 85% casein (Figure 6-7). At lower concentrations of calcium chloride addition (10 mmol L^{-1}), gelation appeared to be favoured in skim milk blends with higher proportion of whey proteins (Figure 6-7). This suggested that in systems where the concentration of calcium ions was lower, a higher proportion of denatured whey proteins promoted gelation, possibly because whey proteins are also able to self-associate and form gels via hydrophobic and disulphide interactions even when there is insufficient calcium ions available for binding to the proteins in the system. On increasing the concentration of calcium chloride up to 20 mmol L^{-1} and 40 mmol L^{-1}, a greater increase in G' during holding at 80°C was observed in skim milk blends with a higher ratio of casein to whey proteins (Table 6-10). This indicated that in systems where the concentration of calcium ions was sufficient, gelation of casein induced by calcium ions may result in the formation of a stronger gel network than gelation of whey proteins. Further, as discussed in Section 6.3.4, the results of the final G' in skim milk compared to 0.6% WPI solutions with 20 to 40 mmol L^{-1} of added calcium salts suggested that casein micelles are primarily responsible for the structure of gel network. Whey proteins likely played a supporting role in enhancing the gel strength, or weakening the gel strength due to competition for the calcium ion binding in the calcium-induced skim milk gel.

The effect of preheat treatment on the skim milk blends with 20 mmol L^{-1} of added calcium chloride appeared to be dependent on the proportion of whey proteins present (Table 6-11, Table 6-12). As mentioned earlier, denatured whey proteins were reported

to bind more strongly to calcium ions (Jeyarajah & Allen, 1994). Preheat treatment may result in denatured whey proteins binding more readily with the calcium ions and forming gels. This may explain why in the non-preheated skim milk blends, the G' decreased with increasing proportion of whey proteins during heating from 20°C to 80°C, but the G' increased with increasing proportion of whey proteins in the preheated skim milk blends (Table 6-11). The results shown in Table 6-11 suggested that with 20 mmol L^{-1} of added calcium chloride, gelation was favoured in skim milk blends with higher proportion of denatured whey proteins during heating from 20°C to 80°C. The decrease in final G' as the proportion of whey proteins increased (or proportion of casein decreased) (Table 6-12) demonstrated that in non-preheated skim milk blends, gelation at 20 mmol L^{-1} of added calcium chloride was mainly driven by casein.

Besides changing the calcium binding properties of whey proteins, preheat treatment can also result in interactions between the casein and whey proteins, which was reported to influence the rheological properties of milk gels (Schorsch et al., 2001; Vasbinder & De Kruif, 2003). Preheat treatment was reported to hinder the formation of calcium bridges and aggregation of casein micelles in rennet-induced milk gels due to steric effects (Dalgleish & Corredig, 2012; Lucey, 2009). Steric hindrance of the whey protein - κ-casein complex on the surface of casein micelles may have resulted in the reduction in final G' when the proportion of whey proteins in the preheated skim milk blends increased from 5% to 10%. Even though further increase in the proportion of whey proteins from 10% to 20% may increase the effect of steric hindrance, the final G' of the calcium-induced skim milk gel increased. In acid-induced milk gels, preheat treatment increased the gel strength as the denatured whey proteins increased the number and strength of contact points, which may occur between the soluble denatured whey

proteins, between the denatured whey proteins and casein micelles, and between the whey protein -κ-casein complex on the surface of casein micelles (Anema et al., 2004a; Dalgleish & Corredig, 2012). A similar mechanism may also be occurring in the calcium-induced skim milk gel where the denatured whey proteins may result in a more complex gel network by increasing the number of contact points for interactions. Thus, it appears that the effect of preheat treatment on the interactions between whey proteins and casein micelles may result in two competing effects on the gel network. In the 15% and 20% whey protein skim milk blends, the higher amount of whey proteins present appears to contribute substantially to the strength of the gel network even though there may be increased whey protein – κ-casein on the surface of casein micelles. Conversely, in the 5% to 10% whey protein skim milk blends, where the amount of whey proteins did not appear to be substantial to enhance the gel strength, preheat treatment may have caused increased steric hindrance on the casein micelle aggregation, thus reducing the final G' of the gel.

6.4 Conclusions

Overall, the results suggest that the gel strength in skim milk with added calcium was the result of interactions of whey proteins, casein, and calcium ions. Different mechanisms dominated the gelation depending on preheat treatment and the concentrations of each component present. The final G' of the 0.6% WPI solutions and skim milk suggested that in native skim milk, casein micelles had a stronger influence than whey proteins on the gelation of the skim milk. During heating of the skim milk from 20°C to 80°C, denatured whey protein association appears to contribute more to the gelation as seen by the preheated skim milk blends with higher whey protein proportions achieving a higher gel strength (G'). Conversely, casein association appeared to be the primary mechanism involved in gel development during holding at

80°C, as the increase in G' during holding was higher in skim milk blends with higher proportions of casein. In preheated skim milk blends, the final G' of the skim milk was dependent on the calcium ions available for binding, and the ratio of casein to whey proteins. Preheat treatment may also affect the properties of the skim milk gel due to changes in the hydrophobicity and calcium binding affinity of the whey protein, as well as steric effect due to formation of whey protein - κ-casein complex on the surface of casein micelles. The $a_{Ca^{2+}}$, pH, and serum calcium were not significantly different across the skim milk blends with different casein to whey protein ratios at the same concentration of calcium salt added.

Chapter 7 - Effect of ionic strength and monovalent ions

7.1 Introduction

The ionic environment plays an important role in the stability of the proteins in milk (Kulozik, 2009; Walstra et al., 2006). Addition of salts which dissociate into ions in solution will change the ionic strength in milk. Alterations in the ionic strength of milk can have a significant effect on the stability of the proteins by modifying the surface charge, hydration and solubility (Damodaran, 1997; Totosaus et al., 2002). An increase in ionic strength may also alter the mineral equilibrium in milk as the activity of individual ions is dependent on the ionic strength of the solution (van Boekel, 2008c; Walstra et al., 1999). These changes may influence the protein-protein interactions and therefore the rheological properties of calcium-induced skim milk gels. In this chapter, a comparison of the effects of monovalent and divalent ions on the gelation in skim milk was investigated by the comparing calcium chloride and sodium chloride addition. The effect of changes in ionic strength and the addition of sodium ions was investigated by addition of sodium chloride to calcium-added skim milk.

7.2 Materials and Methods

7.2.1 Preparation of skim milk with added calcium chloride and sodium chloride

Reconstituted skim milk and calcium chloride stock solutions were prepared as described in Sections 3.2 and 3.3. Stock solutions for sodium chloride were prepared at concentration of 1000 mmol L^{-1}. The skim milks were preheated according to methods described in Section 3.4. The total ionic strength of added salts refers to the calculated ionic strength contributed by the added salts, and does not include the ionic strength contributed by the salts present in native skim milk. The ionic strength in the native skim milk was assumed to be the same in all samples. Calculations of the total ionic strength of the added salts can be found in Appendix 6.

Effect of monovalent ions

The effect of monovalent ions on gelation in skim milk was investigated by complete substitution of calcium chloride with sodium chloride to equivalent ionic strength (i.e. only sodium chloride added to skim milk). The concentrations of sodium chloride added are shown in Figure 7-1.

Table 7-1: The concentration of added sodium chloride and calcium chloride at equivalent ionic strength.

Total ionic strength of added salts (mmol L^{-1}) (calculated)	**Concentration of added calcium chloride (mmol L^{-1})**	**Concentration of added sodium chloride (mmol L^{-1})**
0	0	0
60	20	60
90	30	90
120	40	120

Effect of ionic strength

The effect of ionic strength was investigated by varying the ionic strength in the calcium-added skim milk through addition of sodium chloride. The concentrations of added calcium chloride and sodium chloride, and the total ionic strength of the added salts are shown in Table 7-2.

Aliquots of calcium chloride stock solution and sodium chloride stock solution were added to preheated skim milk to achieve the concentration and ionic strength as shown in Figure 7-1 and Table 7-2. Distilled water was added to achieve final total solids concentration of 9.6% (*w/w*).

Table 7-2: The total ionic strength of the added salts and the concentrations of added calcium chloride and sodium chloride to achieve the ionic strength.

Total ionic strength of added salts (mmol L^{-1}) (calculated)	Concentration of added calcium chloride (mmol L^{-1})	Concentration of added sodium chloride (mmol L^{-1})
30	10	0
60	10	30
	20	0
90	10	60
	20	30
	30	0
120	10	90
	20	60
	30	30
	40	0

7.2.2 Analysis of skim milk

This chapter reports the effect of monovalent ions and ionic strength on the physicochemical and rheological properties of skim milk. The assays and techniques used in this chapter can be found in Sections 3.5, 3.6, 3.8, and 3.10. For the rheological measurement described in Section 3.10, the SS cup was used. The holding temperature was 80°C (sample temperature) for all samples.

7.3 Results and Discussions

7.3.1 Effect of monovalent ions and ionic strength on the calcium ion activity and pH

According to the Debye-Hückel limiting law, addition of salts, monovalent or divalent, results in an increase in the ionic strength of milk which decreases the activity of the ions in the solution (Walstra et al., 2006). Changes in the activity of ions in solution may influence the mineral equilibrium and pH. A comparison between the effect of the

addition of sodium chloride and calcium chloride to skim milk on the $a_{Ca^{2+}}$ and pH in skim milk is shown in Figure 7-1.

Addition of sodium chloride at ionic strength of 60 to 120 mmol L^{-1} did not result in significant changes to the $a_{Ca^{2+}}$, while addition of calcium chloride at the same ionic strength resulted in significant increase in $a_{Ca^{2+}}$ (Figure 7-1a). Addition of calcium chloride resulted in the increase in $a_{Ca^{2+}}$ due to the increase in concentration of calcium ions.

The addition of both calcium chloride and sodium chloride led to a decrease in skim milk pH (Figure 7-1b). Addition of calcium chloride resulted in a larger decrease in skim milk pH compared addition of sodium chloride at an added ionic strength of 60 mmol L^{-1}. On further increase in added ionic strength from 60 to 120 mmol L^{-1}, no further decrease in pH was observed in sodium chloride, but addition of calcium chloride continued to result in a decrease in pH. The difference in the observed decrease in pH for the two salts may be attributed to the different mechanisms involved in the release of the H^+ ions. The decrease in pH with the addition of calcium chloride is explained by the shift in calcium equilibrium which released H^+ ions from phosphates as discussed in Chapter 4. The decrease in pH as a result of the addition of sodium chloride was suggested to be due to increased dissociation of ion pairs as a result of reduction in ion activity with increasing ionic strength, and the exchange of Na^+ with H^+ ions attached to the negative groups on casein (Grufferty & Fox, 1985; van Hooydonk, Hagedoorn & Boerrigter, 1986).

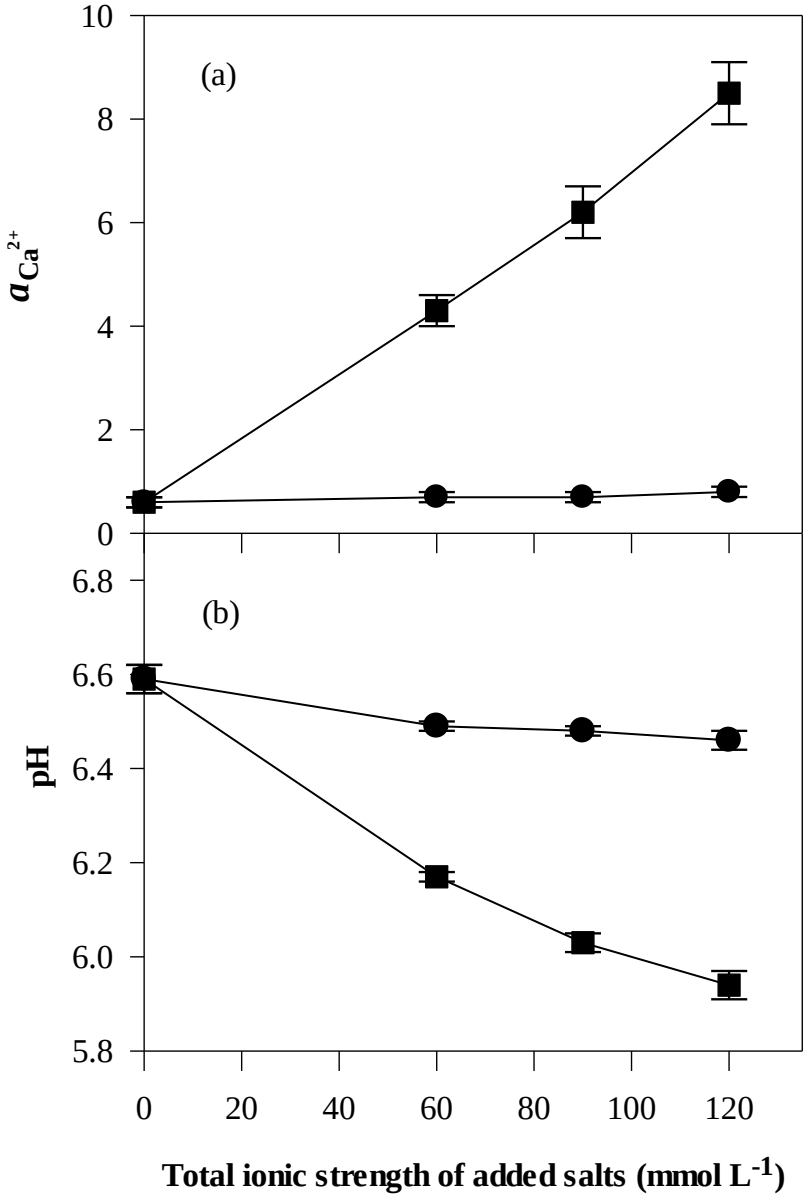


Figure 7-1: The effect of monovalent ions (Na^+) (●) and divalent ions (Ca^{2+}) (■) on the (a) calcium ion activity ($a_{Ca^{2+}}$) and (b) pH of skim milk added at the same ionic strength. Data presented represent solutions with only one type of added salt (either sodium chloride or calcium chloride). Data points are mean values ± standard deviation (n= 6).

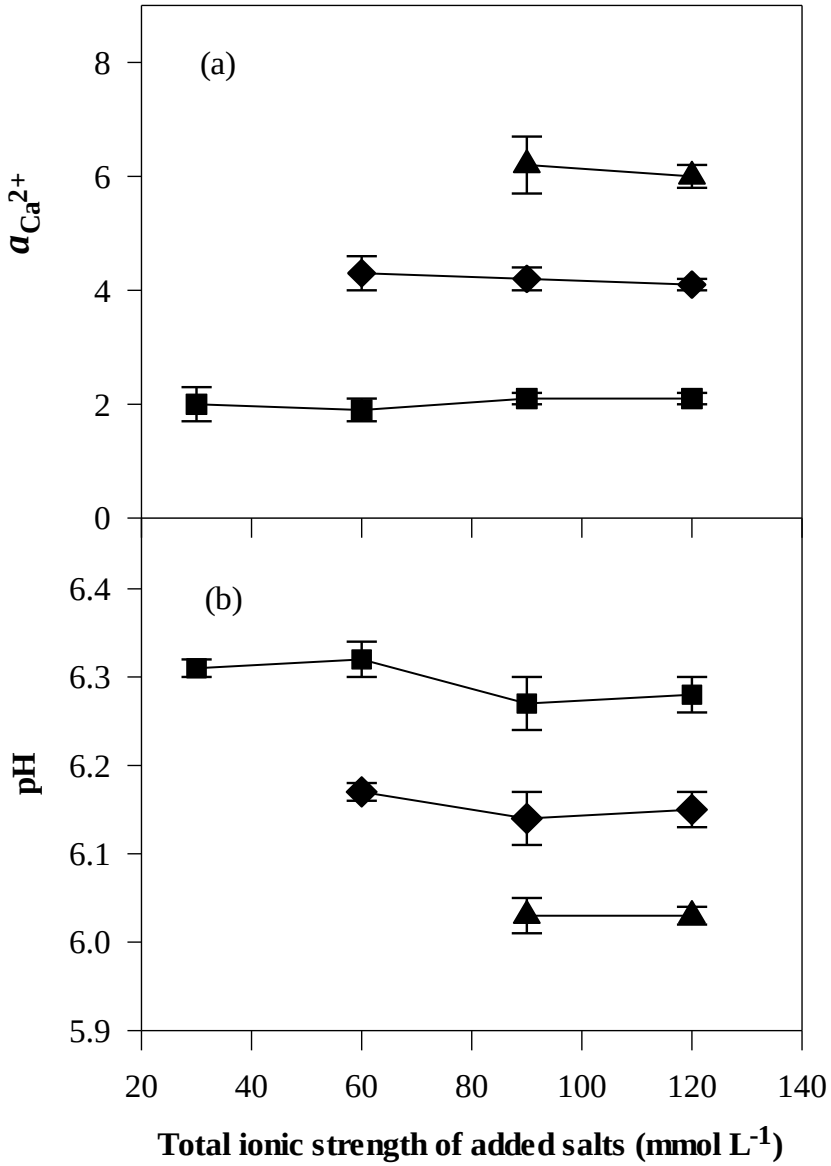


Figure 7-2: The effect of ionic strength on the (a) calcium ion activity (a_{Ca}^{2+}) and (b) pH of skim milk with added calcium chloride, 10 mmol L^{-1} (■), 20 mmol L^{-1} (◆) and 30 mmol L^{-1} (▲). Data points are mean values ± standard deviation (n= 6).

Increasing ionic strength by addition of sodium chloride did not result in significant changes to the a_{Ca}^{2+} in skim milk with 10, 20 and 30 mmol L^{-1} of added calcium chloride (Figure 7-2a). The increase in ionic strength also appeared to have no significant effect on the pH in calcium-added skim milk (Figure 7-1b). The results

showed that the ionic strength of the skim milk within the range studied (30 to 120 mmol L^{-1}) had no significant effect on the a_{Ca}^{2+} and pH of the skim milk.

7.3.2 Effect of monovalent ions and ionic strength on the calcium distribution between the sediment and serum phases in skim milk

Quantification of the mass of calcium in the skim milk and in the serum phase was conducted to provide insights into the changes in the distribution of the calcium between the colloidal and serum phases at different ionic strengths. Statistical analysis of the results shown in Table 7-3 found no significant difference in the concentration of calcium in the serum phase with increase in ionic strength at the same concentration of added calcium salt ($p > 0.05$). Addition of sodium chloride to milk was reported to result in an increase in serum calcium (Grufferty & Fox, 1985; Le Ray et al., 1998). The increase in serum calcium with addition of sodium chloride was attributed to the decrease in activity of calcium ions (due to increased ionic strength). This then leads to an increase in dissociation of ion pairs such as calcium phosphates and calcium citrates (Gaucheron, 2005; Walstra et al., 2006) and the exchange of Na^+ with Ca^{2+} ions which are directly attached to the negative charge on the surface of casein (Gaucheron, 2005; Grufferty & Fox, 1985; Le Ray et al., 1998). An increase in dissociation of ion pairs could be related to the solubility product behaviour in milk. Solubility product is defined as the product of the molar concentrations of the ions which are produced due to dissociation of the compound in a saturated solution (Mazumdar, 2008). As milk is saturated with respect to calcium phosphate, the product of the molar activity of calcium and phosphate ions would be equal to its solubility product (Mazumdar, 2008; Walstra et al., 1999). The solubility product of $CaHPO_4$ is given by the equation (Schwartz & Myerson, 2002):

$$K_{sp} = a_{Ca^{2+}} \times a_{HPO_4} = \gamma_{Ca^{2+}} . c_{Ca^{2+}} \times \gamma_{HPO_4^{2-}} . c_{HPO_4^{2-}}$$

Equation 7-1

where K_{sp} is the solubility product,

a is the activity of the ion,

γ is the activity coefficient of the ion,

and *c* is the concentration of the ion

An increase in ionic strength is known to result in a decrease in the activity of ions due to the decrease in activity coefficient (Walstra et al., 2006). As shown in Equation 7-1, the reduction in activity coefficient would thus allow for increased concentration of Ca^{2+} and HPO_4^{2-} ions in the serum phase before saturation occurs. As a result, increased calcium phosphate and calcium citrates from the casein micelles may dissociate into ions and solubilise in the serum phase, resulting in increased serum calcium (Grufferty & Fox, 1985). However, this result was not found in the present study. The difference in the findings could be due to the concentration of sodium chloride added. Grufferty & Fox (1985) reported that at up to 100 mmol L^{-1} of sodium chloride addition, there was only a small increase in serum calcium (approximately 0.002 mg/ ml). This suggests that within the concentration of sodium chloride added in the present study (up to 120 mmol L^{-1}), possible changes in the concentration of serum calcium may not have been detected as it was outside the limit of sensitivity of the technique used for calcium determination in the present study (0.02 mg/ g). Further, Grufferty & Fox (1985) adjusted the pH of the milk to pH 6.6 following the addition of sodium chloride. As the pH in this study was not readjusted, it may also be the cause of the difference in results between the present study and the work of Grufferty & Fox (1985).

Table 7-3: Concentration of calcium (mg g^{-1}) in skim milk, serum and sediment of skim milk at various concentrations of added calcium chloride (n = 3 to 6).

Concentration of calcium chloride added (mmol L^{-1})	**Concentration of added sodium chloride (mmol L^{-1})**	**Total ionic strength from added salts (mmol L^{-1})**	**Total calcium in skim milk^{+} (mg g^{-1})**	**Serum calcium^{+} (mg g^{-1})**	**Sediment calcium* (mg g^{-1})**
0	60	60	1.18	0.30	0.88
	90	90	1.16	0.30	0.86
	120	120	1.16	0.30	0.86
10	0	30	1.60	0.52	1.08
	30	60	1.58	0.52	1.06
	60	90	1.56	0.50	1.06
	90	120	1.56	0.50	1.06
20	0	60	2.00	0.82	1.18
	30	90	1.98	0.82	1.16
	60	120	1.98	0.80	1.18
30	0	90	2.38	1.14	1.24
	30	120	2.38	1.12	1.26

Pooled standard deviation: ± 0.02

$^{+}$ Concentration determined by EDTA titration

* Concentration determined by difference between milk and serum calcium

7.3.3 Effect of monovalent ions and ionic strength on the rheological properties

The adjustment of ionic strength, typically by the addition of sodium chloride in milk, was reported to influence the rheological properties of milk protein gels (Awad, 2007; Lucey, Van Vliet, et al., 1997; McClements & Keogh, 1995; Schkoda, Hechler & Kessler, 1999). The effect of added sodium chloride on the rheological properties of skim milk was investigated and compared to the effect of added calcium chloride on the rheological properties of skim milk (Table 7-4).

Table 7-4: The final G' of skim milk with added calcium chloride or sodium chloride at various ionic strength, after cooling at 20°C. The skim milk was preheated at 90°C for 10 min, and held in the rheometer at 80°C for 60 min. Data presented are mean values ± standard deviation (n = 2 to 3).

	Final G'			
	Ionic strength of added salts (mmol L^{-1})			
Salt added	0	60	90	120
Calcium chloride	DNG [+]	72.2 ± 1.0	81.7 ± 1.2	86.2 ± 0.2
Sodium chloride	DNG	DNG	DNG	DNG

[+] DNG: did not gel ($G' > 1$ Pa)

Comparisons between calcium chloride (divalent ion) and sodium chloride (monovalent ion) indicated that calcium chloride induced gelation in skim milk on heating, but no gelation was observed when sodium chloride was added at the same ionic strength of 60 to 120 mmol L^{-1} (equivalent to 20 to 40 mmol L^{-1} of calcium chloride and 60 to 120 mmol L^{-1} of sodium chloride) (Table 7-4). Both Ca^{2+} and Na^{+} are capable of screening the charge of the proteins, thus reducing electrostatic repulsion (Bryant & McClements, 1998; Mudgal et al., 2011). The absence of gelation in skim milk with added sodium chloride at the same ionic strength as added calcium chloride indicates that the gelation of skim milk induced by calcium salts was not merely due to a charge screening effect. In whey protein gels, both sodium chloride and calcium chloride were able to induce

gelation, although a lower concentration of calcium chloride was needed to induce gelation than sodium chloride (Croguennec et al., 2004; Mulvihill & Kinsella, 1988; Tang et al., 1995). In contrast, Nguyen et al. (2017) reported that at pH 6.6, addition of sodium chloride (0.1 mol L^{-1}) did not result in gelation in pure micellar casein systems. Further, at pH 6.2 to 6.4, addition of 0.1 mol L^{-1} sodium chloride to pure whey protein systems decreased the minimum protein concentration required for gelation, but addition of sodium chloride to pure micellar casein system significantly increased the minimum protein concentration required for gelation (Nguyen et al., 2017). Nguyen et al. (2017) suggested that the screening of charge repulsion by sodium chloride promoted gelation of whey proteins, but the same charge screening effect may weaken the attraction between the casein micelles. The presence of casein micelles in skim milk may explain why the addition of sodium chloride did not result in the gelation of skim milk in this study. Further, the percentage of whey proteins present in skim milk may have been too low for whey protein gelation to occur.

Gelation observed in skim milk with added calcium chloride could be attributed to the ability of Ca^{2+}, a divalent cation, to act as salt bridges between the carboxyl groups of two neighbouring proteins (Bryant & McClements, 1998; Chung et al., 2013). A similar mechanism between the Ca^{2+} ions and the casein and whey proteins in skim milk may also occur. Ion-specific interactions between the calcium ions and proteins may also occur; these cannot occur with sodium ions. For example, added Ca^{2+} may form calcium phosphate which may become part of the colloidal calcium phosphate (CCP), crosslinking other casein molecules (Choi et al., 2007; Ramasubramanian et al., 2014). The lower pH in the skim milk induced by calcium addition (Figure 7-1) may have also facilitated the gelation of calcium-added skim milk.

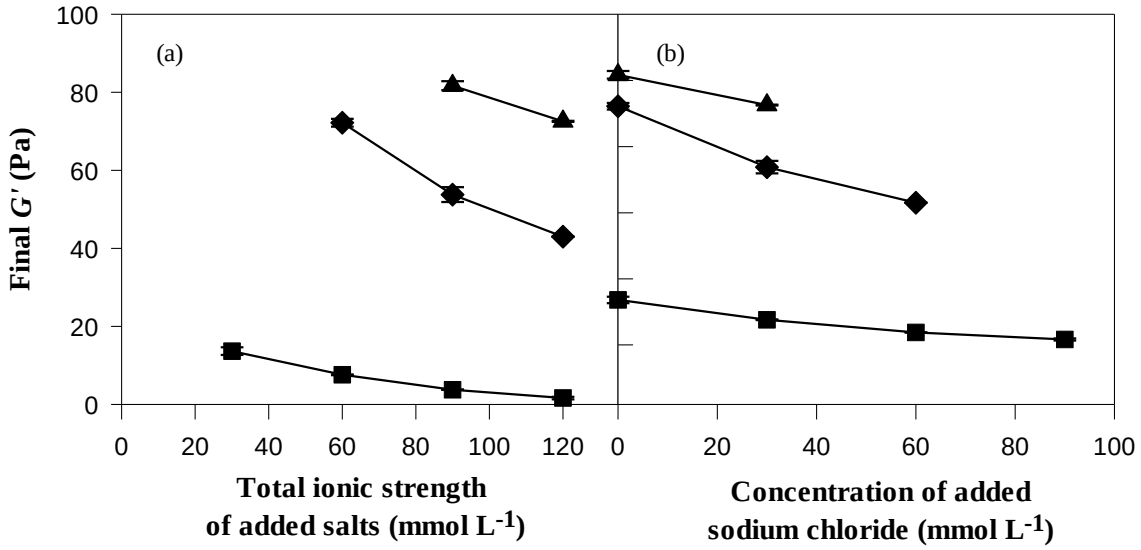


Figure 7-3: Relationship between final G' of calcium-induced skim milk gels with 10 mmol L^{-1} (■), 20 mmol L^{-1} (◆) and 30 mmol L^{-1} (▲) of added calcium chloride, based on (a) total ionic strength of added salts (calcium chloride and sodium chloride) and (b) concentration of added sodium chloride. Skim milk were preheated at 90°C for 10 min prior to salt addition, and held at 80°C for 60 min in the rheometer. Results presented are final G' values after cooling, at 20°C. Data points are mean values ± standard deviation (n= 2 to 3).

At the same ionic strength, skim milk with higher concentrations of added calcium chloride achieved a higher final G' (Figure 7-3a). As discussed earlier, while both Ca^{2+} and Na^{+} are able to screen charges and reduce electrostatic repulsion, Ca^{2+} ions are also capable of forming calcium bridges, which may facilitate more bond formation, leading to a higher G'. Balakrishnan et al. (2018) proposed that Na^{+} ions may screen the negatively charged residues on casein that may otherwise form calcium bridges. This may be the reason for the reduction in final G' when Ca^{2+} ions were replaced with Na^{+} ions, even when the replacement was at the same ionic strength.

At the same concentration of added calcium chloride, the increase in concentration of added sodium chloride resulted in a reduction in the final G' of the calcium-induced skim milk gels at all concentrations of added calcium chloride (Figure 7-3b). Addition

of sodium chloride was reported to have varying effects on the gelation of dairy proteins. In heated and cold-set whey protein gels, increasing the concentration of sodium chloride up to 100 mmol L^{-1} increased the strength of the gels (Mulvihill & Kinsella, 1988; Tang et al., 1995). In acid- and rennet-induced milk gels, addition of sodium chloride was reported to result in increased gelation time and decrease in firmness of the gels (Daviau, Famelart, Pierre, Goudédranche & Maubois, 2000; Lucey, Van Vliet, et al., 1997; van Hooydonk et al., 1986). The reduction in electrostatic repulsion and thereby facilitation of hydrophobic interactions by binding of Na^+ in whey protein systems did not appear to promote gelation in the calcium-induced skim milk gels (McClements & Keogh, 1995; Tang et al., 1995). Nguyen et al. (2017) reported that addition of sodium chloride to pure whey protein systems increased G' of the gels with heating at 80°C, but addition of sodium chloride to pure micellar casein systems decreased the G' with heating at 80°C at pH 6.3. Hence, the reduction in G' in acid-, rennet-, and calcium-induced milk gels on addition of sodium chloride is likely due to the effect of sodium chloride on casein micelles. A possible mechanism could be the competition between the Na^+ ions and Ca^{2+} ions for the binding sites on the negatively charged residues on the casein micelles, or exchange of the Na^+ for Ca^{2+} ions which were attached to casein (Ahmad, Piot, Rousseau, Grongnet & Gaucheron, 2008; Grufferty & Fox, 1985). Attachment of Na^+ instead of Ca^{2+} to the proteins may result in decreased calcium-bridging between the proteins even though the concentration of added calcium chloride remained unchanged, resulting in a lower final G',

However, as discussed in Section 7.3.1 and Section 7.3.2, the $a_{Ca^{2+}}$ and concentration of serum calcium did not increase significantly on addition of sodium chloride. A significant increase in $a_{Ca^{2+}}$ or serum calcium would be expected if the displacement of Ca^{2+} from casein by the Na^+, or solubilisation of the CCP was the reason for the

significant decrease in final G' observed in Figure 7-3. This suggested that addition of Na^+ may involve other mechanisms that hindered gelation of the skim milk. Hydration interactions are short-range repulsive interactions that arise when two hydrated molecules closely approach (Bryant & McClements, 1998). The exact mechanism behind hydration forces are intensely debated, but is generally acknowledged to be present between all surfaces in water, such as surfactants, colloids, biomolecules and proteins (Kowalik, Schlaich, Kanduc, Schneck & Netz, 2017). Famelart, Gauvin, Pâquet & Brulé (2009) found that hydration and solubility of casein increased with increased concentration of sodium chloride addition up to 120 mmol L^{-1}. The increase in hydration of the casein at low ionic strength (< 200 mmol L^{-1}) may be a result of the binding of hydrated Na^+ ions to the proteins. Damodaran (1997) stated that at low ionic strength, although binding of counter-ions to proteins results in screening of charges, it does not affect the hydration shells on the charged group of the proteins, and the increase in hydration could be attributed to the hydration shells of the bound ions. Hydration shells of ordered water molecules surrounding ions may influence the aggregation of proteins in solution by increasing hydration repulsion (Bryant & McClements, 1998; Oakenfull et al., 1997). Binding of hydrated Na^+ to the carboxyl or phosphoseryl residues on casein may increase hydration repulsive forces between the proteins (Bringe & Kinsella, 1991; Saluja & Kalonia, 2008), thereby reducing the tendency for protein aggregation and gelation. Although Ca^{2+} ions are more strongly hydrated than Na^+ ions according to the Hofmeister series (Kunz & Neueder, 2010), it is possible that the attractive electrostatic interactions between Ca^{2+} and the negatively-charged proteins are strong enough to overcome the repulsive hydration forces, thus favouring protein-protein interactions.

7.4 Conclusions

The addition of sodium chloride at ionic strength of 60 to 120 mmol L^{-1} did not result in the gelation of skim milk, even though addition of calcium chloride at the same ionic strength resulted in gelation. This result suggested that promotion of hydrophobic interactions by screening of electrostatic repulsive forces was insufficient to induce gelation in skim milk. Calcium-bridging by the Ca^{2+} ions, increase in colloidal calcium phosphate, and the lower pH induced by calcium chloride addition to skim milk all favoured gelation. The reduction in final G' in calcium-induced skim milk when ionic strength was increased by addition of sodium chloride indicated that Na^{+} ions had an inhibiting effect on the formation of the gel network. The calcium ion activity and serum calcium did not increase significantly with increasing addition of Na^{+}, suggesting that exchange of Na^{+} with casein-bound Ca^{2+} was not the reason for the reduction in final G'. Hydration repulsion between the proteins may increase due to binding of the hydrated Na^{+} ions with the proteins, which could have prevented aggregation and gelation of the proteins. The results showed that a balance between the attractive (calcium-bridging and hydrophobic) and repulsive (electrostatic and hydration) forces between the proteins is critical in the interactions and gelation of the proteins.

Chapter 8 - Overall discussion

The overall aim of this study was to determine the mechanisms involved in the development of calcium-induced skim milk gels through a fundamental understanding of the factors affecting gelation in milk. The skim milk samples were given different treatments, and their chemical chemical properties, such as a_{Ca}^{2+}, pH, and calcium distribution between the serum and colloidal phases, were determined. The chemical properties of the skim milk were related to the rheological properties of the heated calcium-added skim milk to evaluate possible interactions between the proteins that resulted in gelation. Ion-protein interactions were investigated in Chapter 4 and 7. In Chapter 4, the effect of various calcium salts with different dissociation behaviour, was determined. The Hofmeister effect was also investigated by comparing the effect of calcium chloride and calcium iodide addition to skim milk. To determine the effect of monovalent ions and ionic strength, sodium chloride was added to skim milk and the results were compared to the results of divalent calcium ions (Chapter 7). The effects of heat treatment (temperature), pH adjustments, and preheat treatment were investigated in Chapter 5 to determine how processing conditions may affect gelation. The results of the effect of preheat treatment (Chapter 5) suggested that interactions between casein and whey proteins may influence the rheological properties of calcium-added skim milk. Hence, in Chapter 6, a detailed study of the contribution of each protein type to gelation was determined. The type of intermolecular forces involved in protein-protein interactions, and conditions of the skim milk which may promote or inhibit the gelation of a calcium-added skim milk are also discussed.

8.1 Ion-protein interactions in calcium-added skim milk

The addition of ions to milk can affect the stability of milk proteins by directly interacting with the proteins via electrostatic interactions, and/ or by changing the ionic strength which affects hydration and solubility of the proteins (Damodaran, 1997; Totosaus et al., 2002). In Chapter 4, it was found that calcium salts with different dissociation behaviour resulted in different a_{Ca}^{2+} and pH in skim milk. Rheological results showed that calcium-added skim milk with a higher a_{Ca}^{2+} led to formation of gels with higher final G'. This could be attributed to a few possible mechanisms. Firstly, a higher a_{Ca}^{2+} suggested that there may be more free calcium ions (Ca^{2+}) available which may act as calcium bridges between two adjacent proteins (Bryant & McClements, 1998; Dalgleish, 1983). Secondly, a higher a_{Ca}^{2+} would likely result in the calcium equilibrium shifting towards the formation of calcium phosphate. The calcium phosphate may transfer to the casein micelles as colloidal calcium phosphate (CCP) (Philippe et al., 2003). The increase in CCP was proposed to increase crosslinking of casein molecules, possibly with the soluble casein in the serum phase (Horne, 2002; van Boekel et al., 1989). The shift in the calcium equilibrium towards the formation of calcium phosphate also results in the release of H^+ ions from $H_2PO_4^{2-}$, which could destabilise the proteins by reduction in the electrostatic repulsion between the proteins (Chapter 4). For organic calcium salts such as calcium lactobionate, an intermediate cation complex, CaL^+, may also be released besides Ca^{2+} ions (Vavrusova et al., 2014). The positively charged cation, CaL^+, could also interact with the negatively-charged sites, such as phosphate or carboxyl groups on the casein micelles. However, as limited information is available on CaL^+, the hypothesis that CaL^+ may interact with proteins and their effect on protein interactions requires further investigation.

The effect of the electrostatic repulsion on the aggregation and gelation of protein was investigated in Chapter 7 by comparing the rheological properties of skim milk with added monovalent (Na^+) and divalent (Ca^{2+}) ions. Both Ca^{2+} and Na^+ are able to screen charges and reduce electrostatic repulsion, thus favouring hydrophobic interactions between the proteins (McClements & Keogh, 1995; Mulvihill & Kinsella, 1988). However, the addition of sodium chloride to skim milk at the same ionic strength as calcium chloride did not result in gelation in skim milk, indicating that the gelation induced by calcium salt addition was not merely due to charge screening. Addition of sodium chloride to calcium chloride-added skim milk led to a reduction in final G' of the calcium-induced skim milk gel (Chapter 7). This could possibly be due to Na^+ interacting with the negative-charged sites on the proteins, which prevented some Ca^{2+} from interacting with the proteins and forming calcium bridges. However, there were no significant changes in the $a_{Ca^{2+}}$ and serum calcium concentration when sodium chloride was added, suggesting that there were no significant changes in the concentration of Ca^{2+} attached to the casein micelles when Na^+ was added at the concentrations studied in this work. Another possibility for the reduction in final G' of the calcium-induced skim milk gel when sodium chloride was added could be increased hydration repulsion between the proteins. Hydration shells of ordered water molecules surrounding the Na^+ bound onto the surfaces of the proteins could increase the hydration repulsive forces between the proteins, thereby reducing the tendency for aggregation and gelation (Bringe & Kinsella, 1991; Oakenfull et al., 1997).

Most studies involving ion-protein interactions have focused on interactions between cations and the net negatively charged proteins at the natural pH in milk. However, it appears anions may also influence the aggregation and gelation of the milk proteins. In Chapter 4, the final G' values of the calcium-induced skim milk gels with added calcium

iodide were lower than those with added calcium chloride. The anions, Cl^- and I^-, may bind to the positively-charged amino acid residues and on the non-polar surfaces of proteins (Bringe & Kinsella, 1991; Lund et al., 2010). According to the Hofmeister series, I^- ions have a higher affinity for non-polar surfaces of proteins than Cl^- ions. Therefore, I^- ions may have a higher overall affinity than Cl^- ions for proteins. This, in turn, may have led to a higher net negative charge of the iodide-bound proteins, resulting in stronger electrostatic repulsion and thus a lower final *G′*.

8.2 The effect of heat treatment and pH in calcium-added skim milk

Alterations to the pH and temperature of milk are known to have significant effect on the stability of milk proteins. The reduction in pH of skim milk when calcium salt was added appeared to favour gelation because when the pH was readjusted back to the native pH 6.6 after calcium salt addition, the final *G′* decreased. This was attributed to the charge-screening effect of H^+ ions, which may have resulted in reduced electrostatic repulsion between the proteins, favouring aggregation and gelation. In Chapter 5, the results showed that increasing the holding temperature in the rheometer from 70°C to 90°C resulted in increased final *G′*. This was presumably due to the more rapid movement of the molecules as temperature increased, and hence a higher frequency of collision and bond formation among the protein particles (Baldwin, 1986; Hummer et al., 1998).

The final *G′* of the calcium-induced skim milk gel increased when the skim milk was preheated at 90°C for 10 min, which suggested the participation of denatured whey proteins in the gel network (Chapter 5). The pH at which skim milk was preheated at was found to influence the concentration of the casein, β-lactoglobulin and α-lactalbumin in the serum phase, and the rheological properties and strength of the gel. It was hypothesised that the effect of preheating pH on the rheological properties of the

calcium-induced skim milk gel was dependent on the concentration of calcium salt added. In skim milk preheated at pH 6.40, the number of aggregating particles after preheat treatment were presumed to be lower as whey proteins were mostly associated with the casein micelles as analysed by SDS-PAGE. Conversely, in skim milk preheated at pH 6.80, the presence of soluble whey protein aggregates and dissociated whey protein-κ-casein complexes in the serum phase after preheat treatment could provide more sites for aggregation and the potential of a more complex gel network (Anema et al., 2004a). At calcium salt addition of 10 to 15 mmol L^{-1}, the calcium ions available for bond formation may be the limiting factor. Therefore, skim milk preheated at a lower pH (6.40) resulted in higher final *G′*, likely due to the added effect of more H^+ ions present in the system which promoted gelation. As the concentration of calcium salt added increased to 20 and 40 mmol L^{-1}, the number of calcium ions available for interactions with the proteins increased. There may then be sufficient calcium ions to interact with the higher number of aggregating particles in skim milk preheated at higher preheating pH (pH 6.80), thus resulting in formation of a more connected gel network and a higher final *G′*. It should be noted that the results discussed in Chapter 5 applies only to skim milk with casein to whey protein ratio of 4:1. As results in Chapter 6 showed, the effect of preheat treatment may differ depending on the ratio of casein to whey protein present in the skim milk.

8.3 Casein and whey protein interactions in calcium-added skim milk

In Chapter 5, rheological results from the skim milk preheated at different pH suggested that the interactions between casein and whey proteins could influence how the two proteins participated in the gel network. The contribution of casein and whey proteins to the gelation of calcium-added skim milk was then investigated in detail in Chapter 6. In 0.6% WPI solutions (percentage of whey proteins present in native skim milk), gelation

was observed when 10 to 40 mmol L^{-1} of calcium salts (calcium chloride and calcium lactobionate) were added. This indicated that with added calcium, whey proteins were able to form gels in the absence of casein, possibly through calcium bridging. Moreover, denatured whey proteins are also able to associate via hydrophobic and disulphide interactions, which could also cause gelation of the whey proteins (Bryant & McClements, 1998; Chung et al., 2013). However, casein was likely the protein primarily responsible for the formation of the gel network with whey proteins providing a supporting role in enhancing the strength of the gel network. This was indicated by the lower final G' values with the 0.6% WPI solution compared to skim milk at between 20 to 40 mmol L^{-1} of added calcium salt (Chapter 6).

The results for the skim milk blends with different ratios of casein to whey proteins suggested that the contribution of casein and whey proteins towards the strength of a calcium-induced skim milk gel was dependent on the ratio of casein to whey proteins, the calcium ions available, as well as whether any preheat treatment was applied. In preheated skim milk blends, calcium ions appeared to bind predominantly to whey proteins during heating from 20°C to 80°C as observed by the higher G' in skim milk blends with a higher proportion of whey proteins. However, competition between casein and whey proteins for calcium ions may result in differences in gel formation during holding at 80°C (Nguyen et al., 2016). The effect of whey proteins on the final G' of the gel appeared to be dependent on the ratio of casein to whey proteins present. At the same concentration of added calcium chloride, increasing the ratio of whey proteins in the preheated skim milk blends from 5% to 10% decreased the final G', possibly due to the denatured whey proteins competing with casein micelles for binding with calcium ions. On further increase of the whey proteins from 10% to 20%, the final G' increased, presumably due to the ability of whey proteins to self-associate and form gels at

sufficient concentrations. The concentration of calcium ions available also influenced the final G' of the gel. At low concentration of added calcium chloride (10 mmol L^{-1}), the calcium ions available for binding may be limited. As discussed in Chapter 6, the added calcium ions may bind preferentially to denatured whey proteins in the preheated skim milk blends during the initial gelation phase on heating from 20°C to 80°C. This may result in limited calcium ions available for binding with casein micelles in a system with lower concentration of added calcium chloride. The ability of whey proteins to self-associate and form gels appeared to play an important role in the strength of the gel at lower concentrations of added calcium chloride. This may explain why the final G' values of skim milk blends with higher proportions of whey protein (15% and 20% whey protein) was the greatest at 10 mmol L^{-1} of calcium chloride added. At higher concentration of added calcium chloride (20 and 40 mmol L^{-1}), the calcium ions available for binding increased. This could have led to sufficient calcium ions being available for bindings with casein micelles after the initial calcium binding with the denatured whey proteins during heating from 20°C to 80°C. It was postulated that with sufficient calcium ions available, the contribution of the casein micelles to the development of the gel network dominated during holding at 80°C. This could explain why increasing the proportion of casein resulted in a higher increase in G' during holding at 80°C in skim milk blends with 20 and 40 mmol L^{-1} of added calcium chloride.

In non-preheated skim milk blends, the whey proteins were not appreciably denatured prior to heating from 20°C to 80°C in the rheometer. Contrary to the results in the preheated skim milk blends, the G' values after heating from 20°C to 80°C were the lowest in the skim milk blends (20 mmol L^{-1} added calcium chloride) with the highest proportions of whey proteins. This indicated that during heating, denatured whey

proteins promoted gelation, but this was not observed when the whey proteins were not denatured prior to heating in the rheometer. The final G' of the non-preheated skim milk blends decreased with increasing proportions of whey proteins. This suggested that when the whey proteins were denatured prior to heating in the rheometer, the contribution of the denatured whey proteins to the strength of the gel may be greater compared to the whey proteins that are not denatured in the non-preheated skim milk blends. Whether denatured whey proteins would promote gelation was also dependent on the ratio of casein to whey proteins in the skim milk blends. For instance, in 5% whey protein skim milk blends, preheat treatment decreased the final G' of the gel, possibly due to increased competition for calcium binding when the whey proteins were denatured. However, in 20% whey protein skim milk blends, preheat treatment increased the final G' of the gel, which again could be attributed to the ability for whey proteins to contribute to gelation at sufficient concentrations. In systems where there are sufficient calcium ions available for binding, skim milk with higher proportions of casein will form stronger gels as casein is the protein primarily responsible for the gel network, formed through calcium bridging, hydrophobic interactions, and the increase in CCP which may link up the casein molecules in the serum phase. The key factors affecting the gel strength of calcium-induced skim milk gels are summarised in Figure 8-1.

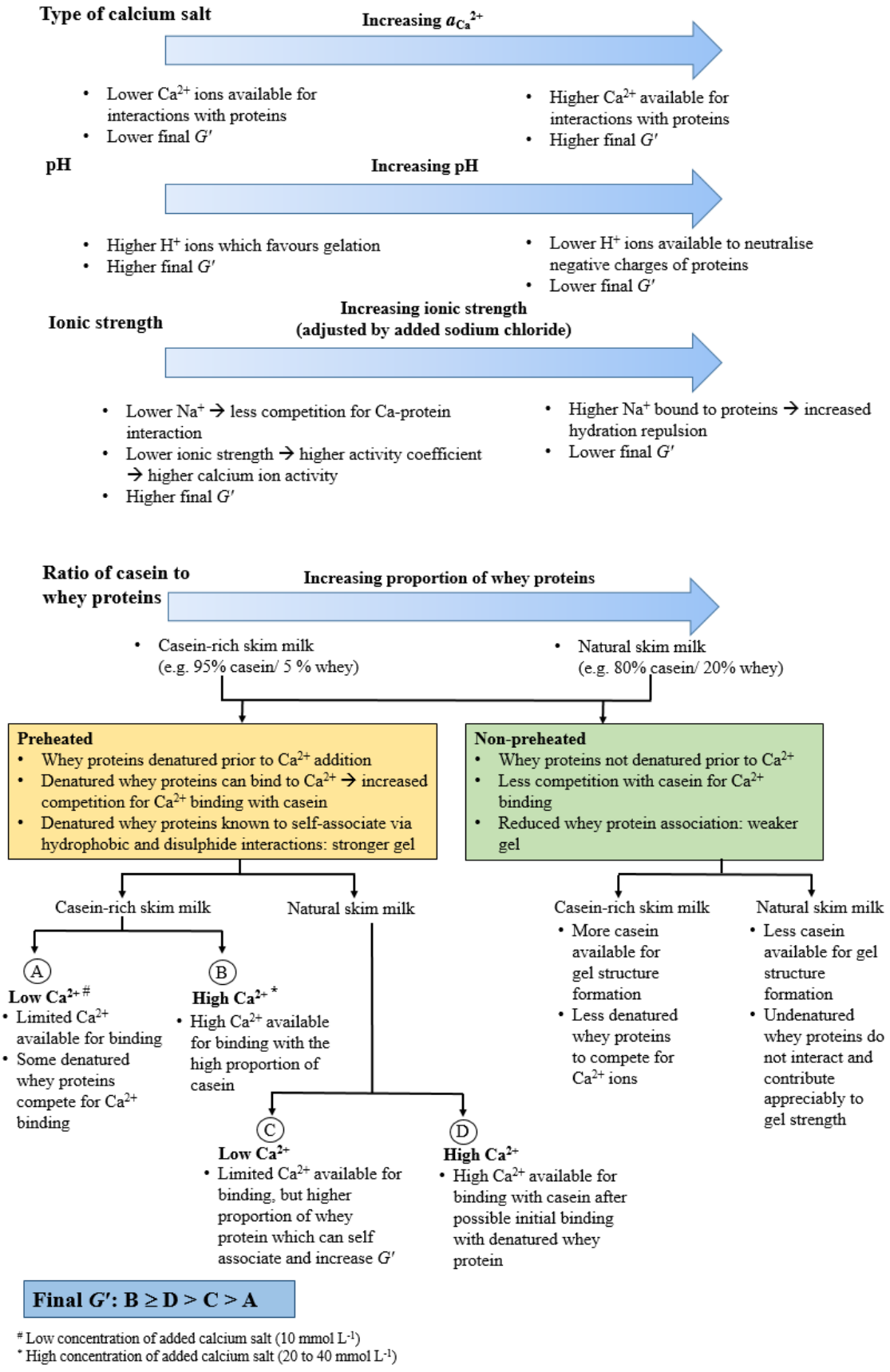


Figure 8-1: Summary of the key factors investigated in this research that were found to affect the gel strength of calcium-induced skim milk gels

Chapter 9 - Conclusions and recommendations

9.1 Conclusions

This work had shown that the addition of calcium salts to skim milk alters the physicochemical properties of the skim milk, such as its a_{Ca}^{2+}, pH, calcium and protein distribution between the serum and colloidal phases, particle size and zeta potential of the casein micelles. These changes may result in decreased stability of the casein and whey proteins in the skim milk, and ultimately aggregation of the proteins and gelation of the skim milk on heating. The effect of treatments that can be applied to skim milk which may influence the strength of a calcium-induced skim milk gel was also explored. It was shown that manipulation of the temperature during gelation, which influences the rate of movement of the particles; the preheat treatment, which influences the casein-whey protein interactions; and the adjustment of the skim milk pH and ionic strength could be used to alter the rheological properties of the gel.

Based on the findings of this study, the characteristics of a calcium-induced skim milk gel is the net result of factors that promoted or inhibited the interactions and aggregation of the proteins. The screening of charges of the proteins, either by Ca^{2+} or H^{+} ions, promoted gelation. This was presumably due to increased hydrophobic interactions due to a reduction in the electrostatic repulsion between the proteins. However, charge screening was not the primary cause of gelation as replacing Ca^{2+} with Na^{+} ions at the same ionic strength, or decreasing the skim milk pH to the pH induced by calcium salt addition, did not result in gelation of the skim milk. The addition of Na^{+} to calcium-added skim milk appeared to inhibit gelation, possibly due to increased hydration repulsion between proteins. The added calcium may have promoted gelation by acting as calcium bridges between the proteins, and/ or by increasing the amount of CCP,

which may crosslink with other casein molecules in the serum phase. The contribution of the casein and whey proteins towards the formation of a calcium-induced skim milk gel was also investigated. Casein was the protein primarily responsible for the structure of the gel network. The presence of denatured whey proteins in the skim milk prior to heating in the rheometer appeared to have two competing effects on the gel network: enhancement of the gel strength by association amongst themselves or with casein micelles via hydrophobic or disulphide bonding and limiting interactions amongst casein micelles due to increased competition for calcium ion binding. An intricate balance between the ratio of the casein to whey proteins present, the amount of calcium ions available for binding, and any preheat treatment applied to denature the whey proteins was crucial in determining the final gel properties. The results demonstrated the complexity in the formation of the gel network of a calcium-induced skim milk gel, and that alterations to any one of the influencing factors can result in different effects on the final gel properties. All in all, this work identified key factors that can be manipulated to produce calcium-induced skim milk gels with different rheological properties.

9.2 Recommendations

One of the key effects of calcium salt addition to skim milk was the change in the calcium equilibrium of the skim milk. Changes in the calcium equilibrium involved mainly the calcium ions, H^+ ions, and phosphate ions. In this work, only the distribution of the calcium between the serum and sediment phases was determined. Determination of the phosphate distribution is recommended to better understand the solubility product behaviour of calcium phosphate, which may provide insights into the interactions between the newly formed calcium phosphate and casein micelles. In addition, the effect of CaL^+ on the calcium equilibrium and interactions between the proteins is also

not known. Hence, further research on the CaL^+ ion may provide valuable information on the effect of organic calcium salts on skim milk.

The effects of pH, temperature, preheat treatment, and preheating pH were studied and discussed as individual factors in Chapter 5. Use of experimental design techniques, such as response surface methodology (RSM), may be useful in elucidating the combined or interaction effects of these factors. RSM may also be used in optimisation of the gel strength of calcium-induced skim milk gels.

At different concentrations of added calcium salt, the order of the final G' for the skim milk preheated at different pH varied. It is hypothesised that different preheating pH may result in different casein and whey interactions, which could lead to different gelation behaviour at different concentrations of added calcium salt. An investigation into the microstructure of the gels formed at different concentrations of added calcium salt and preheating pH could reveal if the association of the casein and whey proteins during gelation differed depending on its preheating pH, and if any structural differences in the gel network may have resulted in the difference in the final G' observed. Further work on varying the preheating pH of the skim milk blends with different casein to whey protein ratio may also provide more information on how the interactions between casein and whey protein during preheat treatment could influence the final G' of the calcium-induced skim milk gel.

The addition of calcium to milk and dairy products remains an important area of interest in dairy research. The findings of this study provided new insights into the calcium fortification and textural modifications of milk. This study showed that the addition of high concentrations of soluble organic calcium salts, the more bioavailable form of calcium compared to soluble inorganic or insoluble calcium salts, is possible in the

manufacture of a calcium-induced skim milk gel. The use of soluble calcium salts to induce gelation in milk allowed the production of a high-calcium dairy product without the undesirable milk protein coagulation during heat processing of milk. The findings of this study also provided an alternative method for textural modification and production of milk protein gels, and the factors that can be manipulated to achieve the desired rheological properties of a high-calcium dairy product.

Chapter 10 - References

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Chapter 11 - Appendices

Appendix 1: Determination of degree of hydration for calcium iodide hydrate

The manufacturer's specification for degree of hydration for calcium iodide hydrate was 4 to 6. Hence, assuming a degree of hydration of 5, and a target calcium stock solution of 250 mmol L^{-1}, the mass of calcium iodide hydrate needed was:

$$\text{Moles} = \frac{\text{Mass}}{\text{Molar mass}}$$

$$0.25 \text{ mol} = \frac{\text{Mass}}{293.887+5(18)}$$

$$= 95.97g$$

where the molar mass of anhydrous calcium iodide is 293.887 g/mol, and H_2O is 18 g/mol.

From EDTA titration, the calcium iodide stock solution was found to be 310 mmol L^{-1} instead of 250 mmol L^{-1}. Therefore, by back calculation, the degree of hydration of the calcium iodide hydrate was:

$$0.31 \text{ mol} = \frac{95.97}{293.887+x(18)}$$

$$x = 0.9$$

Appendix 2: Sample calculations for ionic strength, activity coefficient, and calcium ion activity

Sample calculations for determining the calcium ion activity of 10 mmol L^{-1} calcium chloride standard solution at ionic strength 250 mmol L^{-1}

Ionic strength (I)

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

Where I is the ionic strength, c_i is the concentration of ion *i* (mol L^{-1}), and z_i is the charge of ion *i*,

$$I = \frac{1}{2}\{([Ca^{2+}] \times z_i^2) + ([Cl^-] \times z_i^2)\}$$

Number of Ca^{2+} ions =1, charge of Ca^{2+} ions = +2
Number of Cl^- ions = 2, charge of Cl^- ions = -1

$$I = \frac{1}{2}\{(0.01 \times 1 \times 2^2) + (0.01 \times 2 \times (-1)^2)\}$$

$$= 0.03 \text{ mol } L^{-1}$$

$$= 30 \text{ mmol } L^{-1}$$

The ionic strength contributed by 10 mmol L^{-1} calcium chloride is 30 mmol L^{-1}.

Therefore, to adjust the solution to an ionic strength of 250 mmol L^{-1}, addition of KCl was needed.

Ionic strength needed to be provided by KCl = 250 – 30 = 220 mmol L^{-1}

Number of K^+ ions =1, charge of K^+ ions = +2

Number of Cl^- ions = 1, charge of Cl^- ions = -1

Let *x* be the concentration of KCl needed:

$$I = \frac{1}{2}\{([x] \times z_i^2) + ([x] \times z_i^2)\}$$

$$220 = \frac{1}{2}\{([x] \times (1)^2) + ([x] \times (-1)^2)\}$$

$$220 = 0.5x + 0.5x$$

$$x = 220 \text{ mmol } L^{-1}$$

Concentration of KCl needed = 220 mmol L^{-1}

Activity coefficient of Ca^{2+} ($\gamma_{Ca^{2+}}$) in 250 mmol L^{-1} ionic strength solution

$$\log \gamma_{Ca^{2+}} = -A_{DH} z^2 \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right)$$

where A_{DH} is the Debye-Hückel constant with a numerical value of 0.506 at 20°C, z is the charge of calcium ion (= +2), I is the ionic strength of the solution.

$$\log \gamma_{Ca^{2+}} = -(0.506)(2)^2 \left(\frac{\sqrt{0.25}}{1+\sqrt{0.25}} - 0.3(0.25)\right)$$

$$\log \gamma_{Ca^{2+}} = -2.012 \left(\frac{\sqrt{0.25}}{1+\sqrt{0.25}} - 0.3(0.25)\right)$$

$$\log \gamma_{Ca^{2+}} = -2.012 \left(\frac{0.5}{1.5} - 0.075\right)$$

$$\log \gamma_{Ca^{2+}} = -0.5198$$

$$\gamma_{Ca^{2+}} = 0.302$$

Calcium ion activity ($a_{Ca^{2+}}$) for 10 mmol L^{-1} calcium chloride in a solution with an ionic strength of 250 mmol L^{-1}

$$a_{Ca^{2+}} = c_{Ca^{2+}} \cdot \gamma_{Ca^{2+}}$$
$$a_{Ca^{2+}} = (10) \times (0.3)$$
$$a_{Ca^{2+}} = 3$$

Appendix 3: Sample calculations for protein concentration determination from SDS-PAGE

Percentage of protein in skim milk powder: 32.71% (as analysed by Kjeldahl)

Percentage of protein in reconstituted skim milk: 3.271%

Protein in skim milk = 0.0327 g protein/ g skim milk

Skim milk was diluted 1:40 (*v/v*), therefore 0.0008 g / ml

Density of skim milk = 1.02 g/ ml (measured, n = 6)

Table A1: An example of the band intensities determined from scanning a gel using the densitometer and analysed using the Image Lab™ Software 6.0

	Band intensities	
	Skim milk	Serum phase of skim milk
Casein	122,654,826	26,226,460
β-lactoglobulin	19,990,010	23,087,755
α-lactalbumin	5,137,926	6,218,605
Minor whey proteins	3,228,866	2,276,105

Total intensity in skim milk = 122654826 + 19990010 + 5137926 + 3228866

= 151011628

Total casein in diluted skim milk (40× dilution)

$$= \frac{\text{Intensity of casein in skim milk}}{\text{Total intensity of skim milk}} \times \text{total protein}$$

$$= \frac{122654826}{151011628} \times 0.0008$$

= 6.8×10^{-5} g/ g diluted skim milk

= 0.68 mg/ g diluted skim milk

Total casein in skim milk = 0.68 × 40

= 27.2 mg / g skim milk

Total casein in diluted serum (20× dilution)

$$= \frac{\text{Intensity of casein in serum}}{\text{Total intensity of skim milk}} \times \text{total protein}$$

$$= \frac{26226460}{151011628} \times 0.0008$$

$= 1.4 \times 10^{-5}$ g/ g diluted serum

= 0.14 mg /g diluted serum

Total casein in serum = 0.14 × 20

= 2.8 mg /g serum

Proportion of serum per g of skim milk: 0.87

Total casein in serum per g of skim milk = 2.8 × 0.87

= 2.4 mg / g skim milk

Appendix 4: Results for calcium determination using the EDTA titration and AAS method

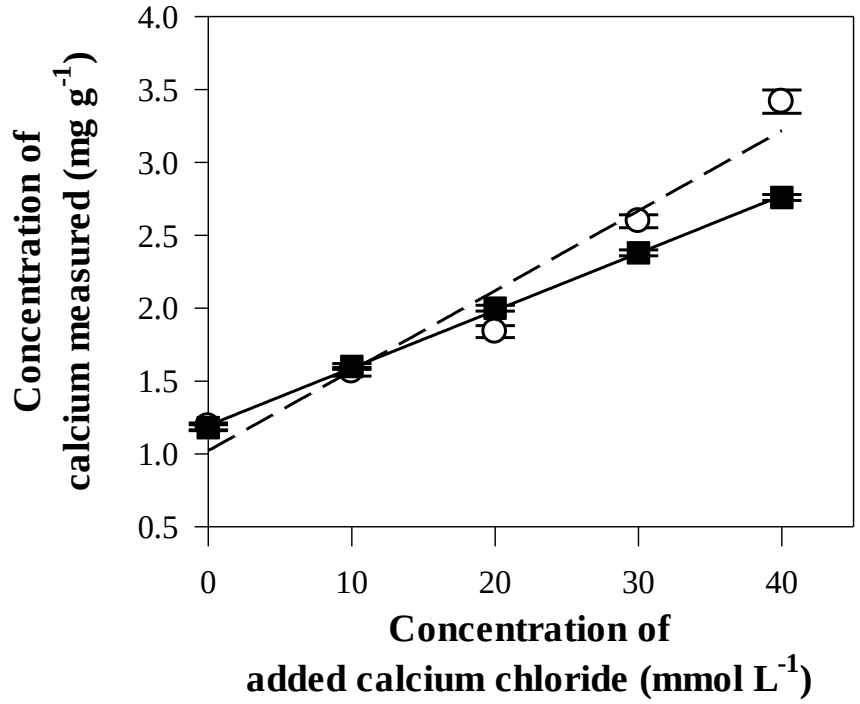

Figure A1: Comparison of the concentration of calcium determined in skim milk with various concentrations of added calcium chloride using the AAS (– –○– –) and EDTA titration method (––■––).

Appendix 5: Sample calculations for rate of increase in G' in the first 20 min of holding

Sample calculation for rate of increase in G' in the first 20 of min of holding for skim milk with 20 mmol L^{-1} of added calcium chloride, preheated at pH 6.40:

Average G' values at t= 0 min during holding (3 replicates): 4.85 Pa

Average G' at t= 20 min during holding: 19.71 Pa

$$\text{Rate of increase in } G' \text{ in the first 20 min of holding} = \frac{19.71 - 4.85}{20}$$

$$= 0.74 \text{ Pa/ min}$$

Appendix 6: Sample calculations for total ionic strength of added salts

Sample calculation for total ionic strength of added salts in skim milk with 10 mmol L^{-1} of added calcium chloride and 30 mmol L-1 of added sodium chloride:

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

Where I is the ionic strength, c_i is the concentration of ion *i* (mol L^{-1}), and z_i is the charge of ion *i*,

$$I = \frac{1}{2}\{([Ca^{2+}] \times z_i^2) + ([Cl^-] \times z_i^2)\} + \{([Na^+] \times z_i^2) + ([Cl^-] \times z_i^2)\}$$

$$I = \frac{1}{2}\{(0.01 \times 1 \times 2^2) + (0.01 \times 2 \times (-1)^2\} + \{(0.03 \times 1 \times 1^2) + (0.03 \times 1 \times (-1)^2\}$$

$$I = \frac{1}{2}(0.06 + 0.06)$$

$I = 0.06$ mol L^{-1}

$= 60$ mmol L^{-1}

Number of Ca^{2+} ions in $CaCl_2$ =1, charge of Ca^{2+} ions in $CaCl_2$ = +2

Number of Cl^- ions in $CaCl_2$ = 2, charge of Cl^- ions in $CaCl_2$ = -1

Number of Na^+ ions in NaCl = 1, charge of Na^+ ions NaCl = +1

Number of Cl^- ions in NaCl = 1, charge of Cl^- ions in NaCl = -1